BBA 65866

# EVIDENCE FOR THE IDENTITY OF ALKALINE PHOSPHATASE AND INORGANIC PYROPHOSPHATASE IN RAT KIDNEY*

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(Received October 7th, 1968)
(Revised manuscript received November 29th, 1968)

SUMMARY

Alkaline phosphatase, extensively purified from rat kidney, was found to hydrolyze inorganic pyrophosphate. The following experimental results suggest that alkaline phosphatase is both a phosphomonoesterase and a pyrophosphatase.

1. The purification of rat kidney alkaline phosphatase was accompanied by a concomitant increase in inorganic pyrophosphatase activity and an almost constant ratio between the two enzyme activities at different purification steps was found. Moreover, both enzymatic activities were eluted together as a single peak after DEAE-Sephadex A-25 chromatography of microsomal proteins solubilized by deoxycholate and lipase.

2. A decrease in the level of inorganic phosphate in the renal cell produces concomitant and significant increases of both enzymatic activities.

3. The amount of phosphate released in the mixed substrate experiments was less than would be expected for independent hydrolysis of the substrates.

4. Both enzyme activities were affected similarly by cysteine, a noncompetitive inhibitor, and by heating in the absence of substrate.

## INTRODUCTION

That mammalian alkaline phosphatase (orthophosphoric monoester phosphohydrolase (EC 3.1.3.1)) is inactive towards inorganic pyrophosphate has been known for many years[2,3]. On the other hand there are several reports that suggest that alkaline phosphatase has inorganic pyrophosphatase activity as well[4–11].

Interest in the problem was intensified by the finding that inorganic pyrophosphate is produced as a by-product in many essential biosynthetic reactions including the reactions of DNA and RNA polymerization, coenzyme synthesis and amino acid and fatty acid activation. The hydrolysis of inorganic pyrophosphate may provide, as suggested by STETTEN[12] and by KORNBERG[13], a control mechanism whereby these biosynthetic reactions are made irreversible.

* A preliminary report was presented at the 5th Meeting of the European Biochemical Society, Prague, 1968 (ref. 1).

In the present work, which was conducted with a highly purified rat kidney alkaline phosphatase, evidence that the same enzyme is responsible for alkaline phosphatase and inorganic pyrophosphatase activities is presented and discussed.

## EXPERIMENTAL

### *Enzyme preparation*

The procedures for purification of rat kidney alkaline phosphatase have been described[14]. In brief, alkaline phosphatase was extracted from rat kidney microsomes by simultaneous treatment with deoxycholate (1% solution of sodium deoxycholate in 0.05 M Tris buffer at pH 7.8) and lipase and was purified successively by chromatography on DEAE-Sephadex A-25, eluting with a linear gradient between 0.03 and 0.15 M NaCl in 0.05 M Tris buffer (pH 8.1), and rechromatography on DEAE-Sephadex as before.

The resultant enzyme preparation gave a single coincident band when stained for enzyme activity and protein after starch-gel electrophoresis. The specific activity was 15 $\mu$moles of *p*-nitrophenol hydrolyzed from *p*-nitrophenylphosphate per min per mg of protein at 25° and pH 10.4. Other phosphomonoesters are hydrolyzed by purified phosphatase[15].

### *Assay methods*

Enzyme activities were assayed at 25°.

Alkaline phosphatase was measured spectrophotometrically at 405 nm by a continuous optical method[16] with 3 mM *p*-nitrophenylphosphate in 0.05 M carbonate–bicarbonate buffer–0.15 mM $MgCl_2$ (pH 10.4), as substrate.

Alkaline inorganic pyrophosphatase was determined using 3 mM sodium pyrophosphate as substrate in 0.05 M Tris–HCl–0.15 mM $MgCl_2$ (pH 8). The procedure consisted of incubating 0.1 ml of enzyme solution with 0.9 ml of reaction mixture for 10 min and stopping the reaction by immersing the tubes in ice; phosphate release was assayed immediately by the method of LOWRY AND LOPEZ[17]. Other specific details are given in the legends of tables and figures. Total protein was determined by the biuret method, according to BEISENHERZ *et al.*[18], except that for individual column fractions protein was estimated from the absorbance at 280 nm as suggested by WARBURG AND CHRISTIAN[19].

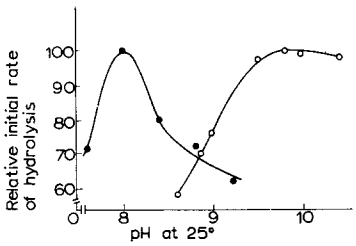
Intracellular inorganic phosphate was estimated by the method of LOWRY AND LOPEZ[17] on a kidney perchloric extract obtained by the quick-freezing technique of HOHORST, KREUTZ AND BÜCHER[20].

## RESULTS

### *Hydrolysis of p-nitrophenylphosphate and inorganic pyrophosphate by alkaline phosphatase*

In Fig. 1 the activity–pH curve for hydrolysis of *p*-nitrophenylphosphate is compared with that for hydrolysis of inorganic pyrophosphate: it is evident that pH has a different influence on the rates of hydrolysis of the two substrates.

The curve for *p*-nitrophenylphosphate shows a maximum value at pH 9.8,



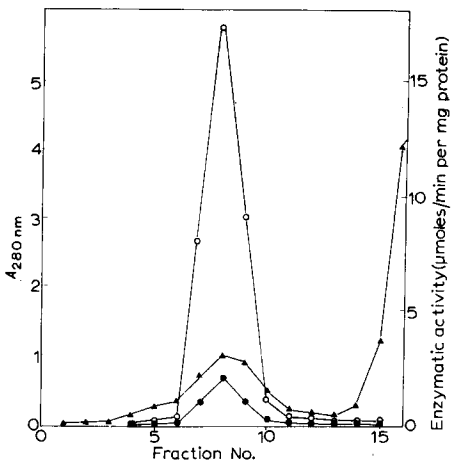




Fig. 1. Activity–pH curves for hydrolysis of *p*-nitrophenylphosphate (○—○) and inorganic pyrophosphate (●—●) by purified alkaline phosphatase from rat kidney. The reaction mixtures contained 3 mM substrate, 1 mM $MgCl_2$ in 0.05 M Tris–glycine buffer (pH 7.5–11). The enzyme activities at optimum pH, although of different values for the two substrates, were arbitrarily assumed as cent per cent (hydrolysis measured by release of orthophosphate).

Fig. 2. Purification of alkaline phosphatase and inorganic pyrophosphatase activities from a microsomal extract containing 30 mg of protein. The extract was prepared from rat kidney microsomes by the procedure described in the text and absorbed to a column of DEAE-Sephadex (20 cm × 2 cm). Elution was achieved with a linear gradient formed from 100 ml of 0.05 M Tris–HCl buffer (pH 8.1) in the mixing chamber and the same buffer + 1 M NaCl in the reservoir flask. The eluate fractions (1.8 ml) were assayed for absorbance at 2800 Å (▲—▲), for alkaline phosphatase (○—○) and for inorganic pyrophosphatase (●—●) activities.

whereas the pH–activity curve for inorganic pyrophosphate rises to a maximum at pH 8 and declines at higher pH values.

*Purification of alkaline phosphatase and inorganic pyrophosphatase*

The results of a typical preparation of alkaline phosphatase and inorganic pyrophosphatase from rat kidney are summarized in Table I. As Table I shows, the

TABLE I

PREPARATION OF ALKALINE PHOSPHATASE AND INORGANIC PYROPHOSPHATASE FROM RAT KIDNEY

Activities are expressed as μmoles of *p*-nitrophenylphosphate or inorganic pyrophosphate hydrolysed per min and per mg of total protein. Abbreviations: A, alkaline phosphatase; B, inorganic pyrophosphatase.

| *Fraction* | *Total units* | | *Specific activity* | | *Purification (fold)* | | *Yield (%)* | | *A/B* |
|---|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *A* | *B* | *A* | *B* | *A* | *B* | |
| Crude extract | 150 | 21 | 0.05 | 0.007 | — | — | 100 | 100 | 7.1 |
| Microsomal suspension in deoxycholate | 96 | 11.2 | 0.6 | 0.07 | 12 | 10 | 64 | 53 | 8.5 |
| Supernatant after lipase digestion | 64 | 8 | 1.7 | 0.21 | 34 | 30.4 | 42 | 38 | 8 |
| First fractionation on DEAE-Sephadex | 36 | 4.9 | 14.5 | 1.99 | 290 | 285 | 24 | 23 | 7.3 |
| Second fractionation on DEAE-Sephadex | 34 | 4.6 | 15.5 | 2.1 | 310 | 300 | 23 | 22 | 7.4 |

TABLE II

EFFECT OF A DIET LACKING IN PHOSPHATE ON THE RAT KIDNEY LEVELS OF ALKALINE PHOSPHATASE, INORGANIC PYROPHOSPHATASE AND INORGANIC PHOSPHATE

The results are means ± standard deviations; in parentheses the number of rats. The content of inorganic phosphate was determined on a kidney perchloric extract obtained by a quick-freezing technique. The enzyme activities were determined on a fraction (15 min at 4000 × *g*, supernatant) of 10% kidney homogenate.

| *Animals* | *Kidney phosphate (μmoles/g per wet weight)* | *Alkaline phosphatase (μmoles/min per g protein)* | *Inorganic pyrophosphatase (μmoles/min per g protein)* |
|---|---|---|---|
| Complete diet | 5.1 ± 0.04 (10) | 82 ± 12 (10) | 11 ± 1.4 (10) |
| Phosphate free diet | 3.3 ± 0.06 (10) | 160 ± 18 (10) | 23 ± 2.2 (10) |

purification of rat kidney alkaline phosphatase was accompanied by a concomitant increase in inorganic pyrophosphatase activity.

A typical elution profile is given in Fig. 2 in which it is clear that alkaline phosphatase and inorganic pyrophosphatase activities run together through the column, both of them being recovered in the few initial fractions at sodium chloride concentrations ranging from 0.03–0.15 M.

*Regulation by phosphate of alkaline phosphatase and inorganic pyrophosphatase activities in rat kidney*

A decrease in the level of inorganic phosphate in the renal cell produced by dietary treatment is accompanied by a concomitant and significant increase in alkaline phosphatase activity[21,22]. Table II shows that in the renal cell there is also a parallel increase of inorganic pyrophosphatase activity.

*Mixed-substrate experiments*

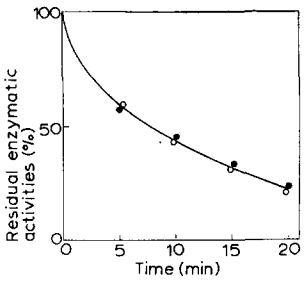
Experiments were carried out in which the rate of phosphate released from

TABLE III

HYDROLYSIS OF *p*-NITROPHENYLPHOSPHATE AND SODIUM PYROPHOSPHATE BY RAT KIDNEY ALKALINE PHOSPHATASE

Enzyme was incubated with *p*-nitrophenylphosphate (3 mM) or with sodium pyrophosphate (3 mM), or with both at pH 9 (0.05 M carbonate–bicarbonate buffer) for 10 min at 25°. The concentration of $Mg^{2+}$ was 0.2 mM throughout. Activities are expressed in μmoles of inorganic phosphate ($P_i$) or *p*-nitrophenol liberated/min per ml of enzyme solution.

| | *Observed* | *Expected for independent hydrolysis* |
|---|---|---|
| Sodium pyrophosphate (3 mM) | | |
| (μmoles of $P_i$/min) | 0.05 | |
| *p*-Nitrophenylphosphate (3 mM) | | |
| (μmoles of $P_i$/min) | 0.32 | |
| (μmoles of *p*-nitrophenol/min) | 0.30 | |
| Sodium pyrophosphate (3 mM) + *p*-nitrophenylphosphate (3 mM) | | |
| (μmoles of $P_i$/min) | 0.25 | 0.37 |
| (μmoles of *p*-nitrophenol/min) | 0.23 | |



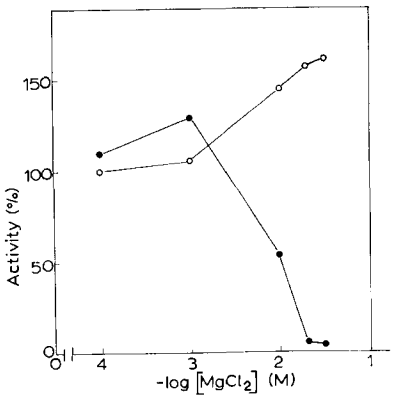




Fig. 3. Effect of incubation at 56° and pH 9 (0.05 M Tris–HCl buffer) on alkaline phosphatase (○—○) and inorganic pyrophosphatase (●—●) activities.

Fig. 4. Effect of $Mg^{2+}$ on alkaline phosphatase (○—○) and inorganic pyrophosphatase (●—●) activities. The reaction mixtures contained 3 mM substrate (*p*-nitrophenylphosphate or inorganic pyrophosphate) buffered at pH 9 with 0.05 M Tris–HCl buffer. Activities, measured by the release of orthophosphate, are given as a percentage of each control with no added $MgCl_2$.

inorganic pyrophosphate and *p*-nitrophenylphosphate present together in the same concentration was compared with that from a single substrate; the concentration of $Mg^{2+}$ was 1 mM throughout. As shown in Table III, the amount of phosphate released in the mixed-substrate experiments was less than would be expected for independent hydrolysis of the substrates.

*Heat lability of enzymes*

Fig. 3 shows the effect of incubation at 56° and pH 9 (0.05 M Tris–HCl buffer) on alkaline phosphatase and inorganic pyrophosphatase activities of purified kidney enzyme; both activities show a constant ratio during heat inactivation at 56°.

*Treatment with amino acids*

COX, GILBERT AND GRIFFIN[23] reported that L-cysteine caused inhibition of the alkaline phosphatase and inorganic pyrophosphatase activities of HeLa cells in tissue

TABLE IV

INHIBITION OF ALKALINE PHOSPHATASE AND INORGANIC PYROPHOSPHATASE ACTIVITIES BY CYSTEINE

The enzyme solution was pre-incubated for 1 min with cysteine or with cysteine + $Zn^{2+}$ in an ice bath. Aliquots were removed and assayed for activities at pH 9 (0.05 M carbonate–bicarbonate buffer).

| *Additions* | *Alkaline phosphatase (μmoles/min per ml enzyme solution)* | *Inorganic pyrophosphatase (μmoles/min per ml enzyme solution)* |
|---|---|---|
| None | 0.33 | 0.045 |
| $2 \cdot 10^{-4}$ M cysteine | 0.12 | 0.015 |
| $2 \cdot 10^{-4}$ M cysteine + $2.5 \cdot 10^{-5}$ M $Zn^{2+}$ | 0.23 | 0.025 |
| $2 \cdot 10^{-4}$ M cysteine + $5 \cdot 10^{-5}$ M $Zn^{2+}$ | 0.34 | 0.05 |

cultures. At a cysteine concentration of $2 \cdot 10^{-4}$ M (pH 9), we observed a strong inhibition of both of these enzyme activities (Table IV). The non-competitive inhibition caused by cysteine was completely removed on the addition of $Zn^{2+}$. Other amino acids tested at the same pH in the concentration range $4 \cdot 10^{-4}$ M and $5 \cdot 10^{-3}$ M were ineffective on either of these enzyme activities.

*Effect of $MgCl_2$ on the enzyme activities*

In the presence of $Mg^{2+}$ the phosphomonoesterase activity of purified kidney enzyme was enhanced; with the pyrophosphate as substrate, however, and $Mg^{2+}$ concentrations up to about 1 mM, there was some activation, but above this concentration $Mg^{2+}$ was strongly inhibitory (Fig. 4). Similar results were obtained with intestinal and liver enzymes[24].

## DISCUSSION

Our own data on the whole support the idea that both phosphomonoesterase and inorganic pyrophosphatase activities of rat kidney are properties of the same enzyme. In fact it is clear from Fig. 1 that purified kidney enzyme hydrolyzes both *p*-nitrophenylphosphate and inorganic pyrophosphate and that kidney preparations had inorganic pyrophosphatase activity at all stages of purification; during the purification procedure (Table I), the ratio of alkaline phosphatase activity (at pH 10.4) to pyrophosphatase activity (at pH 8) remained almost constant at different purification steps (7.1 in crude extracts; 8.5 in microsomal suspension in deoxycholate; 8 in supernatant after lipase digestion; 7.3 after the first fractionation; 7.4 after the second fractionation). Furthermore, the alkaline phosphatase and inorganic pyrophosphatase were eluted together as a single peak after DEAE-Sephadex A-25 chromatography of the microsomal proteins (Fig. 2).

Additional evidence is provided by studies of the mechanism by which inorganic phosphate regulates the level of alkaline phosphatase: a decrease in inorganic phosphate level in the renal cell by dietary treatment is accompanied by concomitant and significant increases in both alkaline phosphatase and inorganic pyrophosphatase activities. ROCHE[25] has reported that alkaline phosphatase activity is less stable than pyrophosphatase activity in extracts of pig liver, but our thermal inactivation experiments with rat kidney enzyme did not reveal any such difference in stability (Fig. 3).

Furthermore, there was no summation of rates of $P_i$ formation when saturating concentrations of *p*-nitrophenylphosphate and inorganic pyrophosphate were mixed (Table III). In addition the inhibition by cysteine of alkaline phosphatase and inorganic pyrophosphatase activities proceeded in a parallel fashion (Table IV). It seems less probable that the two types of phosphatase activity are the result of the presence of two enzymes, each of restricted specificity, since experimental results were obtained with an enzyme preparation which appears substantially homogeneous.

The last experimental result, which requires further mention, is that hydrolysis of inorganic pyrophosphate may be suppressed in a selective fashion by relatively high concentrations of $Mg^{2+}$ (Fig. 4). This inhibition may have been responsible for MORTON's observation[3] that highly purified calf intestinal alkaline phosphatase was free from inorganic pyrophosphatase activity, since his experimental conditions

included a large excess of $Mg^{2+}$ (20-fold) which in our experience would be highly inhibitory.

Our interpretation that in rat kidney both orthophosphatase and pyrophosphatase activities are due to the same enzyme, is in agreement with observations concerning alkaline phosphatase from calf intestine[11] and from several human tissues[8,9,24]. Since purified inorganic pyrophosphatase from yeast[27] and from *Escherichia coli*[28] does not exhibit alkaline phosphatase activity, it is probable that the above conclusions may apply essentially to mammalian enzymes.

ACKNOWLEDGMENT

We are grateful to Prof. VINCENZO BACCARI for his interest and helpful advice in this work.

This work was supported by a grant from the Italian Consiglio Nazionale delle Ricerche.

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