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THE ACTION OF EDTA ON HUMAN ALKALINE PHOSPHATASES

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SUMMARY

The effects of EDTA on the human alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) of bone, intestine and placenta, have been studied by means of automated methods employing controlled concentration gradients. EDTA has three effects on these phosphatases: (a) In the presence of excess substrate, low concentrations of EDTA (10^{-5}–10^{-3} M) cause a loss of phosphatase activity which is the same irrespective of the EDTA concentration. (b) Above 10^{-3} M EDTA bone and intestinal phosphatases display an increasing loss in activity with increasing concentrations of EDTA. Placental phosphatase, however, displays a progressive gain in activity with increasing concentration of EDTA. (c) Preincubation of the phosphatases with EDTA results in a time-dependent inactivation which is not reversed by dilution. This inactivation is also temperature-dependent and pH dependent. Alkaline phosphatases from different tissues show different susceptibilities to the irreversible inactivation by EDTA.

The controlled concentration gradients were also used to study the kinetics of the action of EDTA on these phosphatases. Complex kinetics were observed with all alkaline phosphatases.

INTRODUCTION

Alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) are usually regarded as metalloenzymes[1–4] and, as such[5], they have been the subject of several studies[6,7] employing metal-complexing agents. This paper reports the results of our investigations into the effects of EDTA on human phosphatases*. The automated techniques employed, particularly the controlled concentration gradients of inhibitors and substrates have been described in a previous publication[8].

* The term "phosphatase" in this paper refers to alkaline phosphatase unless otherwise indicated.

MATERIALS AND METHODS

Enzymes

Macroscopically normal human bone and intestine were obtained at autopsy and a human placenta was obtained after a normal confinement. Each tissue was homogenised in a Waring blendor for 2 min with approx. 2 vol. of glass-distilled water. 5 vol. of chloroform water were added to the homogenate and the mixture was allowed to autolyse by standing at room temperature for 3 days. The solid matter was removed by centrifugation for 20 min at 4° (650 × *g*). 3 vol. of cold ethanol were added to the supernatant. This mixture was then stirred continuously for 30 min at −10°. Further centrifugation at 4° for 20 min (10 400 × *g*) produced a precipitate which was washed with a few ml of cold ethanol and recentrifuged. The precipitate obtained was dried over calcium chloride and the powder was stored at −10°.

Crude extracts of placenta and bone were also employed. These were prepared by homogenising the tissues in 0.01 M Tris–HCl buffer (pH 7.4) at 0°. The homogenate was filtered through Whatman No. 1 paper and the filtrate was used without further purification.

Placental homogenate was subjected to the procedure of GORDON *et al.*[9] by the Commonwealth Serum Laboratories (C.S.L.), Melbourne (Australia). The freeze-dried powder obtained by this procedure which consists largely of albumin but which also contains placental alkaline phosphatase was dissolved in Ringer lactate (5%, w/v) and this solution was used as an alternative preparation of placental alkaline phosphatase.

All enzyme preparations, before being used, were dialyzed for 24 h in the cold room against 5 changes of 0.05 M Tris–HCl buffer (pH 7.4).

Assay procedure

The buffers, assay procedure and automated techniques have been described by BIRKETT *et al.*[8] and KITCHENER *et al.*[10]. All glassware and Technicon AutoAnalyzer tubing were washed in 10^{-3} M EDTA followed by numerous rinses in glass-distilled water. No metals were added to the solutions except where indicated.

Assay of enzyme activity in the presence of EDTA

The Technicon AutoAnalyzer was used to assay enzyme activity[11] and to establish several controlled concentration gradients[8] of EDTA which entered the assay mixture together with the substrate solution. The final assay mixture[10] contained $6.5 \cdot 10^{-3}$ M disodium phenylorthophosphate in 0.025 M carbonate–bicarbonate buffer (pH 10) and EDTA in final concentrations varying from 0 to 10^{-1} M. Results were expressed as King–Armstrong units[12] per 100 ml of enzyme solution. The effect of 1,10-phenanthroline was studied in a similar manner. The incubation period was approx. 5 min, a correction factor being applied for minor variations from this figure. Reaction velocities were linear over this period and at this substrate concentration regardless of whether or not inhibitors were present in the assay system.

Pre-incubation with EDTA

Various concentrations of EDTA were prepared in 0.01 M Tris–HCl buffer (pH 7.4) or in 0.025 M carbonate–bicarbonate buffer (pH 10). These EDTA-buffer solutions were placed in a water-bath at 37°, enzyme was added to give an original activity of

15–30 King–Armstrong units per 100 ml, aspiration of the mixture was commenced immediately and continued for 30 min. The method of continuous monitoring of enzyme activity has been previously described[8,13].

In this set of experiments the enzyme–EDTA solution was subject to a 16-fold dilution in the AutoAnalyzer manifold so that the highest concentration of EDTA in the pre-incubation mixture (10^{-3} M) resulted in an EDTA concentration of $6.25 \cdot 10^{-5}$ M during incubation.

Magnesium sulphate or sodium β-glycerophosphate was added to the pre-incubation mixture in some of the experiments so as to give a concentration of 10^{-4} M in the pre-incubation mixture. The volume changes caused by the addition of these substances never exceeded 1%.

Determination of Michaelis constants

This was performed in 0.05 M Tris–HCl (pH 9) by the automated method described by BIRKETT *et al.*[8] at an incubation time of 45 sec. K_m and v_{max} were calculated according to the method of LINEWEAVER AND BURK[14] on an English Electric KDF9 computer by means of an Algol program written by Mr. M. MANTON. The substrate gradient was 0–0.15 mM disodium phenylorthophosphate and the enzyme activities used were in the range 10–15 King–Armstrong units per 100 ml when assayed under optimal conditions[10].

RESULTS

Enzyme inhibition as a function of EDTA concentration

Fig. 1 shows the effect of different concentrations of EDTA in the assay system. In the presence of substrate and 10^{-5} M EDTA these phosphatases exhibited a loss of activity which varied according to the source of enzyme. Bone phosphatase, in 4 experiments, lost a mean of 18.5% of its original activity (range 17–21%) while placental phosphatase (7 experiments) lost a mean of 15.3% of its activity (range 13–18%). Intestinal phosphatase in 5 experiments lost a mean of only 6.6% of its original activity (range 4–10%). When the concentration of EDTA was increased from 10^{-5} M to 10^{-3} M EDTA there was no further loss of activity by any of the phosphatase preparations.

When the concentration of EDTA in the assay system exceeded $3 \cdot 10^{-3}$ M, both intestinal and bone phosphatase exhibited a progressive diminution in activity with increasing concentrations of EDTA. This loss was not affected by changes in incubation time within the range 1–10 min. At 10^{-1} M EDTA, intestinal phosphatase showed 45% of its original activity while only 30% of the original activity of bone phosphatase remained. Placental phosphatase, however, over the range 10^{-3} M–10^{-1} M EDTA displayed a progressive gain in activity so that at $2 \cdot 10^{-2}$ M EDTA the mean activity was 122% of the original. Further increases in EDTA concentrations up to $1.9 \cdot 10^{-1}$ M did not lead to a further increase in placental alkaline phosphatase activity. A similar pattern of activation was obtained in 0.01 M Tris–HCl buffer at pH 9.0. But at pH 8.0 there was only negligible activation. The activation of placental phosphatase which occurred in the presence of 6.5 mM phenylorthophosphate was not seen at a substrate concentration of 0.15 mM. These effects of EDTA were uninfluenced by the mode of preparation of the various phosphatases.

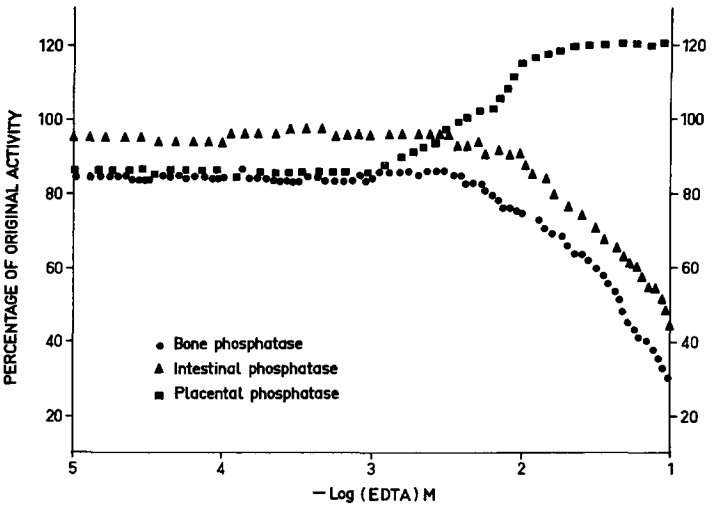

Fig. 1. The change in activity of three human alkaline phosphatases as a function of the concentration of EDTA in the assay mixture. The EDTA concentrations were varied by means of controlled concentration gradients[8] and the composite curve shown for each enzyme is the result of 4 such gradients over 4 concentration ranges (10^{-5}–10^{-4} M, 10^{-4}–10^{-3} M, 10^{-3}–10^{-2} M, and 10^{-2}–10^{-1} M). The substrate was disodium phenylorthophosphate ($6.5 \cdot 10^{-3}$ M) in 0.025 M carbonate–bicarbonate buffer (pH 10) and the incubation temperature was 37°. 100% is the activity of each enzyme preparation under these conditions in the absence of added EDTA. Note the large number of experimental points obtainable by this method.

Pre-incubation with EDTA

Fig. 2 shows the effect of pre-incubation with various concentrations of EDTA on the activity of purified bone, intestinal and placental alkaline phosphatases. In Tris–HCl buffer (pH 7.4) EDTA caused a time-dependent, temperature-dependent exponential inactivation which varied with the concentration of EDTA and according to the tissue of origin of the enzyme, and which was not reversed by dilution.

In the presence of 10^{-3} M EDTA, at 37° the mean half life of placental phosphatase was 6 min (3 experiments, range 5–7 min), that of bone phosphatase 2 min (4 experiments, range 0.5–3 min) and that of intestinal phosphatase less than 1 min (3 experiments). These times were not affected by an increase in the buffer strength from 0.01 M to 0.05 M, or by the degree of purification of the enzymes, although C.S.L. placental alkaline phosphatase was more labile than the other preparations of placental phosphatase. When the pH of the pre-incubation mixture was increased to 10.0 the inhibitory effect of EDTA was reduced. In 10^{-3} M EDTA the half life of placental phosphatase was 38 min, that of bone phosphatase 3.5 min, while intestinal phosphatase had a half life of 6.5 min.

Addition of substrate and magnesium to the pre-incubation mixture

Fig. 3 shows the effect of magnesium sulphate and β-glycerophosphate on the activity of C.S.L. placental phosphatase incubated in 10^{-4} M EDTA at pH 7.4. In the absence of EDTA or other added reagents, C.S.L. placental phosphatase showed a loss of activity of approx. 5% over 30 min at 37°. In the presence of 10^{-4} M magnesium, no loss of activity occurred[15–17]. However, the presence of 10^{-4} M β-glycero-

phosphate did not protect this preparation against the 5% loss of activity over 30 min at this temperature.

The presence of magnesium in the pre-incubation mixture prior to the addition of enzyme, afforded complete protection against EDTA inactivation at pH 7.4. If, instead, the magnesium was added to the pre-incubation mixture 5 min (or 10 min)

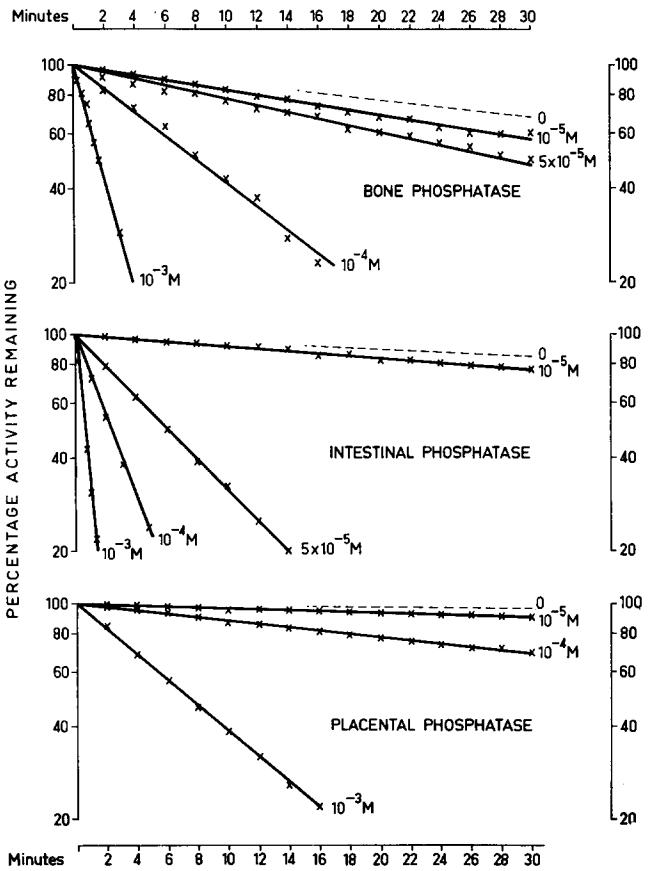

Fig. 2. Decrease in activity of purified human alkaline phosphatases as a function of the EDTA concentration and the duration of pre-incubation with EDTA at 37°. The pre-incubation mixtures contained EDTA at the concentrations indicated , in 0.01 M Tris–HCl (pH 7.4). These were diluted by a factor of 16 before assay. Enzyme activity was monitored continuously[13].

after the addition of enzyme, there was an immediate, though not complete, return of activity (from 60% to 86% at 5 min and from 41% to 59% at 10 min). The activity to which the enzyme had been restored then remained constant throughout the period of observation. A similar protective effect of magnesium in the pre-incubation mixture was observed at pH 10. At this pH, at 37°, and in 10^{-3} M EDTA, the half lives of bone, intestinal and placental phosphatases were increased by magnesium to 30 min, 40 min and no loss at all (over 30 min), respectively.

When β-glycerophosphate was added to the pre-incubation mixture before

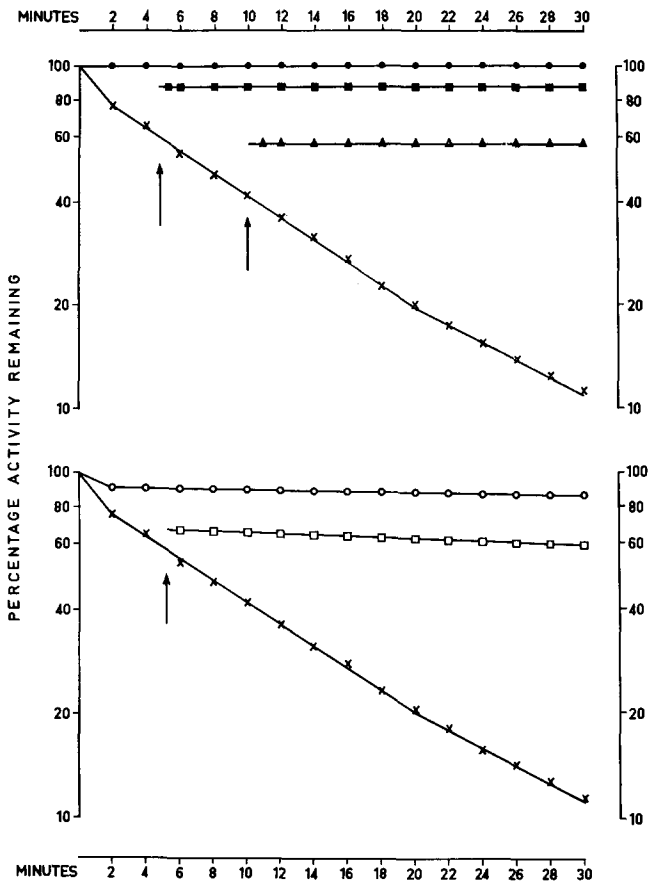

Fig. 3. The effect of magnesium sulphate and β-glycerophosphate on the activity of human placental alkaline phosphatase incubated with 10^{-4} M EDTA. Experimental procedure was the same as outlined in Fig. 2. Final molarities of Mg^{2+} and β-glycerophosphate in the pre-incubation mixture were each 10^{-4} M. Enzyme was added at zero time and the arrows indicate the times at which further additions were made. ×, EDTA only; ●, EDTA + Mg^{2+}; ■, EDTA, Mg^{2+} added at 5 min; ▲, EDTA, Mg^{2+} added at 10 min; ○, EDTA + β-glycerophosphate; □, EDTA, β-glycerophosphate added at 5 min.

placental phosphatase (C.S.L.), the rate of decay of enzyme activity was reduced considerably. In a pre-incubation mixture containing 10^{-4} M EDTA the half life of this enzyme preparation was 8 min. In the presence of β-glycerophosphate this was converted to a half life of 135 min. The addition of β-glycerophosphate to the pre-incubation mixture 5 min after the addition of enzyme also reduced the rate of decay of enzyme activity. Unlike magnesium, β-glycerophosphate caused only a 5% return in enzyme activity. The addition to the pre-incubation mixture at 5 min of magnesium and β-glycerophosphate together, produced results very similar to the effects of magnesium alone.

Effects of phenanthroline

1,10-Phenanthroline in concentrations up to 10^{-3} M had no effect on placental

phosphatase in the presence of substrate and at pH 10.0. Pre-incubation of placental phosphatase with 10^{-4} M and 10^{-3} M 1,10-phenanthroline at pH 7.4 was also without effect.

Lineweaver–Burk plots

Fig. 4 shows double reciprocal plots computed from data obtained by the automated method of BIRKETT *et al.*[8]. Forty five substrate concentrations were used to calculate linear regressions for each EDTA concentration. $5 \cdot 10^{-5}$ M EDTA reduced both $v_{max.}$ and K_m of placental phosphatase. As the concentration of EDTA was

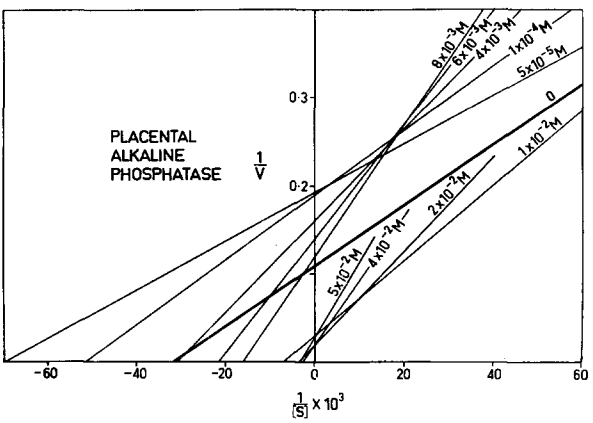

Fig. 4. Lineweaver–Burk plots of the effect of EDTA on human placental alkaline phosphatase. The assay mixture contained EDTA in concentrations as indicated and phenylorthophosphate in 0.05 M Tris–HCl buffer, pH 9.0 at 37°. Metal ions were not added. The Technicon AutoAnalyzer was used for the assay procedure and to establish a controlled concentration gradient of 0–0.15 mM phenylorthophosphate in the assay mixture. Enzyme activities at 45 substrate concentrations were used for each EDTA concentration to compute the linear regressions shown. In the absence of EDTA the K_m of placental phosphatase was 0.033 mM.

increased, both v_{max} and K_m increased, until at $1 \cdot 10^{-2}$ M the v_{max} was in excess of the initial value. Further increases in EDTA concentration produced only increases in K_m without any concomitant increase in v_{max}.

DIXON plots[18] for placental phosphatase were obtained both by replotting the data in Fig. 4 and by using controlled concentration gradients of EDTA at various substrate concentrations. As would be expected from Fig. 4 curved plots were obtained at all substrate concentrations.

DISCUSSION

These results indicate that EDTA has three effects on human alkaline phosphatases. The first, occurring with low concentrations of EDTA in the assay system, is an instantaneous inhibition, apparently independent of variations in the EDTA concentrations within the range 10^{-5}–10^{-3} M. At concentrations of EDTA above 10^{-3} M in the assay system, the second effect is seen. Bone and intestinal phosphatase display an increasing loss in activity with increasing EDTA concentrations, while placental

phosphatase shows a progressive gain in activity. Pre-incubation of the phosphatases with EDTA produces the third effect: a time-dependent inactivation which is not reversed by dilution.

It is suggested that the first effect is due to some activator, possibly a metal[19–21], which is contaminating the enzyme preparations or the commercial reagents employed[22–24]. Such a postulate would explain the reduction in v_{max} of placental and bone phosphatase (Fig. 1) upon the addition of small quantities of EDTA and the absence of any further reduction in spite of a subsequent increase in the EDTA concentration of the assay system. The apparently insignificant effect of small concentrations of EDTA on intestinal phosphatase is attributed to structural differences between this enzyme and other alkaline phosphatases[25–27].

The second effect of EDTA on bone and intestinal alkaline phosphatases commences at EDTA concentrations above 10^{-3} M (Fig. 1). It may be due to the chelation of some divalent cation necessary for enzyme activity[1,28–30], or to a direct effect of EDTA on the enzyme molecule[7].

Placental phosphatase differs from the other phosphatases by being activated by EDTA, a property which it shares with various non-phosphatase enzymes[22,31–33]. A review of the mechanisms whereby these enzymes may be activated by EDTA[22–24,31–40] is beyond the scope of this paper. In the case of phosphatases, the activating effect may be confined to the placental enzyme because of its very stable protein structure. Placental phosphatase differs from other human phosphatases in its amino acid composition[41] and in its stability to heat[16], citrate [10] and urea[8]. Since the phosphatases used in this work were crude preparations the complex kinetics shown in Fig. 4 will not be discussed in this paper.

The third effect of EDTA—*i.e.* the time-dependent inactivation which is not reversed by dilution (Fig. 2)—is seen with all three human phosphatases and has previously been described with other mammalian phosphatases[6,21,30]. This third effect is readily explained by the postulate that EDTA removes a functionally essential metal (probably zinc)[6,21,30] though it is not possible to deduce from this work whether EDTA binds the metal *in situ* and slowly induces conformational changes in the protein structure, or whether the EDTA immediately removes the metal from the protein, leaving an unstable enzyme molecule which then undergoes denaturation[42,43]. AGUS, COX AND GRIFFIN[44] concluded that EDTA probably removes the metal from the metalloenzymes.

The different rates of inactivation displayed by the various enzymes during pre-incubation with EDTA are attributed to structural differences between the various phosphatases[8,16,25–27]. Time-dependent activation by pre-incubation with EDTA, described by MILSTEIN for phosphoglucomutase[31], was not observed in these experiments.

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