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In vivo and *in vitro* Studies of Kinetic Changes of Serum, Liver and Brain High and Low Molecular Weight Alkaline Phosphatase Following Aluminium Exposure in Rat

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Abstract: Alkaline phosphatase is a family of ecto-phosphomonoesterases that can be resolved into two bands on polyacrylamide gel electrophoresis, a high-molecular mass form (Mr>1 000 000) and a low molecular mass form (Mr: 150000). The relationship between aluminium treatment and changes in the activity of serum, liver and brain high and low molecular weight alkaline phosphatase has been investigated in this manuscript. Results obtained from *in vivo* study showed that every other day intrapritoneally injection of 186 μmol kg^{-1} of aluminium ($AlCl_3.6H_2O$), in male rats for 2 weeks resulted in decreasing the level of liver and brain alkaline phosphatase by 14.9 and 9.9%, respectively, whereas an elevation of serum levels of this enzyme by 21.1% was seen in comparison to untreated controls ($p<0.05$). Long-term exposure of 74.5 μmol kg^{-1} of this salt, showed a statistically significant reduction in liver and brain level of alkaline phosphatase by 15.8 and 12.3%, respectively and an increment in serum activity of the enzyme by 30.9% in compared to control group ($p<0.05$). Gel filtration chromatography technique with sephacryl S_{300} showed that, in comparison to control groups, serum and liver homogenates from aluminium treated groups had a significant level of high molecular weight alkaline phosphatase. *In vitro* experiments showed that aluminium inhibited all the isoenzymes non-competitively. Low molecular weight alkaline phosphatases were more heat and urea stable than high molecular weight fractions.

Key words: Aluminium, alkaline phosphatase, high molecular weight alkaline phosphatase, liver, brain

INTRODUCTION

Aluminium (Al) is an element with extensive use in almost all modern industries as well as daily household life. The sources of Al have been reported to be corn, yellow cheese, salt, herbs, spices, tea, cosmetics and utensils. It is also present in medicines and is added to drinking water for purification (Ochmanski and Barabas, 2000).

This metal has toxic effects on many tissues, particularly liver, bone, kidney and blood (Oteiza *et al*., 1993). Impairments of glucose utilization, free radical- mediated cytotoxicity,

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lipid peroxidation, reduced cholinergic function, impact on gene expression and altered protein phosphorylation has been reported following Al toxicity (Strong *et al*., 1996). Meanwhile, Al has also been considered as a toxic environmental factor that may contribute to some neurodegenerative disease such as Alzheimer's disease (Nayak and Chatterjee, 2001). 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 (1972) for the first time using electrophoresis of human serum on cellulose acetate, detected a specific type of ALP and named it high molecular weight fraction of ALP. In addition, various other methods were then used by other investigators to study and characterized this isoenzymes and they named it: fast liver ALP (Wulkan and Leijense, 1986) biliary ALP (Crofton *et al*., 1979) and high molecular mass band ALP (Remaley and Wilding, 1989).

High molecular weight alkaline phosphatase have now been reported in patients with extra-or intra-hepatic cholestasis, malignancy of liver, primary or metastatic carcinoma, Hodgkin's and non-Hodgkin's lymphoma a nd/or leukemia (Wolf, 1990; Bhudhisawasdi *et al*., 2004). This kind of isoenzyme has been suggested as a tumor marker for liver (Moshtaghie *et al*., 1996a) and Colorectal Cancer (Wei *et al*., 1993). Data from this laboratory showed that high molecular weight alkaline phosphatase might be influenced by manganese (Mirhashemi *et al*., 2009). Recent years, it has been reported that aluminium from dialysis fluid enters blood circulation during haemodialysis and bound to serum transferrin (Fenswick *et al*., 2005) and might be accumulated in the liver and may lead to disturbances of this tissue (Moshtaghie *et al*., 1996b). The major aim of this study was to investigate the kinetic behaviors of high- and low molecular weight ALP in male rats exposed to Al *in vivo* and *in vitro*.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Company. This research project was conducted from 1/11/2006 to 1/2/2009.

Twenty eight male Wistar rats (approximate weight 200-220 g) were purchased from Pasteur Institute (Tehran-Iran) and kept in university animal house at standard conditions (22-24°C, 40-60% relative humidity and light cycle coinciding with day-light h) and fed with standard rat food and water *ad libitum* during the entire experimental period. Rats were divided randomly into two groups named: short-term and long-term exposure to Al, 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 weeks, simultaneously treated group was administrated with 186 μmol kg^{-1} of Al ($AlCl_3.6H_2O$) as the same way as controls. Long-term study was carried out using 74.5 μmol 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. Liver tissue was immediately removed, washed with cold (+4°C) saline solution and homogenized (10% w/v) in a buffer solution containing 10 mM tris and 0.25M sucrose, pH:7.4, at +4°C. The homogenates were centrifuged at 13000 g for 20 min at +4°C and the resultant supernatants were carefully removed and 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, with bovine serum albumin as standard (Bradford, 1976).

Gel Filtration Chromatography

In order to separate high and low molecular weight isoenzymes of ALP, gel filtration chromatography on sephacryl S_{300} was used. Sample (0.5 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). The 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. Heat and urea effect on different forms of ALP was carried out at various times interval. The partially purified ALP isoenzymes were poured into glass tubes and were then incubated at 56°C in water bath for 10, 20 and 30 min. The tube were then removed at different time intervals and immediately cooled in ice. Activities of heated enzyme samples were then assayed along with the unheated control samples and the remaining activity of isoenzymes were calculated (Whitby and Moss, 1975). Effects of urea on the isoenzyme activities were examined by incubating the isoenzyme preparations in the presence of urea (3 M) at 37°C. At various time intervals of the incubation, portions of the isoenzyme-urea mixture were withdrawn and their activities were determined and compared with the control samples (El-Demrdash, 2004).

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 AND DISCUSSION

First series of experiments were established to investigate short and long term effects of Al on total serum, liver and brain ALP activities. It was found that administration of Al (186 μmol kg^{-1}) every other day for 2 weeks lead to the elevation of serum total ALP activity by 21% in comparison to normal health controls. Significant reduction in the liver total ALP activities by 14.9% was seen when rats were treated with same amount of Al every other day for 2 weeks (Table 1). Elevation of 30.9% in total serum ALP and reduction of 15.8 and 12.3% in liver and brain ALP was seen following every other day i.p. administration of Al for 7 weeks (Table 1). Gel filtration chromatography experiments were established to separate high and low molecular weight ALP from sera, liver and/or brain homogenates of both treated and untreated animals. To do this gel filtration chromatography technique was used. Fractionation of serum from aluminium treated animal show that the elevation in the serum total ALP activity was mostly related to the high molecular weight ALP (Fig. 1). When liver homogenate from both control and aluminium treated rat was chromatographed, it was found that there was significant reduction by 28.9% in the concentration of low molecular weight ALP and elevation in high molecular weight alkaline

Table 1: Effect of Al on the activity of serum, liver and brain ALP specific 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	2.35±0.13*	2.91±0.20*	1.82±0.11*
B			
Control	2.10±0.10	3.16±0.26	2.27±0.13
Treated	2.75±0.08*	2.60±0.17*	1.99±0.15*

Rats were injected with Al as ($AlCl_3.6H_2o$) 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 Al treated animals and their controls ($p<0.05$). Data are presented as Mean±SD

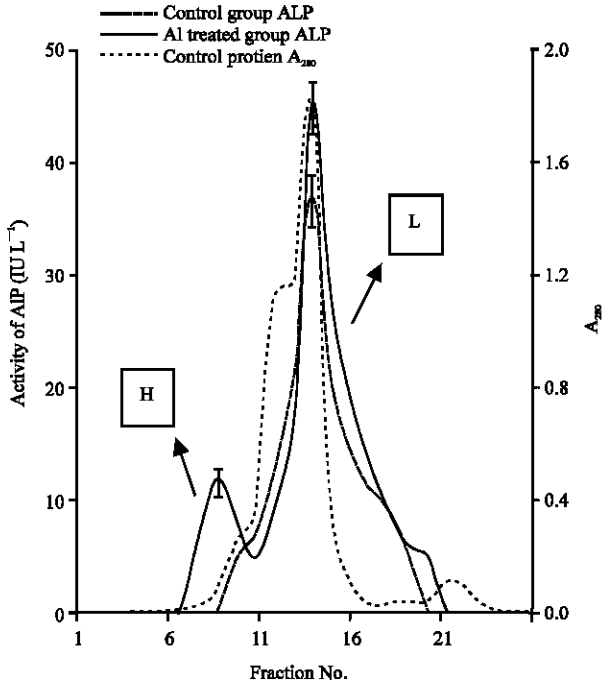

Fig. 1: The elution profile of serum of control and Al treated groups. Serum was diluted with buffer and loaded on the top of the column containing sephacryl S_{300} and eluted with buffer(tris-HCl, 50 mM, pH 7.4) at 10 mL h^{-1} rate. ALP activity of fraction and A_{280} were measured High molecular weight ALP was increased significantly ($p<0.05$) in Al treated group when compared with controls

phosphatase (Fig. 2). Figure 3 shows that significant reduction of low molecular weight by 33.3% in brain ALP following Al treatment and no changes in high molecular weight ALP was seen.

For *in vitro* experiments, separated high and low molecular fractions of alkaline phosphatase were purified using ammonium sulfate, DEAE-cellulose and column chromatography. Our data represented that 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, respectively. The effect various concentrations of aluminium on the activity of high and low molecular forms of alkaline

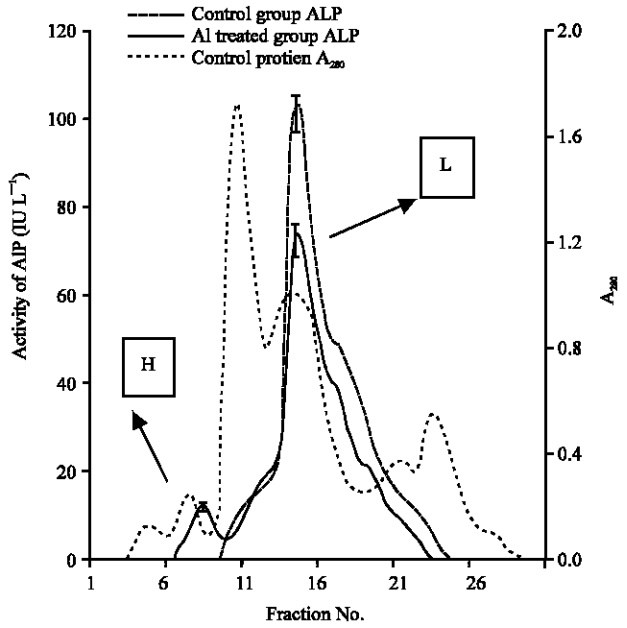

Fig. 2: The elution profile of liver of control and Al treated groups. Liver from Al and untreated animals were homogenate as mentioned earlier and loaded on the top of column and eluted as mentioned with method

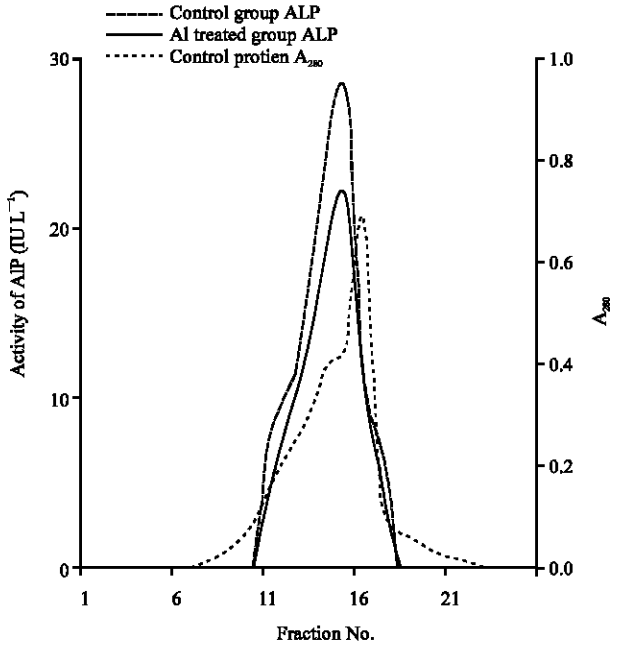

Fig. 3: The elution profile of brain of control and Al treated groups. Brain from Al and untreated animals were homogenate as mentioned earlier and loaded on the top of column and eluted as mentioned with method

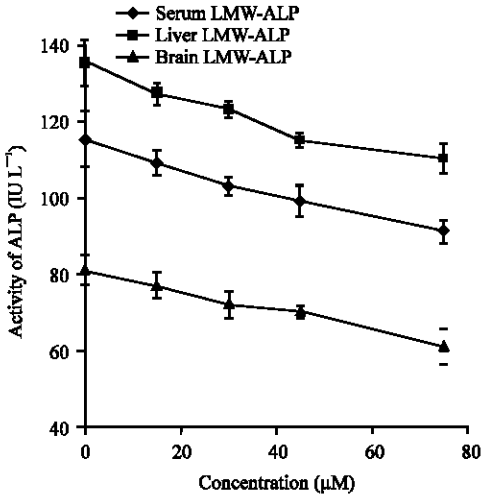

Fig. 4: Aluminium effects on the activity of Low molecular weight alkaline phosphatase isoenzymes Different concentration (15, 30, 45 and 75 µM) of Al was used. Zero concentration of this element was considered as control In each concentration of Al, the activity of serum, liver and brain low molecular ALP was determined 4 times and the result was shown as Mean±SD. LMW-ALP: Low molecular weight ALP HMW-ALP: High molecular weight ALP

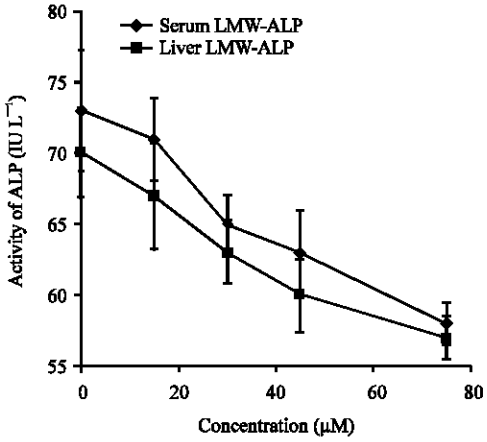

Fig. 5: Aluminium effects on the activity of high molecular weight alkaline phosphatase isoenzymes. Al was used with various concentrations (15, 30, 45 and 75 µM). Activity of serum and liver high molecular weight ALP was measured in the absence of Al and considered as control in each concentration, the activity was determined 4 times and the result was shown as Mean±SD LMW-ALP: Low molecular weight ALP HMW-ALP: High molecular weight ALP

phosphatase were investigated (Fig. 4, 5). These findings showed that aluminium with concentration of 75 µM significantly ($p<0.05$) decreased the activity of serum, liver and brain low molecular weight alkaline phosphatase by 20.9, 18.5 and 24.7% and serum and liver high

molecular weight isoformes by 19.2 and 17.1%, respectively. The K_m and V_{max} values for each type of the enzyme was calculated in the presence and absence of aluminium (75 μM) and amino acids (10 mM) based on Lineweaver-Burk plot (Table 2).

Heat and urea denaturation of different forms of ALP was carried out. The results showed that serum and liver high molecular weight forms were more stable than their low molecular forms and so the serum high molecular weight ALP is more resistant than liver high molecular form to heat denaturation (Fig. 6). Just as the Fig. 7 shows the effect of urea on the high and low molecular activities were similar to those obtained from heat denaturation study.

Measurement of the activity 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

Table 2: Effects of aluminium (75 μM) and amino acids (10 mM) on high and low molecular ALP

	Serum HMW-ALP	Liver HMW-ALP	Serum LMW-ALP	Liver LMW-ALP	Brain LMW-ALP
Control	7.70	5.70	24.40	32.30	10.90
	0.31	0.24	0.20	0.16	0.11
Al	4.76	3.70	19.60	25.64	8.00
	0.31	0.24	0.20	0.16	0.11
Leucine	6.90	5.10	22.70	29.10	9.30
	0.31	0.24	0.20	0.16	0.11
Cysteine	7.10	4.80	23.10	31.20	8.90
	0.31	0.24	0.20	0.16	0.11
Phenylalanine	6.50	5.20	20.10	29.70	10.10
	0.26	0.21	0.15	0.11	0.08

Each cell of the table has 2 numbers, that represents V_{max} and K_m as following manner, V_{max}, K_m, V_{max} and K_m were expressed as μmol/min/mg of protein and mM, respectively

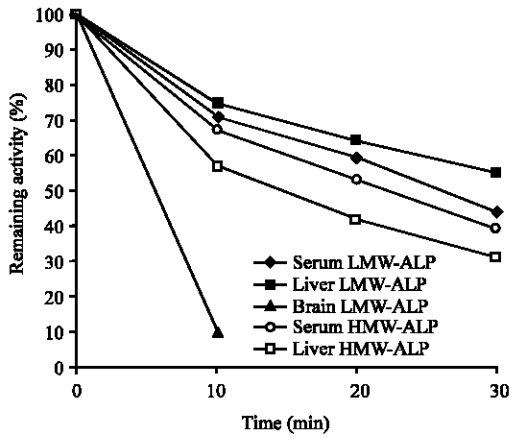

Fig. 6: Effect of heat denaturation on high and low molecular weight ALP. Serum, liver and brain high and low molecular weight ALPs were incubated at 56°C in water bath for 10, 20 and 30 min. Serum and liver high molecular weight ALP isoenzymes were more labile than serum and liver low molecular weight ALPs brain low molecular weight ALP was denatured more rapid than serum and liver high and low molecular weight isoenzymes. LMW-ALP: Low molecular weight ALP HMW-ALP: High molecular weight ALP

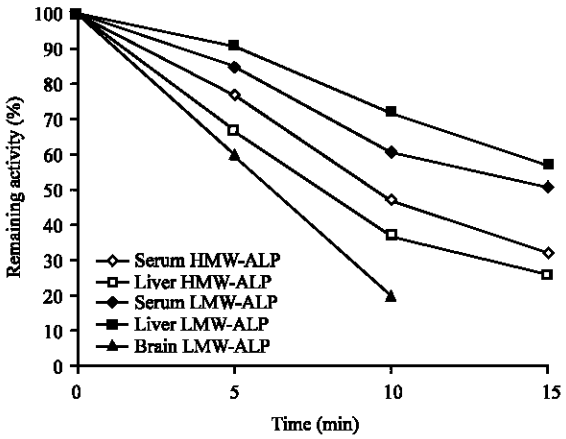

Fig. 7: Effect of urea on high and low molecular weight isoenzymes of ALP. The isoenzymes of alkaline phosphatase were incubated at 37°C for various time intervals (10, 20 and 30 min) by urea 3 M. Serum and liver high molecular weight ALP isoenzymes were more labile than serum and liver low molecular weight ALPs. Brain low molecular weight ALP was denatured at the first. LMW-ALP: Low molecular weight ALP HMW-ALP: High molecular weight ALP

weight ALP could be considered as a tumor marker or liver and colorectal cancers (Moshtaghie *et al*., 1996a, b; Wei *et al*., 1993). This isoenzyme could be influenced by manganese (Mirhashemi *et al*., 2009). However, to our knowledge no data has been presented in the literature concerning aluminium toxicity and the induction of high molecular weight alkaline phosphatase in sera of patients with aluminium overload. Results obtained from *in vivo* studies, revealed the relationship between aluminium toxicity and changes in the sera, liver and/or brain high and low molecular weight alkaline phosphates. Present findings showed that following short and long terms of aluminium administration to rats, total alkaline phosphatase activity in serum increased significantly ($p<0.05$), whereas liver and brain total alkaline phosphatase activities decreased ($p<0.05$). These changes were dose and time dependent processes. El-Demrdash (2004) demonstrated that other enzymes such as aspartate aminotransferase, alanine aminotransferase, were significantly decreased in the liver, while the activities of these enzymes increased in serum following aluminium treatment. Decreased activity of alkaline phosphatase may be due to binding of aluminium to DNA and RNA and inhibiting low molecular fraction of the enzyme (Ochmanski and Barabas, 2000). Yosef (2004) also showed that aluminium administration to rat reduced total activity of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase. Chronic aluminium exposure leads to oxidative stress, cholestasis and impairment of hepatic handling of organic anions by decreasing both sinusoidal uptake and canalicular excretion (Gonzalez *et al*., 2004). Also, 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 (Rahman *et al*., 2000). When serum total alkaline phosphatase activity was fractionated, it showed that, elevation of serum ALP activity was mostly due to the high molecular weight alkaline phosphatase in aluminium treated group in comparison to control group. Elevated high molecular weight alkaline phosphatase was also found in liver homogenates in aluminium exposed group, but there was no high molecular weight ALP in brain

homogenates. The elevated high molecular weight alkaline phosphatase in serum may originate from the liver and/or other tissues producing this enzyme. This may also be due to either damage to bile duct and/or synthesis of new high molecular weight alkaline phosphatase. Alternatively association of low molecular weight ALP with other enzymes including 5'-nucleotidase, γ-glutamyltranspeptidase and nucleotide-pyrophosphatase, could lead to in the formation of high molecular weight alkaline phosphatase (Remaley and Wilding, 1989; Wulkan and Leijense, 1986). Kinetic behaviors of 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 aluminum. 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 (Harkness, 1968; Sussman and Gottlieb, 1969) and those which need both Zn^{2+} and Mg^{2+}, represented by kidney, liver, brain and to lesser extent by bone alkaline phosphatase (Brunel and Cathala, 1973; Rosnblum *et al*., 1970). Inhibition of low and high molecular weight ALP by aluminium may result from binding of aluminium to the isoenzymes and changing in its native conformation. The experimental data demonstrated that the inhibition of low and high molecular weight alkaline phosphatase by phenylalanine is uncompetitive, whereas leucine and cysteine inhibits these isoenzymes non-competitively (Table 2). The inhibitory effects of amino acids on high and low molecular weight alkaline phosphatase isoenzymes may be considered as a result of either the formation of a poorly dissociable isoenzyme-inhibitor-substrate complex, or the inhibitor prevented the breakdown of the subsequently formed phosphoryl-enzyme intermediate rather than acting on the enzyme-substrate complex, however, the conversion of the enzyme-substrate-inhibitor complex into the phosphoryl-enzyme-inhibitor complex may represent the blocked step in the hydrolysis (Frenley and Walker, 1970; Ghosh and Fishman, 1966).

CONCLUSION

In conclusion *in vivo* study demonstrated that, aluminium could make pathophysiological damage to liver tissue, particularly bile ducts leading to the production and secretion of high molecular weight alkaline phosphatase and the appearance of this isoenzyme in the sera could be considered as a suitable tool in the diagnosis of toxicity of the exposed elements. 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 aluminium and/or amino acids such as phenylalanine. These isoenzymes were inhibited by heat and urea but serum and liver high molecular fractions were more labile than the serum and liver low molecular forms and the brain low molecular fraction was the most labile to heat denaturation tests.

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