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# ON THE RELATION OF HEAVY METALS TO THE ACTIVITY AND HEAT STABILITY OF ALKALINE PHOSPHATASE FROM HUMAN PLACENTA

F. R. HINDRIKS[a], A. GROEN[b] AND A. M. KROON[b]

[a]*Laboratory of Clinical Chemistry, University Hospital and*[b] *Laboratory of Physiological Chemistry,* State University, Groningen (The Netherlands)*

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SUMMARY

It is well established that human placentae contain high amounts of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) and that the rise in alkaline phosphatase activity in serum during pregnancy is to be ascribed to the presence of this placental enzyme. The placental isoenzyme is characterized by its stability at temperatures up to 65 °C. The enzyme kinetics of alkaline phosphatase extracted from human placentae and its sensitivity to various inhibitors were studied and a search for the divalent cation requirements with respect to activity and heat stability was undertaken. The activity of placental alkaline phosphatase could be abolished by treatment with EDTA. From reactivation studies with different divalent cations it appears that $Zn^{2+}$ or $Mg^{2+}$ are necessary for both the activity and the resistance to heat treatment. $Hg^{2+}$ can replace either of the two former cations in both aspects, albeit to a lesser extent.

## INTRODUCTION

It has already been known for a long time that the activity of alkaline phosphatase in serum of pregnant women rises as pregnancy proceeds[1,2]. The elevated activity is significant from the 20th week of amenorrhoea. Beck and Clark[3] were the first who concluded that this rise in alkaline phosphatase activity was due to a liberation of placental phosphatase into the maternal circulation. These authors based their conclusions on their observation that alkaline phosphatase in serum during pregnancy was fairly resistant to inhibition by sodium taurocholate, isoenzymes from other sources (liver, bone) being much more sensitive to this inhibitor. However, the intestinal isoenzyme appeared to be quite resistant too. More recently, Neale *et al.*[4] offered a better differential criterium to distinguish between the placental enzyme and alkaline phosphatases from other sources. They showed that the activity of the

* Postal address: Bloemsingel 10, Groningen, The Netherlands.

placental enzyme withstands heating at 56 °C for 30 min, whereas it was already known from the work of Moss and King[5] that the alkaline phosphatase activity in extracts from bone, intestinal tissue, kidney and liver is abolished by such a heat treatment. This heat stability was shown to hold likewise for the extra activity in the serum during pregnancy by Zuckerman and Sadovsky[6]. Since this work, these observations have been confirmed by many others[7–9] and the estimation of heat-stable alkaline phosphatase activity has been introduced in clinical chemistry as a means of measuring placental function in the course of pregnancy[10]. We were interested in exploring the basis for the heat stability of the placental enzyme in more detail. In this paper experiments regarding the divalent cation requirements of the placental enzyme will be described in relation to its enzymatic activity and its stability at high temperature.

## MATERIALS

### *Human placentae*

Human placentae were obtained from vaginal deliveries after uncomplicated pregnancies. After removing maternal and fetal blood as much as possible, material from the paracentral part of each placenta was freed from major strands of connective tissue, cut into pieces and either used for extraction directly or stored at −20 °C.

### *Blood*

Blood samples were drawn from children with osteoblastic hyperfunction, from men suffering from liver diseases accompanied with elevated liver alkaline phosphatase activity in the serum and from pregnant women. For some experiments serum from different samples of each category was pooled. Serum was separated from the cellular elements within 2 h after collection of the blood and stored at −20 °C until use.

## METHODS

### *Extraction procedure*

Placental tissue (6 g) was immersed in 12 ml cold 40 mM Tris–HCl buffer (pH 8.0), containing 0.15 M NaCl. All subsequent steps were at 0–4 °C. The sample was cut into small pieces and homogenized in a Virtis homogenizer (The Virtis Co., Gardener, N.Y.). The homogenate was centrifuged at 1500 × *g* for 15 min; centrifugation was repeated twice using the supernatant of the former run. The final supernatant was used for further experiments directly or stored at −20 °C.

### *Determination of alkaline phosphatase activity*

Alkaline phosphatase activity was determined by the modification by Bowers and McComb[11] of the method of Bessey *at al.*[12]. The samples to be assayed for enzyme activity were incubated for 15 min at 37 °C in a total volume of 1.1 ml, containing 230 mM 2-methyl-2-amino-1-propanol, 30 mM HCl and 8.3 mM disodium 4-nitrophenylphosphate and either 0.1 ml serum or 20–40 μg of protein extract. The final pH was 10.7. The reaction was stopped with 10 ml 0.02 M NaOH. The absorbance at 410 nm of the 4-nitrophenol produced was measured in a Beckman DU-2 spectro-

photometer. Under these experimental conditions the reaction is linear with time for up to 30 min, and with an enzyme concentration up to 2–4-fold of that of the activity normally present.

*Heat stability of alkaline phosphatase*

For estimation of the heat-stable activity of alkaline phosphatase the enzyme samples were kept at 56 °C for 30 min either at pH 8 (sera), or at pH 10 (placental extracts).

*Dialysis*

Dialysis was carried out as follows: placental extract in 40 mM Tris–HCl buffer, containing 0.15 M NaCl (pH 8.0) was diluted with 40 mM 2-methyl-2-amino-1-propanol–HCl buffer (pH 10) until the alkaline phosphatase activity corresponded with about 1.2 $\mu$moles substrate converted per min per ml. The diluted extract was centrifuged once at 1200 $\times$ $g$ for 10 min after heating at 56 °C for 30 min. Three ml of the supernatant was incubated with 18 ml 100 mM EDTA in 40 mM 2-methyl-2-amino-1-propanol–HCl buffer (pH 10) at 37 °C for 15 min and, thereafter, dialysed at 4 °C for 48 h against 800 ml 40 mM 2-methyl-2-amino-propanol–HCl buffer (pH 10). After 12 and 30 h the dialysis buffer was replaced with 500 ml fresh buffer. After 48 h all alkaline phosphatase activity in the EDTA-treated extract had disappeared. A control dialysis was run through the same procedure with the addition of 18 ml 40 mM 2-methyl-2-amino-1-propanol–HCl buffer, not containing EDTA.

*Reactivation experiments*

A sample of the EDTA-treated placental extract was incubated for 10 min at 37 °C in a total volume of 0.9 ml containing 278 mM 2-methyl-2-amino-1-propanol, 36 mM HCl and either $ZnCl_2$, $MgCl_2$ or $HgCl_2$ in a concentration range from $10^{-3}$ to $10^{-8}$ M (pH 10.7). The enzymatic reaction was started with 0.2 ml 45.6 mM 4-nitrophenyl phosphate. After incubation for 15 min at 37 °C, the reaction was stopped with 10 ml of 0.02 M NaOH. The absorbance at 410 nm of the 4-nitrophenol produced was measured as described above.

*Protein*

Protein was determined by the method of Cleland and Slater[13] using a serum with a known protein concentration as the standard.

## RESULTS AND DISCUSSION

*Heat stability and some enzymological properties of alkaline phosphatase in extracts of human placentae*

To ensure that the enzyme activity of the alkaline phosphatase in the placental extracts used was indeed heat stable and to allow a critical choice of experimental conditions, the stability of the enzyme at 56 °C was determined as a function of the time of heat treatment. The results are given in Fig. 1. It can be seen that the enzyme in the placental extract was not inactivated during the first hour of incubation at 56 °C. Even after a 24-h treatment of the extract at the elevated temperature, more than 70% of the phosphatase activity was retained. For comparison, different sera

having alkaline phosphatase activity were included as controls. The serum of men, containing alkaline phosphatase derived mainly from the liver and the serum of children, containing mainly the osteoblastic isoenzyme, are inactivated by about 90% during a 30 min incubation at 56 °C. The activity in the serum of pregnant women showed a rapid fall to about 70% in the first 50 min of incubation and only a very slow further inactivation. These observations are fully consistent with those of others[10,14,15] and show in the first place that the activity in the placental extracts used is indeed to be ascribed completely to the heat-stable enzyme and, furthermore, that under the conditions of the heat treatment chosen for our experiments (30 min; 56 °C) at least two other isoenzymes of alkaline phosphatase are fully inactivated.

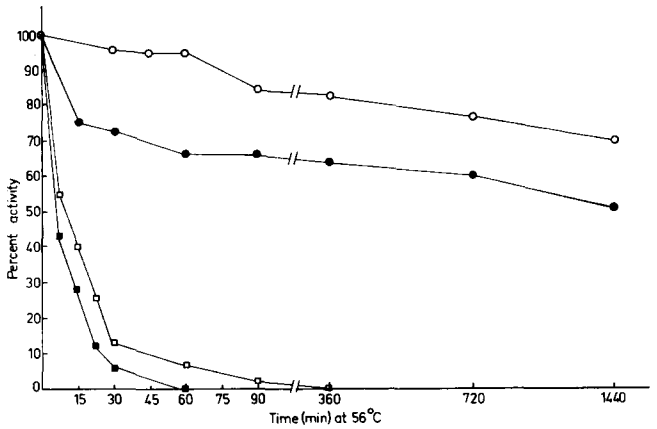


Fig. 1. Heat stability of alkaline phosphatase from different origins as a function of the treatment of the enzyme at 56 °C. ○—○, placental extract; ●—●, serum during pregnancy; □—□, serum of men suffering from liver disease; ■—■, serum of children with osteoblastic hyperfunction. Further conditions are given in Methods.

It should be mentioned that some authors prefer a treatment at 65 °C because, as they argue, nonplacental phosphatase activity is not completely inactivated during a treatment for 30 min at 56 °C (refs 16–18). With our experiments, treatment of the placental enzyme at temperatures of 65 °C and higher leads to a slow inactivation. Contamination with isoenzymes other than the heat-stable phosphatase was not a serious problem since our experiments were not performed with serum but with placental extracts.

The properties of the enzyme in the placental extract were further studied by investigating the sensitivity to inhibition by L-phenylalanine, inorganic phosphate and EDTA. The results are given in the Lineweaver–Burk plots of Fig. 2. The $K_m$ for 4-nitrophenyl phosphate of the enzyme in the extracts was 1.3 mM. The inhibition by L-phenylalanine was uncompetitive in nature and the $K_i$ was calculated to be about 6 mM. Inorganic phosphate showed competitive inhibition with a $K_i$ of 0.5 mM, whereas the pattern of inhibition by EDTA was clearly noncompetitive ($K_i$ = 6 mM). This spectrum of inhibitions of the placental isoenzyme is similar to that of the intestinal isoenzyme[19] but clearly different from that of the osteoblastic and liver isoenzymes because the latter two are resistant to inhibition by L-phenylalanine[20]. The only obvious difference between the intestinal and placental phosphatase remains,

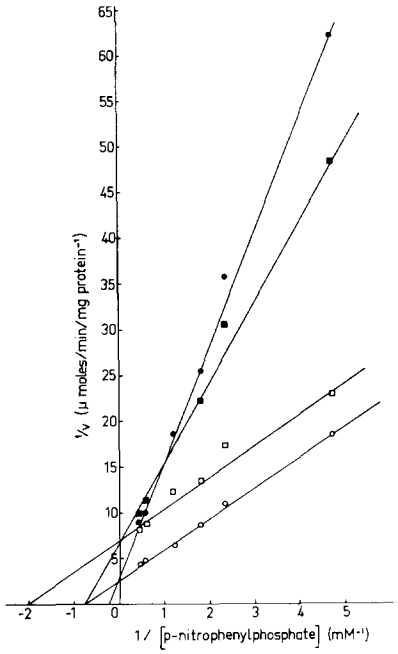


Fig. 2. Lineweaver–Burk plot of the activity of alkaline phosphatase in placental extracts as a function of the concentration of 4-nitrophenyl phosphate in the presence and absence of various inhibitors. After preincubation with the inhibitor for 5 min at pH 10.7, the alkaline phosphatase activity was measured as described under Methods. The reaction velocity ($v$) was expressed in $\mu$moles/min per mg extracted protein. ○—○, uninhibited; ●—●, 1.5 mM disodium hydrogenphosphate; ■—■, 10 mM EDTA; □—□, 4 mM L-phenylalanine.

therefore, the difference in heat stability between the two. These observations confirm and extend similar results from other laboratories[9,21,22].

*The divalent cation requirements of alkaline phosphatase in placental extracts*

In view of the strong inhibition of alkaline phosphatase activity by EDTA it may be assumed that the activity of the placental alkaline phosphatase is dependent on the presence of divalent cations, just as with the alkaline phosphatases of other mammalian[23] and bacterial origins[24,25]. Conyers *et al.*[26] also investigated the action of EDTA on human alkaline phosphatase from bone, intestine and placenta. Preincubation of the enzymes with EDTA resulted in an inactivation which was dependent on time, temperature and pH. Since extensive dialysis of the placental extract does not alter the activity of the alkaline phosphatase, it may be concluded that these divalent cations are quite strongly bound.[27] This is completely in line with the observations that purified alkaline phosphatase from different sources contains $Zn^{2+}$ (refs 25, 28–31). The possibilities of reactivating alkaline phosphatase after treatment with EDTA by divalent cations have been investigated by different authors. For mammalian phosphatases these experiments have met with moderate success only. Smith *et al.*[32] were unable to reactivate alkaline phosphatase isoenzymes from different tissues recovered from polyacrylamide gels after these enzymes had been treated with EDTA. Harkness[27] obtained a maximal reactivation of 40% in the pres-

ence of $10^{-3}$ M $Zn^{2+}$ after dialysis of purified placental alkaline phosphatase against a buffer containing 0.05 M acetate and 5 mM EDTA (pH 5.0). The latter study, however, provides only scanty information about recovery of the enzyme and of total protein. Furthermore, one may argue that $Zn^{2+}$ in a concentration of $10^{-3}$ M is extraordinarily high and most likely not reflecting physiological conditions. We decided, therefore, to search for better conditions for inactivation and reactivation.

We altered the procedure of dialysis in the following way. EDTA was added to the placental extract to a final concentration of 0.1 M, the pH was kept at 10 and the buffer used was methylaminopropanol. This extract was dialysed against excess 40 mM methylaminopropanol buffer, pH 10 (see Methods) to get rid of the divalent cations by chelation to the EDTA, and of the EDTA chelates by the extensive dilution during dialysis. The obvious advantage of this procedure should be that the EDTA content of the placental extract after dialysis was negligible. The results of a series of experiments are given in Table I. It can be seen that an EDTA treatment as described above abolishes the alkaline phosphatase activity of the extracts completely. Similar treatment of the extracts, except for the omission of the EDTA during dialysis, led to complete recovery of the alkaline phosphatase activity. The

TABLE I

RECOVERY AND HEAT STABILITY OF ALKALINE PHOSPHATASE IN PLACENTA EXTRACT AFTER DIALYSIS
Percentages are related to the total recovered activity of the enzyme. Means ± S.E. are given.

| *Treatment of extract* | *Number of experiments* | *Spec. act. (μmoles/min per mg extracted protein)* | *Recovery (%)* | *Heat stability (%)* |
|---|---|---|---|---|
| Extract before dialysis | 10 | 0.26 ± 0.01 | 100 | 96 ± 1 |
| EDTA-treated extract after dialysis | 10 | 0.0031 ± 0.0003 | 1 ± 0.03 | — |
| Extract after control dialysis | 10 | 0.32 ± 0.02 | 98 ± 1 | 98 ± 1 |

specific activity became even somewhat higher, for reasons not completely understood as yet. Apparently some low molecular weight material interfering with the protein determination was present in the original extracts during dialysis. It can further be seen that the treatment without EDTA had no effect whatsoever on the heat stability of the alkaline phosphatase in the extracts.

In an attempt to assess the divalent cation requirements of the placental alkaline phosphatase, reactivation studies were performed. In Fig. 3 the results of a series of reactivation experiments with 7 different divalent cations are shown. Within the concentration range of $10^{-3}$–$10^{-6}$ M, $Co^{2+}$, $Fe^{2+}$, $Mn^{2+}$ and $Sn^{2+}$ were unable to restore the activity of alkaline phosphatase in the EDTA-treated extracts. $Zn^{2+}$, $Mg^{2+}$ and also $Hg^{2+}$ were able to reactivate the enzyme to a considerable degree; in some instances with $Zn^{2+}$ and $Mg^{2+}$ complete reactivation was obtained. With $Hg^{2+}$ the extent of reactivation never exceeded 70% of the values in control extracts. Optimal reactivation was obtained at $2 \cdot 10^{-5}$–$5 \cdot 10^{-5}$ M for $Zn^{2+}$, between $5 \cdot 10^{-4}$ and $5 \cdot 10^{-5}$ M in the case of $Mg^{2+}$ and at about $5 \cdot 10^{-5}$ M for $Hg^{2+}$. Combinations of $Zn^{2+}$ and $Mg^{2+}$ at a lower concentration than that necessary for optimal reactivation by either of

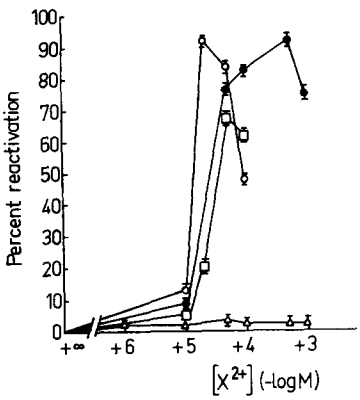


Fig. 3. The reactivation by divalent cations of placental alkaline phosphatase in extracts after extensive dialysis against EDTA. All experimental details are given in Methods. Reactivation is expressed as % of the phosphatase activity of control extracts treated in the same way but with the omission of EDTA during dialysis. 100% varies from 0.21 to 0.36 μmoles/min per mg extracted protein in the different experiments. Means ± S.E. are given; the number of experiments was 8 for $Zn^{2+}$, $Mg^{2+}$ and $Hg^{2+}$ and 2–3 for the other ions. ○—○, $Zn^{2+}$; ●—●, $Mg^{2+}$; □—□, $Hg^{2+}$; △—△, $Co^{2+}$, $Fe^{2+}$, $Mn^{2+}$, $Sn^{2+}$.

these ions, never led to more than an additive effect. Apparently $Zn^{2+}$ and $Mg^{2+}$ can replace each other at their site(s) on the enzyme from which they have been removed by the EDTA treatment. It should be stressed that this observation does not necessarily mean that all $Zn^{2+}$ can be replaced by $Mg^{2+}$ and *vice versa* because it has

TABLE II

HEAT STABILITY OF ALKALINE PHOSPHATASE IN PLACENTA EXTRACT AFTER DIALYSIS BEFORE AND AFTER REACTIVATION BY DIVALENT CATIONS

Percentages are related to the total activity of the enzyme recovered.

| *Treatment of extract* | *Activity* | *Additions for reactivation* | | | | | |
|---|---|---|---|---|---|---|---|
| | | $5 \cdot 10^{-5}$ *M* $Zn^{2+}$ | | $5 \cdot 10^{-5}$ *M* $Mg^{2+}$ | | $2 \cdot 10^{-5}$ *M* $Hg^{2+}$ | |
| | | *mean* ± *S.E.* | *n* | *mean* ± *S.E.* | *n* | *mean* ± *S.E.* | *n* |
| Dialysis with EDTA | Alkaline phosphatase activity (μmoles/min per mg protein extracted) | 0.27 ± 0.02 | 9 | 0.28 ± 0.02 | 7 | 0.22 ± 0.02 | 4 |
| | Recovery of alkaline phosphatase activity (% of control dialysis) | 84 ± 3 | 9 | 88 ± 4 | 7 | 68 ± 5 | 4 |
| Dialysis with EDTA and heat treatment before addition of the divalent cations | Alkaline phosphatase activity (μmoles/min per mg protein extracted) | 0.008 ± 0.001 | 4 | 0.008 ± 0.001 | | 0.007 ± 0.001 | 2 |
| | Heat stability (% of control dialysis) | 2 ± 1 | 4 | 2 ± 1 | 2 | 2 ± 1 | 2 |
| Dialysis with EDTA and heat treatment after addition of the divalent cations | Alkaline phosphatase activity (μmoles/min per mg protein extracted) | 0.25 ± 0.03 | 5 | 0.26 ± 0.03 | 5 | 0.21 ± 0.06 | 2 |
| | Heat stability (% of control dialysis) | 81 ± 3 | 5 | 80 ± 3 | 5 | 64 ± 2 | 2 |

not been shown as yet that the EDTA treatment removes all of the metal ions present on the enzyme. Our observations are different from those of Harkness[27], who obtained moderate reactivation by $Co^{2+}$. The reason for this discrepancy remains unknown.

Having conditions available for complete reactivation of the alkaline phosphatase, we could examine whether the heat stability of the "apoenzyme"* is retained during the treatment with EDTA. It can be seen in Table II that this is not the case. Heating of the dialysed extract for 30 min at 56 °C altered the enzyme in such a way that reactivation by the addition of $Zn^{2+}$, $Mg^{2+}$ or $Hg^{2+}$ became impossible. If, however, the divalent cations were added to the dialysed extracts before heating for 30 min at 56 °C, reactivation by the divalent cations occurred to the same extent as in the corresponding untreated extracts. With regard to inactivation and reactivation and to the loss of heat stability after EDTA treatment, the fraction of the alkaline phosphatase in serum during pregnancy that is heat stable behaved exactly the same. These results have led us to the conclusion that both the activity and the heat stability of placental alkaline phosphatase are dependent on the presence of divalent cations. In separate experiments it appeared that the enzyme is not protected from inactivation by heat treatment after dialysis in the presence of EDTA, if $Mn^{2+}$ is added during the incubation at 56 °C. It is clear, therefore, that the same divalent cations influence activity and heat stability. The physiological ion will most likely be $Zn^{2+}$ in view of the fact that the latter has been found in preparations of placental alkaline phosphatase of high purity[31].

To investigate whether the heat stability is a general property of placental alkaline phosphatase or is restricted to human placenta, placentae of the monkey *Macaca mulatta* were examined. Monkeys were chosen because they are evolutionary closely related to man and especially because they too have a hemochorial type of placenta. In a series of 5 experiments the alkaline phosphatase activity in the homogenates and extracts of the monkey placentae was $0.021 \pm 0.003$ and $0.027 \pm 0.003$ $\mu$mole/min per mg protein. This is an order of magnitude lower than the corresponding values for human placentae (*cf.* Table I). Moreover, this activity was completely lost when the extracts were kept at 56 °C for 30 min. The heat stability appears, therefore, on the one hand to be confined to the placental enzyme among the isoenzymes of different tissues and on the other hand this peculiar but diagnostically useful property is not even shared by the phosphatase from the placentae of monkeys.

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* Apoenzyme has been put between quotation marks because the presence of prosthetic groups other than the divalent cations and of residual cations cannot be excluded.

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