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TITLE

Mercury and other heavy metal toxicity and mitocheondral dysfunction, (part of a coordinated programme of isotopic tracer-aided studies of the biological side-effects of foreign chemical residues in food and agriculture)

FINAL REPORT FOR THE PERIOD

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FINAL REPORT
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Title:

Mercury and other heavy metal toxicity and mitochondrial dysfunction.

Research Institute:

Universiti Kebangsaan Malaysia,
Kuala Lumpur, Malaysia.

Chief Scientific Investigator:

Poedijono Nitisewojo.

Period of Contract:

1974-07-01 to 1977-07-01

Scientific Background and Scope of Project:

The problem of polution appears to be found in every part of the world; it is not merely the monopoly of the developed countries. In the developing countries as well, the problem of polution is increasing in magnitude due to the booming industrial developments.

Little information is available concerning the molecular basis of mercury and other heavy metal toxicity. The primary biochemical lesion responsible for the toxicity of heavy metals are not much known, although a great deal is known about the effects of these substances on a number of subcellular systems, metabolic pathways and isolated enzymes.

ATP, produced by mitochondrial catalysed reaction is critical to the maintenance and integrity of aerobic cells. Some of the mitochondrial functions are perturbed by a small amount of mercury and other heavy metals (Lee et al., 1971; Brierly et al., 1968; Scott et al., 1970; Goyer et al., 1968; Goyer & Krall, 1969). The relationship between heavy metal toxicity in animals, perturbation of mitochondrial functions and the role of mitochondria in the maintenance of cellular integrity suggest that the suppression of mitochondrial oxidative phosphorylation may be the basis of mercurial and other heavy metal toxicity in men and animals.

Kidney mitochondria isolated from mercury poisoned rats lost their phosphorylating capabilities (Southard & Nitisewojo, 1973).

The scope of the project was to find the relationship between the effect of heavy metals on the mitochondrial functions and their toxicities.

Toxicity of some heavy metals have been intensively studied almost in every aspect (Friberg & Vostal, 1972; Clarkson, 1972; Friberg et al., 1971; Passow et al., 1961; Berglund & Berlin, 1969; Goyer, 1968). Although heavy metals have different toxicological properties, they share a common property that appears responsible for their toxicity, i.e. they all have a high affinity for sulfur and sulfhydryl groups. The difference in toxicological properties of heavy

metals is partly due to their differences of distribution in the body. Mercuric chloride for example is rapidly absorbed and accumulated by the kidney (Friberg & Vostal, 1972; Clarkson, 1972; Bedstrup, 1972; Swensson & Ulfvarson, 1968; Rothstein & Hayes, 1960). Methyl mercury appears uniformly distributed throughout the body with relatively high levels in the brain compared to mercuric chloride (Clarkson, 1972). Cadmium accumulates initially in the liver with the kidney level increasing and surpassing the level in the liver with time (Friberg et al. 1971).

The subcellular distribution of mercury has been studied, and it appears that mercury is found in all subcellular fractions (Grief et al., 1956; Nechay et al., 1967; Norseth, 1969). Mitochondrial and lysozomal fractions contain the highest level of mercury in liver cells from rats (Norseth, 1967; Norseth, 1968). The effects of mercury on mitochondrial function in vitro (Lee et al., 1970; Brierly et al., 1968; Scott et al., 1970; Knight et al., 1968), as well as in vivo (Southard & Nitisewojo, 1973) show that mitochondria dysfunction may be the cause of mercury toxicity. Kidney mitochondria isolated from lead poisoned rats also showed sign of functional damage and partial uncoupled oxidative phosphorylation (Goyer et al., 1968; Goyer & Krall, 1969).

The functions of the mitochondria isolated from the animal poisoned by the heavy metal were studied to find the mechinism by which heavy metal affected the mitochondria.

Experimental Method:

Mercury.

A lethal dose of mercury ($HgCl_2$), 4 mg Hg^{++}/kg body weight, was injected to albino rat (200-250g) intravenously through the lateral vein of the tail. Several hours after the injection, the rat was sacrificed by decapitation and the neck of the decapitated rat was flushed with running water to draw out the blood as much as possible. The kidney mitochondria were isolated by the procedure of Johnson & Lardy (1967) in a medium of 0.25 M in sucrose and 10 mM in Tris-HCl buffer, pH 7.4. Kidney mitochondria were selected in this study, since in the acute poisoning, most of the mercury was accumulated by the kidney and the kidney mitochondria lost their capacity to phosphorylate (Southard & Nitisewojo, 1973). Liver mitochondria isolated from the mercury poisoned rat showed no demonstrable loss of the capacity for oxidative phosphorylation.

The control functions of the isolated kidney mitochondria then were tested in a closed reaction cell. The amount of ATP (mmoles) synthesized per amount of oxygen (natoms) consumed, i.e. the P/O ratio, was determined at 30° C in a medium containing 80 mM KCl, 50 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM potassium succinate, 10 mM potassium phosphate (^{32}P)K_2HPO_4, 1 mM ADP, 5 mM glucose, 5 Units of hexokinase per ml, 10 uM rotenone and 1 mg mitochondrial protein per ml. The raction was stopped by the addition of trichloroacetic acid (final concentration 2%). Glucose-1-phosphate (^{32}P) was separated from (^{32}P)K_2HPO_4 by the

procedure of Lindberg & Ernster (1956) and the P/O ratio was calculated by the method of Lee et al. (1971). Oxygen consumption (respiration) was monitored with a Beckman oxygen analyzer (Model Fieldlab) and oxygen sensitive electrode and recorded with a Beckman ten inch potentiometric recorder. The respiratory control ratio (RCR) was determined by dividing the rate of mitochondrial respiration with ADP by the rate of respiration without ADP. Mitochondrial protein was determined by the Biuret procedure of Gornall et al. (1949).

To study the effect of selenium on the toxicity of mercury in the kidney mitochondria, one group of rats were given 1 ppm selenium (as sodium selenate) in the diet for 1 week and another group for 2 weeks prior to the injections of mercury. The kidney mitochondria were then isolated and tested as described above. Mortality study of the mercury poisoned rats under selenium diet was also carried out.

Most of the reagents and biochemicals were purchased from Sigma and the radioactive phosphate was purchased from the Bandung Atomic Reactor Center, Indonesia.

Cadmium.

A lethal dose of cadmium ($CdCl_2$), 4 mg Cd^{++}/kg body weight was injected intravenously to rat (200-250g) and the kidney and liver mitochondria from the cadmium poisoned rat were isolated and tested as in the mercury study. To measure the P/O ratio at the phosphorylation site III, ascorbate-tetramethyl paraphenylene diamine (TMPD) were used for the substrates

(Southard et al., 1973). Cadmium concentration in the isolated mitochondria was determined using atomic absorption spectrophotometer, Varian Techtron, Model AA-6. The cadmium was extracted from mitochondrial protein (20-50 mg) with 2 M perchloric acid. After centrifugation, the supernatant was diluted with water and then measured in the atomic absorption spectrophotometer. For normal mitochondria, the supernatant used directly without dilution.

In the lethal dose determination study, some rats died very shortly after the injections of cadmium, and there were apparent symptoms of acute cardio-vascular effects. A study of the effect of the intravenous injection of cadmium on cardio-vascular system was carried out. Rabbit (approximately 1.5 kg) was anaesthesized with nembutal and the blood pressure was recorded from the carotic artery using a Physiograph, Projector model, Type PMP-4B, with Pressure Transducer P-1000-A, from Narco-Bio-System Inc., Houston, Texas. Cadmium chloride was injected slowly through the femoral vein.

The effect of the addition of Mg^{++} and ethylene glycol bis (2-aminoethyl)-N, N-tetraacetate (EGTA), which is a powerful chelating agent for Ca^{++}, was investigated, since in mercury poisoning, the inhibition of oxidative phosphorylation in mitochondria by mercury was due to the decrease of Mg^{++} and the increase of Ca^{++} concentration in the mitochondria.

The effect of selenium on the mitochondria isolated from cadmium poisoned rat was also investigated, similar to that

in mercury study.

Lead.

The effect of lead on the oxidative phosphorylation of the kidney and liver mitochondria isolated from lead poisoned rat was also studied. Rat was given lead acetate at 80 mg Pb^{++}/kg body weight intravenously or 120 mg Pb^{++}/kg body weight every two days for 2 weeks subcutaneously. It was very hard to establish the dose of a sigle intravenous injection to obtain about 90% mortality.

To determine the P/O ratio, the phosphate concentration was varied, since a preliminary study indicated that phosphate concentration added in the reacting medium had a marked effect on the phosphorylating capability of the isolated mitochondria from lead poisoned rat.

To study the effect of lead on mitochondrial respiration, 2,4-dinitrophenol (DNP) was used to stimulate the respiration.

In vitro study, the minimum concentration of lead which inhibited completely the stimulation of respiration by DNP was determined. Phosphate then was added to see if phosphate could reverse the inhibition of DNP respiration by lead.

Results Obtained.

Mercury.

A lethal dose of mercury given intravenously to rat, indeed suppressed almost completely the oxidative phosphorylation in kidney mitochondria isolated from the mercury poisoned rat.

When rat was given 1 ppm selenium in the diet for 1 or 2 weeks, the mitochondria had some of their capability to phosphorylate. Table 1 shows that the P/O ratio (using succinate as the substrate) of the mercury poisoned mitochondria was 0.05. The P/O ratio of the normal mitochondria was 1.50. When rat was given selenium in the diet for 1 week, selenium somewhat protected the mitochondria from mercury toxicity. The P/O ratio was 0.50. The protection by selenium increased when the rat received selenium for 2 weeks. The P/O ratio was 1.09.

Table 1 also shows that the respiratory control ratio (RCR) of the mercury poisoned mitochondria was almost 1, indicating that the mitochondria did not have any coupling capability. The protection of the mitochondria by selenium against the toxicity of mercury was also shown here based on the mitochondrial coupling capability. The RCR after the rat received 1 week of selenium in the diet was 1.8 and after 2 weeks it was 2.3.

Table 1. The effect of selenium on the P/O ratio and respiratory control ratio of kidney mitochondria isolated from mercury poisoned rat.

Dose of mercury (mg Hg^{++}/kg)	Time on 1 ppm selenium (days)	P/O (succinate)	RCR (succinate)
0	0	1.50	4.5
4	0	0.05	1.1
4	7	0.50	1.8
4	14	1.09	2.3

Figure 1 shows that selenium in the diet prolonged the death time in rat poisoned with mercury.

Cadmium.

The target organ in cadmium poisoning is liver. Table 2 shows that kidney mitochondria isolated from cadmium poisoned rat had a normal P/O ratio. Liver mitochondria however, isolated from the same rat showed low P/O ratio, which was between 0.6 to 0.8. Three out of ten experiments, rats died very shortly after the injections of cadmium, which seemed to be due to the effect of cadmium on the cardio-vascular system. The P/O ratio of cadmium poisoned liver mitochondria was obtained from 5 experiments. One experiment was discarded, since the P/O ratio was approximately 0.05, which was too low compared to the other data, and was thought to be due to the experimental error.

Table 2. The effect of a lethal dose of cadmium (4mg Cd^{++}/kg) on the P/O ratio of the isolated kidney and liver mitochondria.

Treatment	Source of mitochondria	P/O (succinate)
None	kidney	1.4 - 1.6
	liver	1.5 - 1.7
$CdCl_2$	kidney	1.4 - 1.5
	liver	0.6 - 0.8

The poisoned liver mitochondria also showed slower rate in succinate respiration and that the respiratory control ratio was almost 1, compared to at least 4 in normal liver mitochondria (Figure 2).

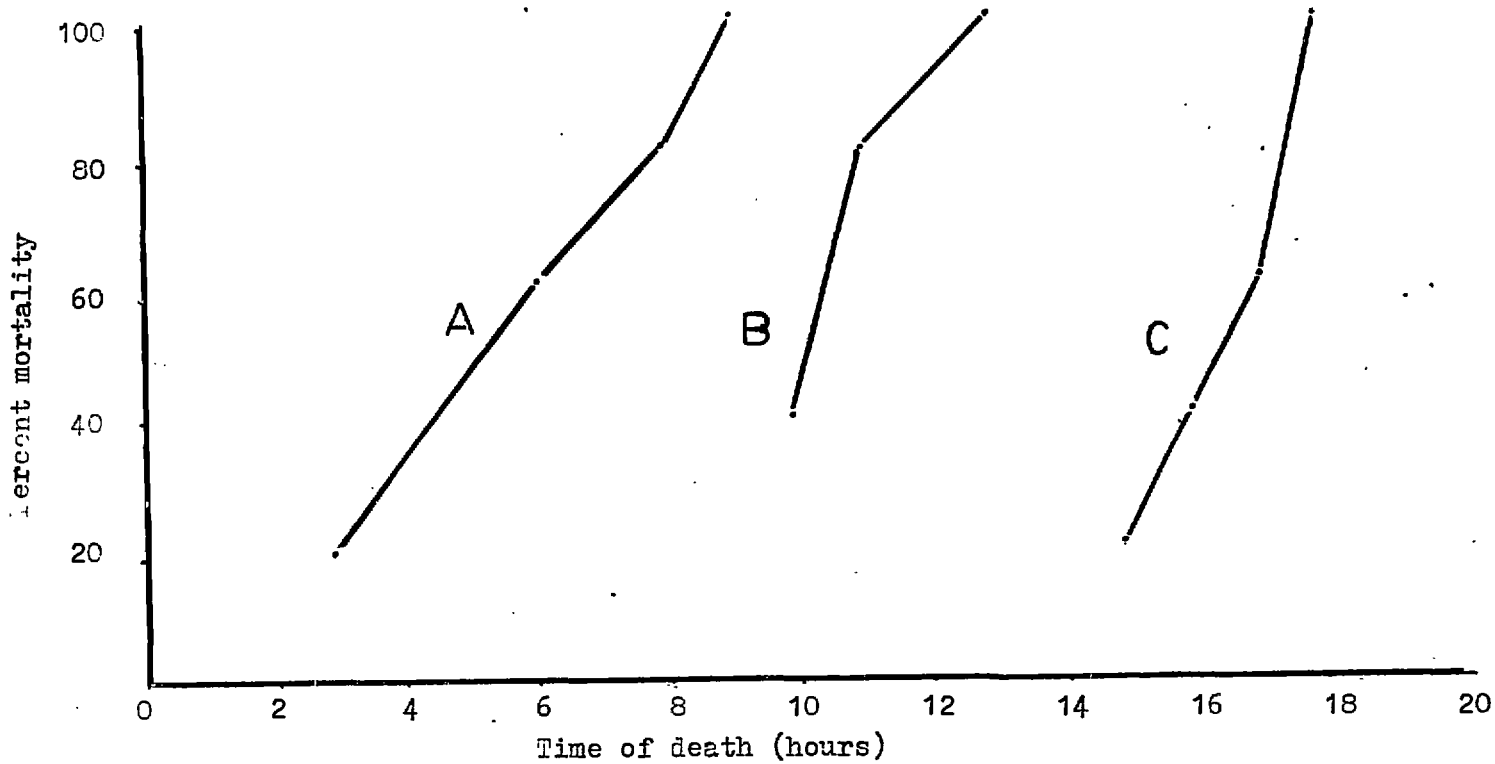

Figure 1. Effect of selenium on the mortality of mercury poisoned rats.
A: No selenium in the diet,
B: After 7 days on 1 ppm selenium diet,
C: After 14 days on 1 ppm selenium diet.

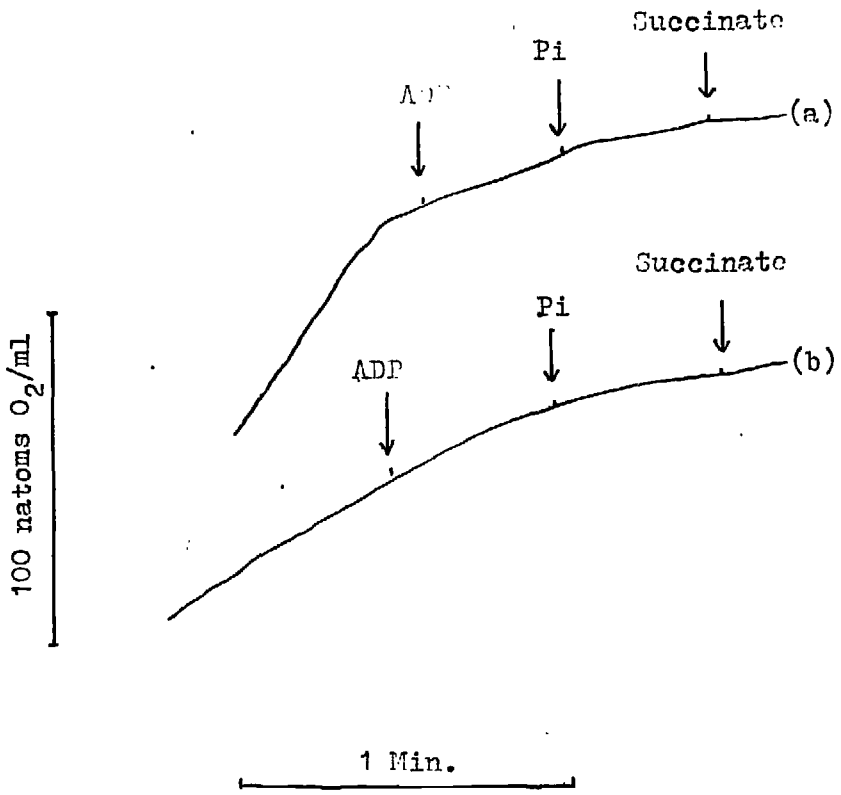

Figure 2. The effect of cadmium on the respiration of the isolated rat liver mitochondria. (a) control rat, (b) cadmium poisoned rat, 4 mg Cd^{++}/kg, intravenous.

The oxidative phosphorylation of the mitochondria, either isolated from liver or from kidney at site III was not affected by the cadmium (Table 3). This indicates that the effect of the intravenous injection of cadmium at 4 mg Cd^{++}/kg body weight was mostly on the oxidative phosphorylation mechanism at site II. This does not mean that site III is not sensitive to cadmium. In vitro, Southard et al. (1974) reported that 10 nmoles Cd^{++}/mg mitochondrial protein depressed the P/O ratio (succinate) from 1.5 to 0.05. The concentration of cadmium in the liver mitochondria isolated from cadmium poisoned rat was only 3 to 5 nmoles Cd^{++}/mg mitochondrial protein (Table 3). Table 3 also shows that kidney mitochondria contained very low amount of cadmium. This correlates well with the P/O data, indicating that the presence of cadmium in the mitochondria caused the mitochondrial dysfunction. Normal mitochondria contained undetectable cadmium.

Addition of Mg^{++} and EGTA to the medium containing liver mitochondria isolated from cadmium poisoned rat increased the P/O to approcimately 1.4, which was very close to normal P/O of liver mitochondria.

Intravenous administration of cadmium to rabbit caused the increase in the amplitude (the difference between the systolic and the diastolic presseure) as well as the frequency of the heart beat (Table 4). No furthur studies on the mechanism of action of the effect of cadmium on the cardio-vascular system were conducted in this project. However, in a separate project,

Table 3. The effect of cadmium on the P/O ratio (ascorbate-TMPD) of kidney and liver mitochondria isolated from cadmium poisoned rat.

Treatment	Source of mitochondria	P/O (ascorbate-TMPD)	Cd^{++} (nmoles/mg
None	kidney	0.8 - 0.9	undetectable
	liver	0.8 - 0.9	undetectable
$CdCl_2$ (4 mg Cd^{++}/kg)	kidney	0.7 - 0.9	0.5 - 0.8
	liver	0.6 - 0.8	3.0 - 5.0

Table 4. The effect of intravenous injection of cadmium on the carotic arterial blood pressure in rabbit.

	Normal	After injection of $CdCl_2$ (I.V., 5 mg Cd^{++}/kg)
Systolic pressure (mm Hg)	150	160
Diastolic pressure (mm Hg)	100	80
Frequency of heart beat/min.	235	280

preliminary results showed that the effect of intravenous injection of cadmium on blood pressure was dependent on the dose of cadmium as well as the rate of cadmium administration.

The results on the study of the interaction of cadmium and selenium were similar to those in mercury-selenium interactio

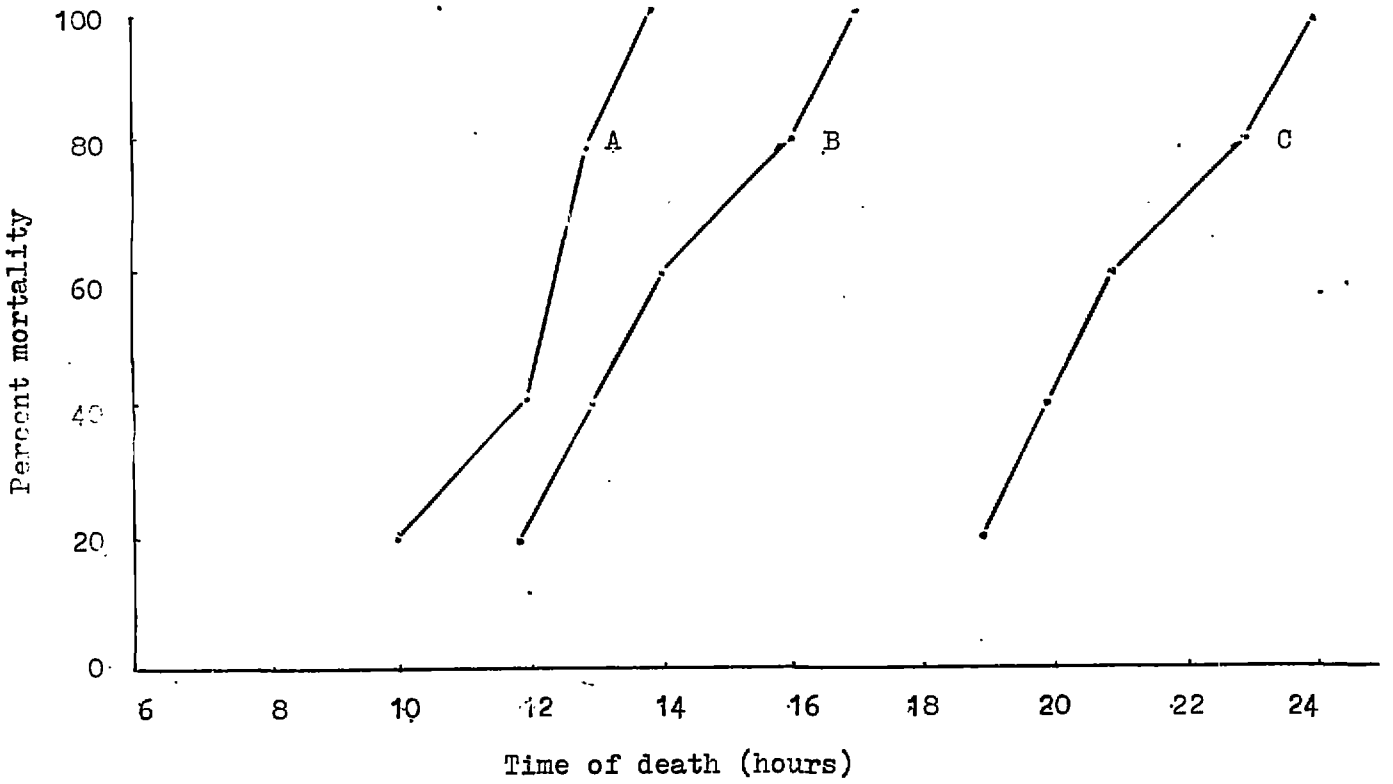

Figure 3. Effect of selenium on the mortality of mercury poisoned rats.

A: No selenium in the diet.

B: After 7 days on 1 ppm selenium diet.

C: After 14 days on 1 ppm selenium diet.

study. Rat which was exposed to selenium showed some resistance from the effect of cadmium. Liver mitochondria isolated from that rat had higher P/O ratio compared to that in rat without selenium (Table 5). Mortality study also showed the protection of selenium against cadmium toxicity (Figure 3).

Table 4. The effect of selenium on the P/O ratio and respiratory control ratio of liver mitochondria isolated from cadmium poisoned rat.

Dose of cadmium (mg Hg^{++}/kg)	Time on 1 ppm selenium (days)	P/O (succinate)	RCR (succinate)
0	0	1.60	4.0
4	0	0.06	1.1
4	7	0.50	1.5
4	14	1.19	2.7

Lead.

Liver and kidney mitochondria isolated from lead poisoned rat given intravenously or subcutaneously, showed normal respiration, P/O ratio and respiratory control ratio. However, the stimulation of mitochondrial respiration (succinate) cause by the addition of DNP was depressed by lead, either *in vivo* (subcutaneous injection) or *in vitro* (2 ug Pb^{++}/mg mitochondrial protein) (Figures 4, 5 and 6).

The results indicate that lead inhibits electron transport, but it does not depress the phosphorylation. Goyer *et al.* (1968) however, have reported a partially uncoupled oxidative phosphorylation in mitochondria from lead poisoned

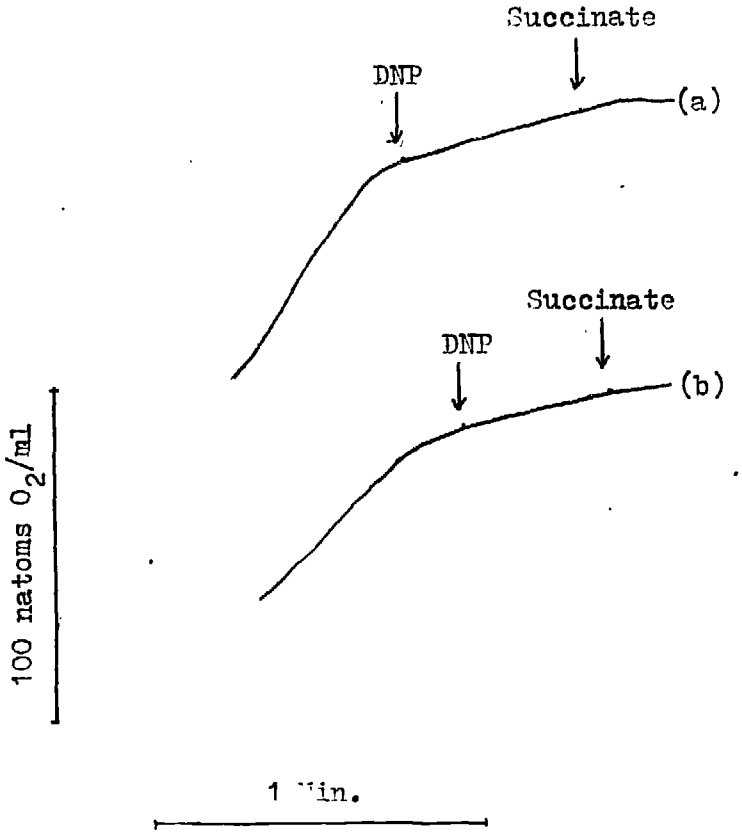

Figure 4. The effect of lead on the respiration of the isolated rat liver mitochondria. (a) control rat, (b) lead poisoned rat, 120 mg Pb^{++}/kg, subcutaneous, every 2 days for 2 weeks.

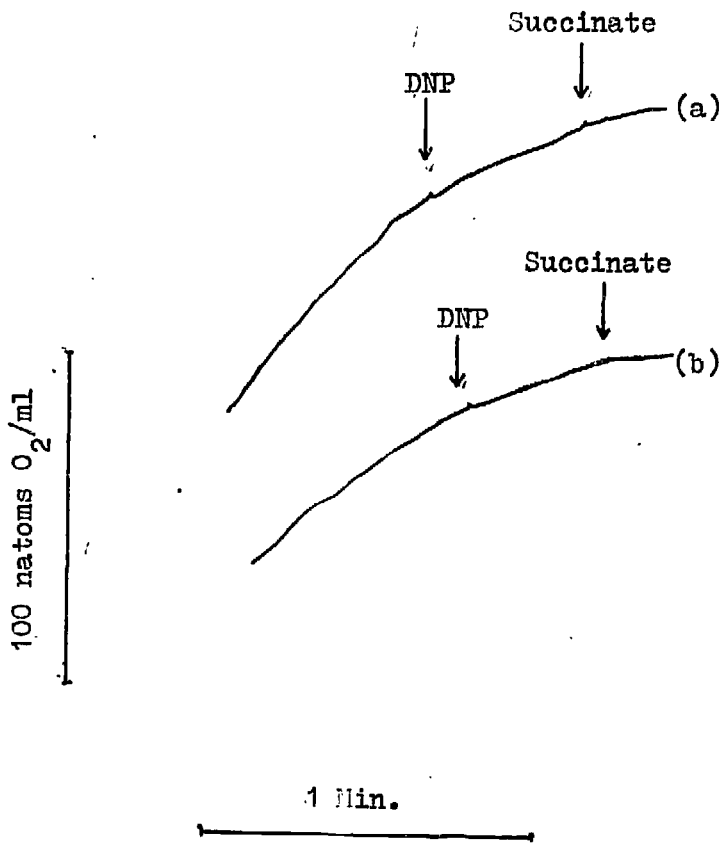

Figure 5. As in Figure 4, kidney mitochondria.

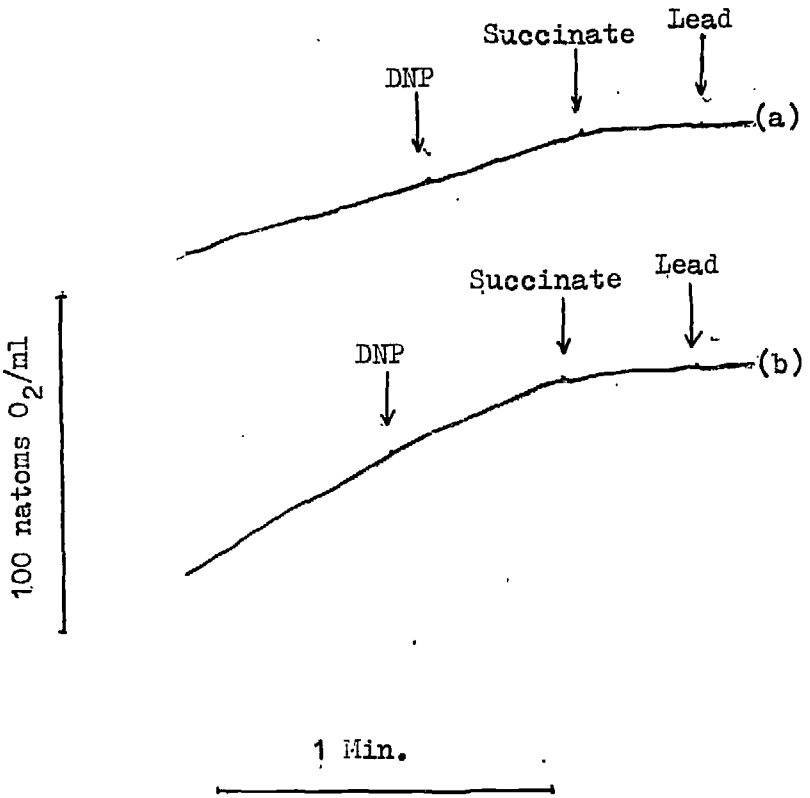

Figure 6. The effect of lead on the mitochondrial respiration in vitro, 2 ug Pb^{++}/mg.
(a) Isolated liver mitochondria,
(b) isolated kidney mitochondria.

rat. Later, it was apparent that the inorganic phosphate concentration in the mitochondria suspension for the determination of P/O and respiratory control ratios was critical, because phosphate could bind lead in the mitochondria. Table 6 shows that subcutaneous injection of lead (120 mg Pb^{++}/kg every 2 days for 2 weeks) lowered the respiratory control ratios in both kidney and liver mitochondria, when phosphate concentration was less than 5 umoles/mg mitochondrial protein. P/O ratios however, were not significantly depressed.

Table 6. The effect of lead on the P/O and respiratory control ratios in kidney and liver mitochondria isolated from lead poisoned rat.

Phosphate Added umoles/mg	P/O (Succinate)		Respiratory Control Ratio			
			Normal		Lead	
	Kidney	Liver	Kidney	Liver	Kidney	Liver
0.63	-	-	4.0	3.0	1.3	1.4
1.25	0.9-1.4	1.0-1.2	4.2	3.5	1.6	1.9
2.50	-	-	4.5	4.0	2.0	2.9
5.00	1.2-1.6	1.0-1.6	4.5	4.0	3.2	4.2

More study on the interaction of lead and phosphate in the mitochondria was then carried out <u>in vitro</u> using DNP as the uncoupler to stimulate the mitochondria respiration (succinate as the substrate). Rat liver mitochondria were used, since liver yields more mitochondria than does kidney. The stimulation of mitochondrial respiration by DNP was mixmum when the concentration of DNP was approximately 2×10^{-4} nmoles/

mg mitochondrial protein. Lead at a concentration of 70 nmoles/mg blocked completely the stimulation of respiration caused by DNP. When phosphate was added to the mitochondrial suspension after the addition of lead, the mitochondria responded again to the addition of DNP. The minimum concentration of phosphate necessary to restore the respiration of lead poisoned mitochondria (70 nmoles/mg) was 20 umoles/mg mitohondrial protein (Figure 7).

Conclusions.

Mercury.

In acute mercury poisoning, it seems that the death of the animals is due to the kidney failure which is caused by the depression of the oxidative phosphorylation in the kidney mitochondria. Since no or very little ATP can be synthesized in the mitochondria, practically no active biological and biochemical reactions can occur for the maintenance and integrity of the cells, since ATP is needed for those reactions.

Some of the mechanism of actions of mercury toxicity on the kidney mitochondria are the dapletion of Mg^{++} and the accumulation of Ca^{++} in the mitochondria. Addition of Mg^{++} and the addition of EGTA (to remove Ca^{++}) to the isolated kidney mitochondria restored the oxidative phosphorylation capability of the mitochondria. Removal of the effects of the toxic agent, beside the removal of the toxic agent itself, is a normal procedure to treat the animal from a poisoning. Injection of Mg^{++} and EGTA to the rat however, would not be indicated, since

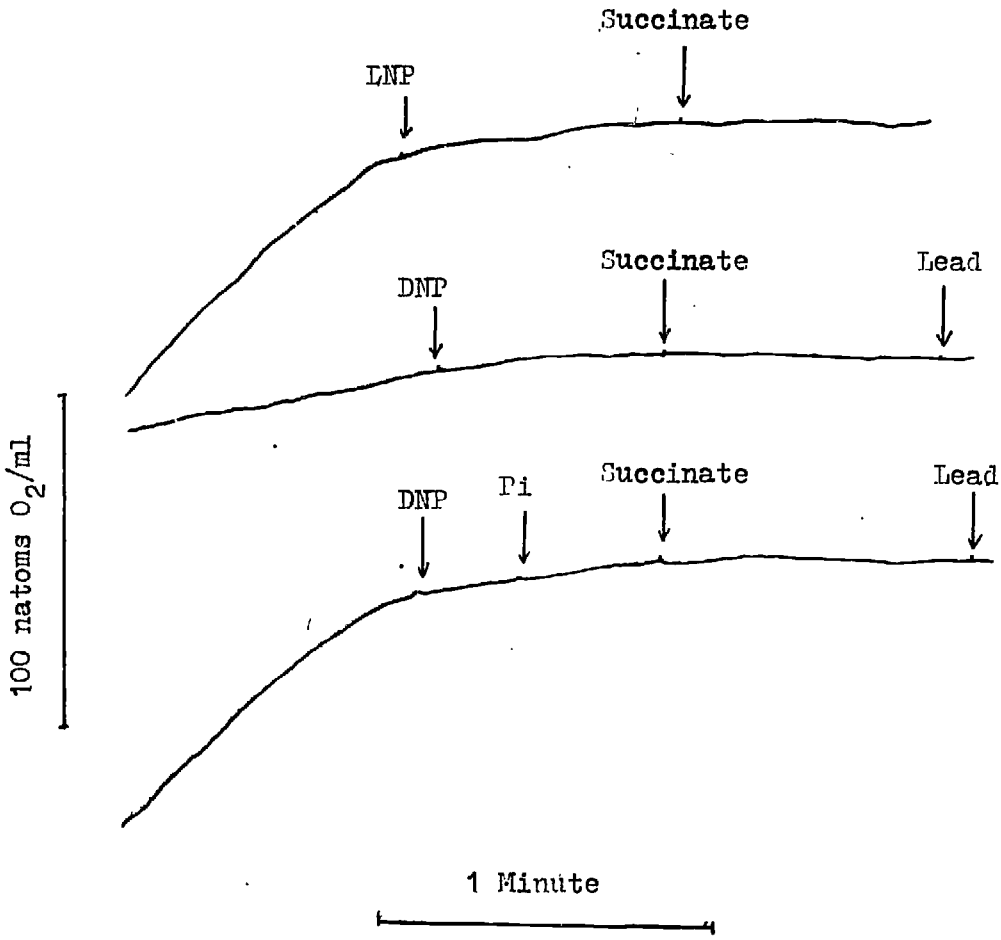

Figure 7. The interaction of lead and phosphate on rat liver mitochondrial respiration in vitro.

the effects of Mg^{++} and EGTA in the animal would be very complex. Besides, Mg^{++} and EGTA might not be able to get into the mitochondria. Similarly so the administration of EDTA (ethylene diamine tetraacetate) to remove the mercury. Selenium on the other had, has been reported to have a protective effect on mercury toxicity (Potter & Matrone, 1974; Ansari & Britton, 1974; Ganther & Sundee, 1974). Selenium then was given to the rat prior to the administration of mercury to see if the protection of mercury poisoning by selenium would cause the restoration of mitochondrial oxidative phosphorylation capability. The results of the preliminary experiments were rather disappointing, but after repeated experiments the results showed that indeed selenium protected the mitochondria from mercury poisoning.

Cadmium.

The conclusions which can be drawn from cadmium studies are similar to those drawn from mercury studies. The main difference is that in cadmium poisoning, the target organ is liver, so that it can be said that the death of the animal in acute cadmium poisoning is probably due to the liver failure which is caused by the depression of the oxidative phosphorylation in the liver mitochondria.

Lead.

Phopshate reversed the effects of lead in the mitochondria. The toxicity of lead was found to be very dependent upon the availability of phosphate to the mitochondria.

Further study on the interaction of phosphate and lead may be worth-while. For example, the effect of high and low phosphate feeding on lead poisoned animal, the mortality study, the concentration of phosphate and lead in kidney or liver mitochondria isolated from such animal, etc., might show if external high phosphate intake is really affecting lead toxicity.

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2. P. Nitisewojo, D. Sastradipradja, H. Permadi and Ishak T. Kechik (1976). The effect of cadmium and lead on the oxidative phosphorylation of liver and kidney mitochondria of rat. Technical Document IAEA - 193.

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