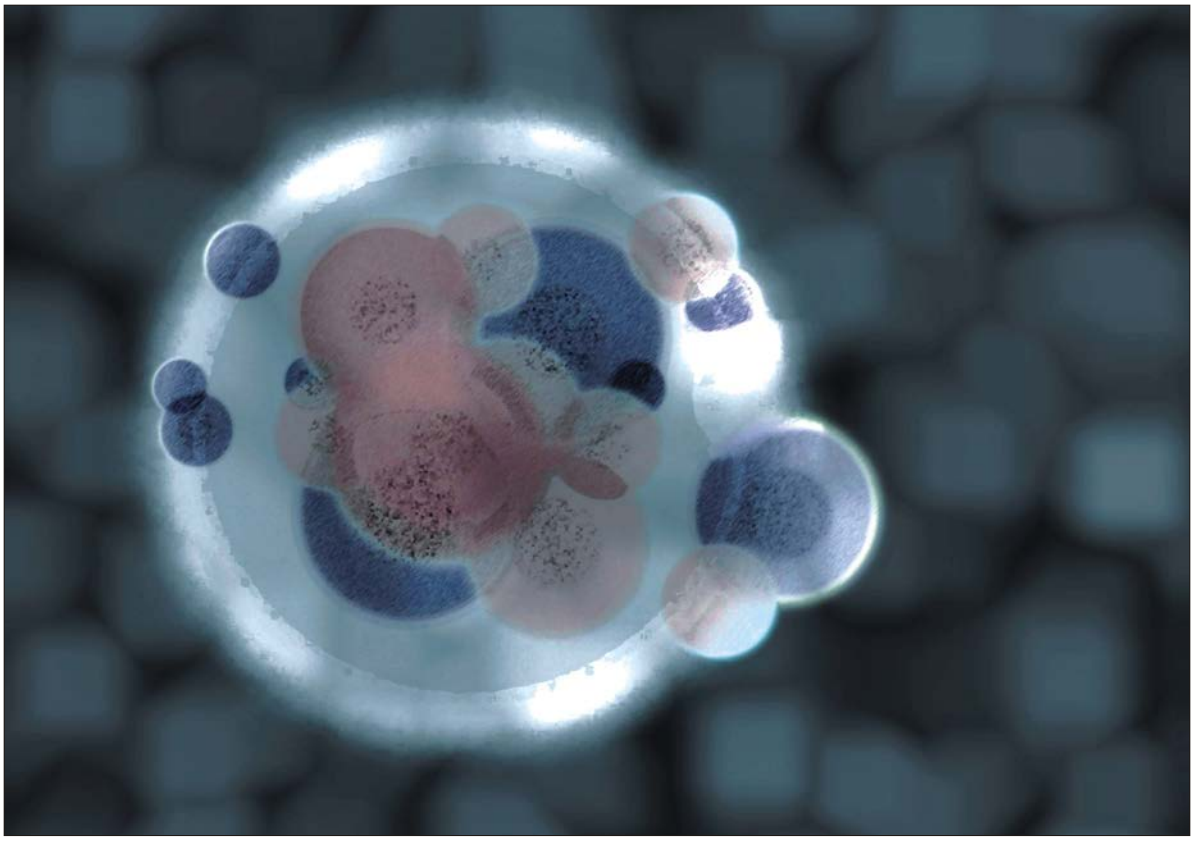

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Lead Toxicity on Kinetic Behaviors of High and Low Molecular Weight Alkaline Phosphatase Isoenzymes of Rat, *in vivo* and *in vitro* Studies

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Abstract: The relationship between lead (Pb) toxicity and changes in the kinetic characteristics of serum, liver and brain high and low molecular weight alkaline phosphatase isoenzymes has been examined in this document. Alkaline phosphatase is a family of phosphomonoesterases that was measured in serum, liver and brain using paranitrophenol phosphate (pNPP) as substrate and 2-amino-2-methyl-1-propanol as buffer. Protein concentration was determined as described by Bradford. Results obtained showed that every other day intrapritoneally injection of 39.5 μg kg^{-1} of lead as (Pb $(CH_3COO)_2 3H_2O$), in male rats for 2 consecutive weeks resulted in decreasing level of liver and brain alkaline phosphatase by 16.7 and 10.9%, respectively, whereas an elevation of serum enzyme activity by 28.4% was seen in comparison to untreated controls ($p<0.05$). Long-term exposure to 13.2 μg kg^{-1} of this salt, showed a statistically significant reduction in liver and brain levels of alkaline phosphatase by 18.7 and 13.2% respectively and an increment in serum activity of the enzyme by 37.6% in compared to control group ($p<0.05$). Using gel filtration chromatography technique with sephacryl S_{300} showed that, in comparison to control groups, serum and liver homogenate from lead treated groups had a significant level of high molecular weight alkaline phosphatase, which might be considered as a potential biomarker for lead toxicity. *In vitro* experiments showed that lead inhibited all the isoenzymes.

Key words: Lead acetate, enzyme kinetics, alkaline phosphatase, liver, brain

INTRODUCTION

Lead (Pb) is one of the most ubiquitous heavy metal which has been demonstrated to be neurotoxic (Dabrovaska-Buuta *et al*., 2004). The direct neurotoxic action of Pb include: apoptosis, excitotoxicity, influences on neurotransmitter storage and release process, cerebrovascular endothelial cells and astroglia (Lindsky and Schneider, 2003). Exposure to low level of Pb has been associated with behavioral abnormalities, learning impairment and impaired cognitive function in human and experimental animals (Adonaylo and Oteiza, 1999). This metal is dispersed through environment, in ambient air, in a number of food stuffs, in drinking water and finally in dusts (Shalan *et al*., 2005). Pb is absorbed through the gastrointestinal tract or lungs, the absorbed Pb is distributed into the liver, kidney and bone marrow, causing a direct toxic effect in these organs (Satyalatha and Vardhani, 2005). This metal can cause liver damage and may disturb the normal biochemical process in the hepatobiliary system (Sipos *et al*., 2003). Undesired effects on heme biosynthesis, interference with catecholaminergic and particularly dopaminergic function (Nour Eddine *et al*., 2005), lipid peroxidation and free radical mediated cytotoxicity (Mateo *et al*., 2003), altered proliferation and differentiation of neural stem cells (Hung and Schneider, 2004) and carcinogenic effects in human (Fracasso *et al*., 2002), has been reported following Pb toxicity. On the other hand, alkaline phosphatases (ALP) are a large group of cell surface glycoprotein that exists in the majority of species from bacteria to higher vertebrates (Moss, 1997). Fritische and Adams-Park by electrophoresis of human serum on cellulose acetate detected a specific type of ALP and named it "high molecular weight fraction of ALP (Fritische and Adams-Park, 1972). High molecular weight alkaline phosphatase have now been reported in patients with extra- or intra- hepatic cholestasis, malignancy of liver,

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primary or metastatic carcinoma, Hodgkin's and non-Hodgkin's lymphoma, and/or leukemia (Wolf, 1990; Bhudhisawasdi *et al*., 2004). This kind of isoenzyme has been suggested as a tumor marker for liver (Moshtaghie *et al*., 1996) and colorectal cancer (Wei *et al*., 1993). Data from our laboratory showed that high molecular weight alkaline phosphatase might be influenced by manganese and aluminium (Mirhashemi *et al*., 2009, 2010). The existence of hepatobiliary dysfunction in those patients with Pb overload (Sipos *et al*., 2003) lead us to investigate the probable occurrence of high molecular weight ALP in sera, liver and brain of rats treated with Pb and determine the kinetic characteristic of these isoenzymes.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Company. Twenty-eight male Wistar rats (approximate weight 200-220 g) were purchased from Pasteur Institute (Tehran-Iran) and kept in the university animal house at standard conditions (22-24°C, 40-60% relative humidity and light cycle coinciding with day-light hours) and fed with standard rat food and water ad libitum during the entire experimental period. This research project was conducted from 1/11/2006 to 1/11/2009. Rats were divided randomly into two groups named: short-term and long-term exposure to Pb, respectively. Each group had its specific control group. In short-term study, control group received every other day intrapritoneally (i.p.) injection of sterile normal saline (0.1 mL) for 2 consecutive weeks, simultaneously treated group was administrated with 39.5 μg kg^{-1} of Pb ($Pb(CH_3COO)_2.3H_2O$) as the same way as controls. Long-term study was carried out using 13.2 μg kg^{-1} of this salt for duration of 7 weeks, as described method for the short- term groups.

Rats were then killed by decapitation at the end of their treatment periods. Blood samples were collected and sera were separated from cells by centrifugation and were used for enzyme and protein assay. Brain and liver tissues were immediately removed, washed with cold (+4°C) saline solution and homogenized (10% w/v) in a buffer solution containing 10 mM tris and 0.25 M sucrose, pH:7.4, at +4°C. The homogenates were then centrifuged at 13000 g for 20 min at +4°C and the resultant supernatants were carefully removed and were used for the enzyme and protein determination (Yazar and Tras, 2001). Alkaline phosphatase activity was measured at 410 nm and 37°C by the formation of paranitrophenol (pNP) from paranitrophenol phosphate (pNPP) as substrate and 2-amino-2-methyl-1-propanol (AMP) as buffer (Bomers and McComb, 1975). Protein concentration was determined as described by Bradford (1976), with bovine serum albumin as standard. In order to separate high and low molecular weight isoenzymes of ALP, gel filtration chromatography on sephacryl S_{300} was used. Each sample (0.2 mL) was diluted with equal volume of tris buffer (50 mM, pH 7.4) and was then applied to a column (50×0.9 cm) loaded with sephacryl S_{300} and was then eluted at 10 mL h^{-1} with tris-HCl buffer (50 mM, pH 7.4). Fractions of 1 mL were then collected (Moshtaghie *et al*., 1995). ALP activity and protein concentrations in each fraction were determined according to the methods mentioned earlier (Bomers and McComb, 1975; Bradford, 1976). *In vitro* experiments were established to study kinetic behaviors of separated high and low molecular weight isoenzymes of alkaline phosphatase and For this purpose, partially purification of these isoenzymes was carried out using protein precipitation with ammonium sulfate (Green and Hughes, 1995), Dialysis technique (McPhie, 1971) and ion- exchange chromatography by DEAE-cellulose (Crofton and Smith, 1979), respectively. The activities of high and low molecular weight alkaline phosphatase isoenzymes were measured in various concentrations of lead and compared with the control (zero concentration of the element).

High and low molecular weight isoenzymes of alkaline phosphatase were incubated for 30, 60, 90 and 120 min with constant concentration of lead. For doing these tests, appropriate volume (2 mL) of the isoenzyme preparations were incubated with AMP buffer containing 75 μM of lead for duration time of 120 min in water bath at 37°C. At various time intervals (for example after 30 min) of the incubation, portions of the enzyme-element mixture were withdrawn, the activity of the enzyme was measured and compared with the activity of the enzyme in the presence of the element without incubation time that considered as control. Percent of the remaining activity of the isoenzymes was calculated and was drawn against the incubation times.

Statistical analysis: Analysis of data was accomplished using SPSS statistical software package. Between-groups comparisons were performed with t-test. All results were presented as Mean±SD and so were statistically significant at $p<0.05$.

RESULTS

It was found that short term administration of Pb lead to the significant ($p<0.05$) elevation of serum total ALP activity by 28.4% in comparison to normal healthy controls. Significant ($p<0.05$) reduction in the liver and brain total ALP activities by 16.7 and 10.9% was seen

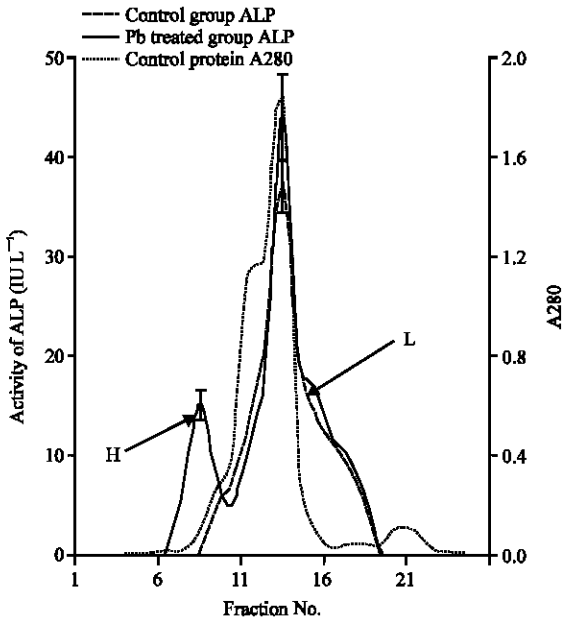

Fig. 1: The elution profile of serum of control and Pb treated groups. Animals were treated with Pb. High molecular weight ALP was increased significantly ($p<0.05$) in Pb treated group when compared with controls. H: High molecular weight ALP, L: Low Molecular weight ALP

Table 1: Lead Effect on serum, liver and brain total ALP activity

	ALP (IU mg^{-1} tissue protein)		
Group	Serum	Liver	Brain
A			
Control	1.94±0.10	3.42±0.26	2.02±0.12
Treated	2.49±0.18*	2.85±0.13*	1.80±0.07*
B			
Control	2.10±0.10	3.16±0.26	2.27±0.13
Treated	2.89±0.12*	2.57±0.17*	1.97±0.09*

Rats were injected with Pb as $(Pb(CH_3COO)_2.3H_2O)$ every other day for two weeks (A) and for seven weeks (B). Animal were killed and ALP activity was determined. *Indicates statistically significant difference of ALP activity between Pb treated animals and their controls ($p<0.05$). Data are presented as Mean±SD

when rats were treated with same amount of Pb every other day for two weeks (Table 1). Long term study was performed by injection of Pb every other day for seven weeks. Significant ($p<0.05$) elevation of 37.6% in total serum ALP and significant ($p<0.05$) reduction of 18.7% and 13.2% in liver and brain ALP were seen (Table 1). Comparing the data obtained from short and long term effects of Pb on the activity of the enzyme in serum, liver and brain showed that changes in the enzyme activity was dose and time dependent processes. Gel filtration chromatography technique was established to separate high and low molecular weight ALP from sera, liver, and/or brain homogenates of both treated and untreated

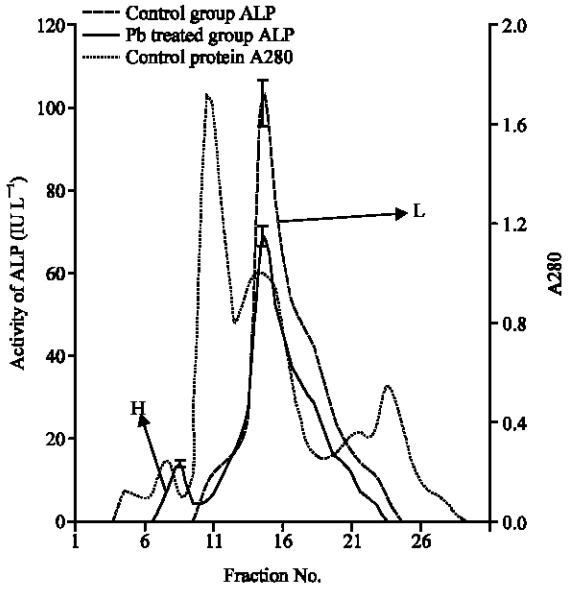

Fig. 2: The elution profile of liver of control and Pb treated groups. Animals were treated by Pb. High molecular weight ALP was increased, whereas low molecular weight ALP was decreased. H: High molecular weight ALP, L: Low Molecular weight ALP

animals. Fractionation of the serum from Pb treated animals showed 18.9% elevation in low molecular weight ALP and 16 times increase in high mass ALP related to control group (Fig. 1).

When liver homogenates from Pb treated rat was chromatographed, a significant reduction of 31.25% in the activity of low molecular weight ALP and a significant ($p<0.05$) elevation in high molecular weight alkaline phosphatase (Fig. 2) was found.

Figure 3 shows significant ($p<0.05$) reduction in low molecular weight by 28.1% in brain ALP following Pb treatment, but no changes was observed in the level of high molecular weight ALP when compared with control values.

For *in vitro* experiments, separated serum, liver and brain low molecular weight alkaline phosphatase were purified by 22.6, 20.3 and 12.7 fold, whereas, this value for serum and liver high molecular weight ALP was 21.3 and 27.3 fold, respectively.

Serum, liver and brain low molecular weight isoenzyme activities were reduced significantly ($p<0.05$) by 23.5, 25.2 and 19.8%, respectively, with the reaction medium containing 75 μM concentration of lead (Fig. 4). The reduction percent for serum and liver high molecular weight alkaline phosphatase was 21.9 and 22.8% ($p<0.05$), respectively (Fig. 5).

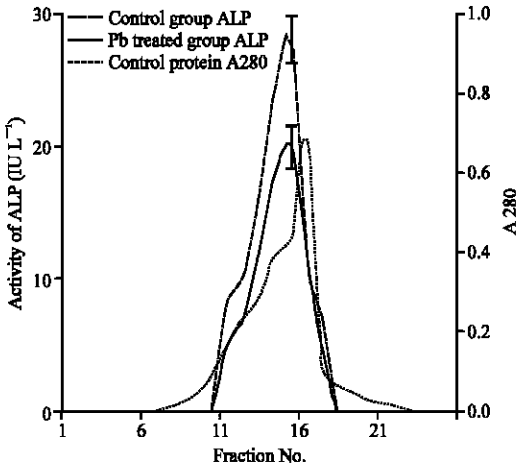

Fig. 3: The elution profile of brain of control and Pb treated groups. Pb was administrated every other day for 7 weeks. Regarding considerable reduction in activity of low molecular weight ALP, high molecular ALP was not produced in this group

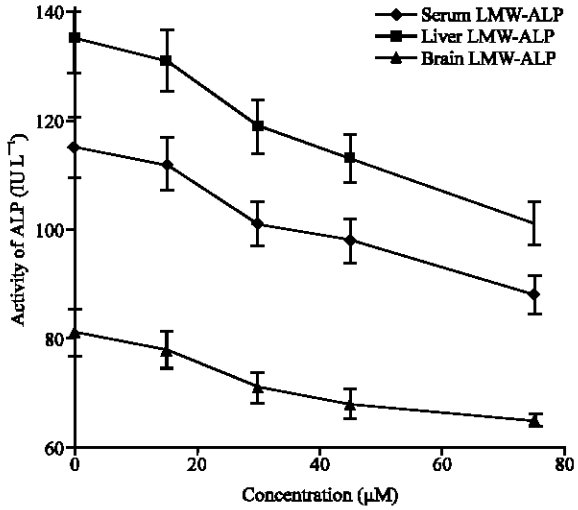

Fig. 4: Effects of lead on the activity of low molecular weight alkaline phosphatase isoenzymes. Activity of serum, liver and brain low molecular weight Alp was measured in the absence (as control) and presence of diverse concentration (15, 30, 45 and 75 µM) of Pb was measured. In each concentration, the activity was determined 4 times and the result was shown as mean±SD. LMW-ALP: Low Molecular Weight ALP

High and low molecular weight isoenzymes of alkaline phosphatase were incubated for 30, 60, 90 and 120 min with constant concentration of lead. It has been

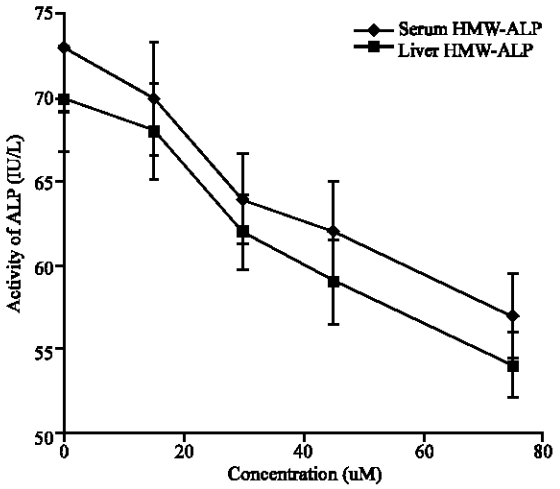

Fig. 5: Effects of lead on the activity of high molecular weight alkaline phosphatase isoenzymes. Activity of serum and liver high molecular weight ALP was measured in 0, 15, 30, 45 and 75 µM of Pb separately. Zero (0) concentration was considered as control. In each concentration, the activity was determined 4 times and the result was shown as Mean±SD. HMW-ALP: High Molecular weight ALP

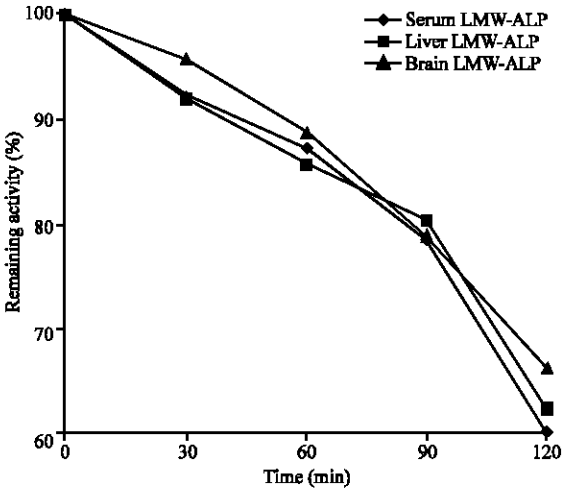

Fig. 6: Effects of incubation times on the activity of low molecular weight ALP at constant concentration of Pb. Serum, liver and brain low molecular weight alkaline phosphates isoenzymes were incubated with AMP buffer containing Pb (75 µM) for 30, 60, 90 and 120 min. The remaining activity of the isoenzymes was calculated at each time intervals. 0 time of incubation was considered as 100% of remaining activity

demonstrated (Fig. 6, 7) that by increasing the incubation time, the activity of high and low molecular weight

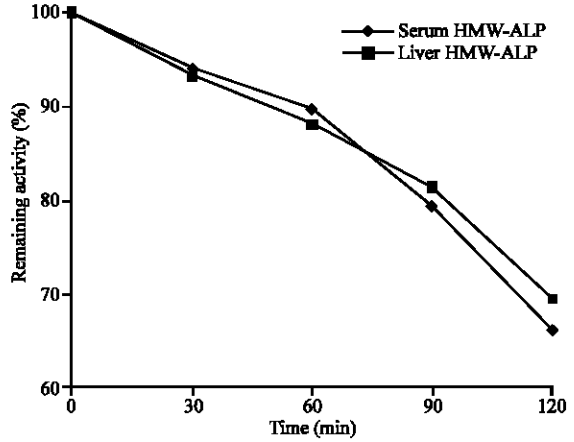

Fig. 7: Effects of incubation times on the activity of high molecular weight ALP at constant concentration of Pb. Serum and liver high molecular weight alkaline phosphates isoenzymes were incubated with AMP buffer containing Pb (75 μM) for 30, 60, 90 and 120 min. The remaining activity of the isoenzymes was calculated at each time intervals. 0 time of incubation was considered as 100% of remaining activity

isoenzymes of alkaline phosphatase were reduced, especially these changes were more obvious after 120 min of incubation ($p<0.05$).

DISCUSSION

Measurement of the activities of alkaline phosphatase isoenzymes has been used for the identification and monitoring of diseases associated with the isoenzymes. Biliary ALP or high molecular ALP has been found in the sera of patients with biliary obstruction and metastatic liver cancer (Bhudhisawasdi *et al*., 2004). Previous studies showed that high molecular weight ALP could be considered as a tumor marker for liver and colorectal cancers (Moshtaghie *et al*., 1996; Wei *et al*., 1993). This isoenzyme could be influenced by manganese and aluminium (Mirhashemi *et al*., 2009, 2010). However, up to our knowledge no data has presented in the literature concerning Pb toxicity and the induction of high molecular weight ALP in the serum of patients with Pb overload.

Results obtained from *in vivo* studies, revealed that short and long terms Pb administration to rat increased total serum ALP activity significantly ($p<0.05$), whereas, liver and brain total ALP activities decreased (Table 1). These changes were a dose and time dependent processes.

It has been also demonstrated that other enzymes such as acid phosphatase, ATPase and acetylcholine esterase were decreased in brain (Antonio *et al*., 2003; Antonio and Leret, 2000) and liver (Corpas *et al*., 2002), while the activity of alkaline phosphatase, aspartate aminotransferase, alanin aminotransferase and γ-glutamyltranspeptidase were increased in serum following lead toxicity (Shalan *et al*., 2005). Present findings agree with the above mentioned reports. Decreased in the activity of ALP in liver and brain may be due to decrease in hepatic RNA (Shalan *et al*., 2005), and/or inhibiting the synthesis of ALP activity by the replacement of zinc substitution, or indirectly decreasing zinc availability for ALP synthesis (Antonio *et al*., 2003). Also, Rahman *et al*. (2000) suggested that the decrease in the activities of alkaline phosphatase in different tissues might be due to the increased permeability of plasma membrane or cellular necrosis and this showing the stress condition of treated animals. When serum total ALP was fractionated, it showed 16 times elevation in the high molecular weight ALP activity in Pb treated group in comparison to control group (Fig. 1). Elevated high molecular weight ALP was also found in liver homogenate in the Pb exposed group (Fig. 2). The elevated high molecular weight ALP in serum may be originated either from the liver and/or other tissues producing this enzyme. This may also be due to either damage of bile duct and/or synthesis of new molecules of high molecular weight ALP. Alternatively association of low molecular weight ALP with other enzymes including 5'-nucleutidase, γ-glutamyltranspeptidase and nucleotide- pyrophosphates, could be resulted in the formation of high molecular weight ALP (Wulkan and Leijense, 1986; Remaley and Wilding, 1989).

Lead effect on high and low molecular weight alkaline phosphatase were examined *in vitro*. Partially purified serum, liver and brain high and, low molecular weight alkaline phosphatase fractions were prepared. It was not possible to highly purify the preparations, because large quantities of samples were not available, nor was this attempted. On the other hand, our aim was sufficiently to purify the isoenzymes that they could be separated from one another and from most of the contaminating proteins and allow kinetic experiments to be performed.

It has been shown that high and low molecular weight alkaline phosphatase fractions were inhibited by Pb. Obviously there are two classes of mammalian phosphatases, those which only need one type of element, Zn^{2+}, for their activities; they are represented by intestinal and placental alkaline phosphatase (Sussman, and Gottlieb, 1969; Harkness, 1968) and those which need both Zn^{2+} and Mg^{2+}, represented by kidney, liver, brain

and to lesser extent by bone alkaline phosphatase (Rosnblum *et al*., 1970; Brunel and Cathala, 1973). Inhibition of low and high molecular weight ALP by Pb may result from binding of Pb to the isoenzymes and changing in its native conformation and so Zn^{2+} replacement by Pb at its binding site.

In summary *in vivo* study demonstrated that lead could make pathophysiological damage to liver tissue, particularly bile ducts leading to the production and secretion of high molecular weight alkaline phosphatase. From *in vitro* experiments, it may be concluded that high and low molecular weight alkaline phosphatase from, serum, liver and brain were similar (where tested) with regard to mechanism of inhibition by lead.

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