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# THE ZINC-STIMULATED ACID AND NEUTRAL *p*-NITROPHENYL PHOSPHATASE ACTIVITIES OF CHICK LIVER AND DUODENUM

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SUMMARY

1. Some properties of the soluble $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatases active at acid and neutral pH have been studied with $(NH_4)_2SO_4$-precipitated preparations from the soluble cytoplasm fraction of homogenates from chick liver, duodenum and metanephros. Analogous $Zn^{2+}$-stimulated phosphatase activity was not found in the particulate fraction from these tissues or in any fraction from homogenates of chick brain or heart muscle.

2. The pH optima of these soluble, $Zn^{2+}$-stimulated enzymes were at pH 4.9, 6.2 and 6.7, respectively, for duodenum, liver and metanephros. The enzymes responsible for the low level of soluble activity without added $Zn^{2+}$ or with added $Mg^{2+}$ showed maximal activity at pH 5.4–5.8 in all three tissues.

3. The soluble $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase enzymes of liver and duodenum were similar in most other properties examined. With preparations from both tissues, a marked (10- to 20-fold) stimulation of *p*-nitrophenyl phosphatase activity was elicited only by $Zn^{2+}$, but $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ caused some stimulation. The optimal concentration of $Zn^{2+}$ varied over the range 3–10 mM, depending on pH and type of buffer used. A marked and specific stimulatory effect of $Zn^{2+}$ was seen only for hydrolysis of *p*-nitrophenyl phosphate, of the many phosphate compounds tested. Hydrolysis of ATP by these preparations was moderately stimulated by both $Mg^{2+}$ and $Zn^{2+}$. Indirect evidence suggests that the $Zn^{2+}$-stimulated hydrolysis of *p*-nitrophenyl phosphate was not due to the inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1), fructose-1,6-diphosphatase (fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11), or ATPase (ATP phosphohydrolase, EC 3.6.1.3) present in these preparations.

## INTRODUCTION

Zinc has been identified as an essential component or potent activator of various alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1)

Abbreviation: MES, 2(*N*-morpholino) ethanesulfonic acid.

preparations from microbial[1] and animal [2–5] sources, which usually show substantial activity with a variety of substrates. Zinc has also been implicated in the function of certain phosphatases that are active at neutral or acid pH and that usually show more restricted substrate specificity, *e.g.* 5'-nucleotidase (EC 3.1.3.5) and cyclic phosphodiesterase from *Escherichia coli*[6], and cyclic phosphodiesterase[7], glyceryl-phosphoryl choline diesterase (EC 3.1.4.2)[8] and dinucleotide pyrophosphatase (EC 3.6.1.9)[9] from mammalian tissues. Some phosphatases that either have no apparent metal requirement or are activated by $Mg^{2+}$ are inhibited by 1–5 mM $Zn^{2+}$ [10–12]. It is, therefore, of interest that chick liver[12,13] and chick duodenum[12] contain substantial levels of soluble *p*-nitrophenyl phosphatase (*p*-nitrophenyl phosphate phosphoric monoester hydrolase) activity at neutral or acid pH that is activated by 5 mM $Zn^{2+}$, whereas the particulate *p*-nitrophenyl phosphatase activities of these tissues at the same pH values are inhibited by this concentration of $Zn^{2+}$. This study was undertaken to characterize further the $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatases of chick liver and duodenum. Other chick tissues were also examined for the presence of similar enzymatic activities.

## METHODS

### *Tissue preparations*

Homogenates and cell fractions were prepared from organs of 4-day white Leghorn chicks, essentially by the procedures previously described[12]. The degree of homogenization was adjusted to give about 75–90% cell breakage for the various tissues. The initial homogenates, after being strained through gauze, were made up with the 250 mM sucrose–1 mM EDTA (pH 7.0) homogenizing medium (sucrose–EDTA medium) to contain 100 mg wet wt tissue per ml. After removal of the fraction containing nuclei and whole cells by centrifugation at 700 × *g* for 10 min, the supernatant fluid was centrifuged at 100 000 × *g* for 60 min. The resulting precipitate was resuspended in 10 mM Tris–maleate buffer (pH 7.0) containing 1 mM EDTA (Tris–maleate–EDTA medium) to give a "particulate fraction" (P), and the supernatant solution was made up with Tris–maleate–EDTA to the original volume of homogenate used, to give a "soluble fraction" (S). Samples of Fraction S were taken to 75% saturation with $(NH_4)_2SO_4$ by addition of saturated $(NH_4)_2SO_4$ solution at 0–2 °C; after standing 15 min, the mixture was centrifuged at 20 000 × *g* for 10 min, and the precipitate resuspended in the Tris–maleate–EDTA medium to give the "soluble-precipitated fraction" (SP). Homogenates and fractions routinely were frozen immediately after preparation and were stored at −15 °C until thawed for assay or additional treatment.

### *Assays*

Phosphatase activities were assayed either by determination of inorganic phosphate released, as previously described[12,14], or by determination of *p*-nitrophenol formed[15]. For either procedure the standard incubation mixture contained in a total volume of 1.0 ml: buffer, usually 50 mM acetate (pH 5.0), 50 mM Tris–maleate (pH 6.2), or 100 mM 2-amino-2-methyl-1-propanol (pH 9.5); substrate, usually 3 mM *p*-nitrophenyl phosphate; metal ion, either 10 mM $MgCl_2$ or 10 mM $ZnSO_4$, as noted; and 0.1 mM EDTA. The SP fractions of liver and duodenum gave maximal $Zn^{2+}$-

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TABLE I

*p*-NITROPHENYL PHOSPHATASE ACTIVITIES IN HOMOGENATES AND CELL FRACTIONS FROM TISSUES OF 4-DAY CHICKS

Homogenates (H), particulate fractions (P) and soluble-precipitated fractions (SP) were prepared and assayed for *p*-nitrophenyl phosphatase activity by determination of inorganic phosphate released and for protein content as described under Methods. Enzyme assays were carried out with 3 mM *p*-nitrophenyl phosphate as substrate at pH 5.0 in acetate buffer, at pH 6.2 in Tris–maleate buffer and at pH 9.5 in 2-amino-2-methyl-1-propanol buffer. Where indicated, $MgCl_2$ and Zn $SO_4$ were added, each in 10 mM concentration. The results tabulated are for typical preparations. The enzyme activities are expressed as $\mu$moles inorganic phosphate released/min per g wet wt of original tissue and protein content as mg/g wet wt of original tissue.

| *Tissue* | *Fraction* | *Enzyme activity per g original tissue* | | | | | | | | | | *Protein content (mg/g)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *pH 5.0* | | | *pH 6.2* | | | *pH 9.5* | | | | |
| | | — | $Mg^{2+}$ | $Zn^{2+}$ | — | $Mg^{2+}$ | $Zn^{2+}$ | — | $Mg^{2+}$ | $Zn^{2+}$ | $Zn^{2+} + Mg^{2+}$ | |
| Liver | H | 9.9 | 12.8 | 9.0 | 9.0 | 10.6 | 19.4 | 2.1 | 2.5 | 1.2 | 1.6 | 181 |
| | P | 8.1 | 9.1 | 1.3 | 6.7 | 7.2 | 1.3 | 1.0 | 1.5 | 0.5 | 1.0 | 94 |
| | SP | 1.8 | 2.1 | 10.0 | 0.9 | 1.3 | 17.9 | 0.4 | 0.6 | 0.4 | 0.4 | 55 |
| Duodenum | H | 7.6 | 9.1 | 7.3 | 5.1 | 7.4 | 6.1 | 14.4 | 54.1 | 12.2 | 42.0 | 155 |
| | P | 4.0 | 4.8 | 1.4 | 2.7 | 3.8 | 1.8 | 3.6 | 38.0 | 7.3 | 31.0 | 68 |
| | SP | 0.9 | 1.2 | 9.3 | 0.6 | 1.3 | 3.9 | 0.5 | 2.0 | 0.2 | 0.5 | 36 |
| Metanephros | H | 8.2 | 9.9 | 8.1 | 4.0 | 7.2 | 9.7 | 4.7 | 10.0 | 6.0 | 11.0 | 152 |
| | P | 6.8 | 8.5 | 3.4 | 4.1 | 5.9 | 3.2 | 3.9 | 7.5 | 5.2 | 9.7 | 99 |
| | SP | 1.3 | 1.9 | 5.3 | 1.1 | 1.5 | 5.8 | 0.3 | 0.7 | 0.6 | 0.6 | 39 |
| Heart muscle | H | 2.2 | 2.8 | 0.9 | 1.7 | 3.1 | 0.5 | 1.0 | 2.2 | 0.1 | 0.1 | 125 |
| | P | 1.4 | 1.9 | 0.6 | 1.1 | 1.7 | 0.3 | — | — | — | — | 85 |
| | SP | 0.3 | 0.5 | 0.1 | 0.1 | 0.3 | 0.1 | — | — | — | — | 20 |
| Brain | H | 4.9 | 5.7 | 3.1 | 2.4 | 4.9 | 0.5 | 1.1 | 1.5 | 0 | 0.1 | 100 |
| | P | 3.3 | 4.3 | 2.2 | 1.5 | 3.9 | 0.4 | — | — | — | — | 75 |
| | SP | 0.3 | 0.6 | 0.6 | 0 | 0.2 | 0 | — | — | — | — | 16 |

stimulated *p*-nitrophenyl phosphatase activity in Tris–maleate buffer (pH 6.2) and acetate buffer (pH 5.0), respectively, and these buffers were routinely used for the characterization studies on the enzymes in these fractions. The reaction was initiated by addition of enzyme to the pre-warmed incubation mixture, and incubation continued for 15 min at 37 °C. The reaction was stopped by addition of either 0.5 ml cold 10% trichloroacetic acid for the inorganic phosphate assay, or 1.0 ml 0.2 M NaOH, for the *p*-nitrophenol assay. After centrifugation of the cooled mixture, samples of the supernatant fluid were assayed for inorganic orthophosphate released by the procedure of Lowry and Lopez[14] or for *p*-nitrophenol formed by reading absorbance at 410 nm.

The protein content of enzyme preparations was determined by the micromethod of Lowry *et al.*[16], with bovine serum albumin as standard.

*Chemicals*

Substrates, most of them in the form of sodium salts, were obtained from Sigma Chemical Company, as were 2-amino-2-methyl-1-propanol, 2(*N*-morpholino) ethanesulfonic acid (MES), diethylenetriaminepentaacetic acid and bovine serum albumin. Other chemicals were reagent or certified grade materials obtained from Fisher Chemical Company. All solutions were made with double glass-distilled water.

## RESULTS

*$Zn^{2+}$- and $Mg^{2+}$-stimulated p-nitrophenyl phosphatase activities in chick tissues and cell fractions*

Table I summarizes the distribution of *p*-nitrophenyl phosphatase activities at acid, neutral and alkaline pH, in the particulate fraction (P) and soluble, $(NH_4)_2SO_4$-precipitated fraction (SP) of homogenates prepared from various tissues of 4-day chicks, and the effect of 10 mM $Zn^{2+}$ and $Mg^{2+}$ on these activities. Liver, duodenum, metanephros and brain contained substantial activity at acid or neutral pH which was slightly stimulated by addition of $Mg^{2+}$ and was localized primarily in the particulate fraction (P). These particulate activities were strongly inhibited by 10 mM $Zn^{2+}$, and activities in the presence of 10 mM $Mg^{2+}$ plus 10 mM $Zn^{2+}$ (not tabulated) were similar to the activities with 10 mM $Zn^{2+}$ alone. The substantial activities at alkaline pH in duodenum and metanephros were also primarily associated with the particulate fraction; these activities were substantially stimulated by $Mg^{2+}$ and moderately stimulated by 10 mM $Zn^{2+}$, while the combination of $Mg^{2+}$ and $Zn^{2+}$ resulted in activities higher than those seen with $Zn^{2+}$ alone. The soluble-precipitated (SP) fractions from the 5 tissues studied showed only low levels of *p*-nitrophenyl phosphatase activity with or without added $Mg^{2+}$, at acid, neutral or alkaline pH. However, Table I shows that the SP fractions from liver, duodenum and metanephros contained substantial levels of $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activity at acid or neutral pH.

*Effects of preparative procedures on $Zn^{2+}$-stimulated activities*

The data in Tables I and II suggest that the soluble-precipitated fraction (SP) of liver and duodenum can account for all of the $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activity at acid and neutral pH found in homogenates of these tissues. As

TABLE II

SPECIFIC ACTIVITY OF *p*-NITROPHENYL PHOSPHATASE IN SOLUBLE FRACTION AFTER VARIOUS TREATMENTS

Homogenates, soluble fraction (S) and soluble-precipitated fraction (SP) were prepared from 4-day chick liver and duodenum as described under Methods. The soluble, twice precipitated fraction was obtained from SP fraction by a second precipitation with 75% saturated $(NH_4)_2SO_4$. The 50–65% $(NH_4)_2SO_4$ fraction was that obtained between 50 and 65% saturation with $(NH_4)_2SO_4$ by stepwise additions of saturated $(NH_4)_2SO_4$ at 0–2 °C to SP fraction suspended in 0.02 M Tris–maleate (pH 6.5)–1 mM EDTA (10 mg protein/ml) and centrifuging 15 min after each addition. Dialyzed SP fraction was obtained by dialysis at 2 °C for 18 h against 20 mM Tris–maleate (pH 6.5)–1 mM EDTA, then for 6 h against 20 mM Tris–maleate (pH 6.5). The *p*-nitrophenyl phosphatase activity was determined with and without 10 mM added $ZnSO_4$, by the *p*-nitrophenol assay described under Methods, at pH 6.2 for preparations from liver and at pH 5.0 for the preparations from duodenum. Specific activities are expressed as nmoles *p*-nitrophenol formed/min per mg protein in the preparation, and protein content of the preparation as mg protein in the preparation obtained from 1 g wet wt of liver or duodenum. The results given are for a typical experiment.

| *Preparation and treatment* | *Liver* | | | *Duodenum* | | |
|---|---|---|---|---|---|---|
| | *Specific activity* — $Zn^{2+}$ | | *Protein content (mg/g)* | *Specific activity* — $Zn^{2+}$ | | *Protein content (mg/g)* |
| Homogenate | 49 | 99 | 180 | 53 | 48 | 152 |
| Soluble fraction (S) | 25 | 253 | 57 | 36 | 130 | 40 |
| Soluble-precipitated fraction (SP) | 15 | 305 | 53 | 30 | 255 | 35 |
| Soluble, 2 × precipitated | 12 | 300 | 52 | 28 | 260 | 33 |
| 50–65% $(NH_4)_2SO_4$ fraction | 32 | 1020 | 12 | 30 | 510 | 10 |
| Dialyzed SP fraction | 12 | 270 | 52 | 20 | 92 | 35 |

may be calculated from data in Table II, the total activity in the SP fraction is greater than in the original soluble fraction (S), particularly in the case of the duodenum; the mechanism responsible for the apparent increase has not been identified. Fractionation of liver and duodenum homogenates by a classical differential centrifugation procedure[17] resulted in high recovery and high specific activity in the S and SP fractions (data not tabulated) similar to that obtained by the fractionation procedures routinely used in this study. Minor purification of the $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase enzyme was obtained by fractionation of the SP preparations with $(NH_4)_2SO_4$ (Table II).

Omission of EDTA from the media for homogenization and for resuspension of fractions and omission of EDTA from the standard assay medium did not alter the activities of SP preparations more than ± 10%. Dialysis of SP fractions of either liver or duodenum against 1 mM EDTA in 20 mM Tris–maleate buffer (pH 6.5) (Table II), or against 10 mM EDTA, 1 mM diethylenetriaminepentaacetic acid or 1 mM 8-hydroxyquinoline in Tris–maleate (pH 6.5), did not substantially reduce the activity without added $Zn^{2+}$. The major loss of $Zn^{2+}$-stimulated activity in the duodenal SP preparation during dialysis (Table II) may reflect thermal lability of the duodenal enzyme, since similar losses of activity occurred when dialysis was carried out in the absence of chelating agent, and since the duodenal SP preparation lost 30% of its activity when stored at 2 °C for 24 h.

On the basis of these results, the soluble-precipitated preparations (SP) were used for initial characterization of the $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatases of chick liver and duodenum. The enzymes in SP preparations from both tissues were

stable for several months if stored at −15 °C as either a dry pellet or suspended in Tris–maleate–EDTA medium (pH 7.0). Under the standard conditions of assay, the *p*-nitrophenyl phosphatase activities in these preparations from liver and duodenum were linear for at least 20 min, and there was no initial lag period.

*Effect of pH on activity*

The effect of pH on *p*-nitrophenyl phosphatase activity in the SP preparations from 4-day chick liver, duodenum and metanephros, in the presence or absence of added $Zn^{2+}$, is summarized in Fig. 1. The pH profiles of the high activities with added $Zn^{2+}$ were significantly different for the three tissues, with sharp peaks at pH 4.9 and 6.2 for the duodenum and liver enzymes, respectively, while the metanephros preparation showed a peak at pH 6.7 and a pronounced shoulder at lower pH. For each tissue, the low activity of the SP preparation without added $Zn^{2+}$ showed a broad

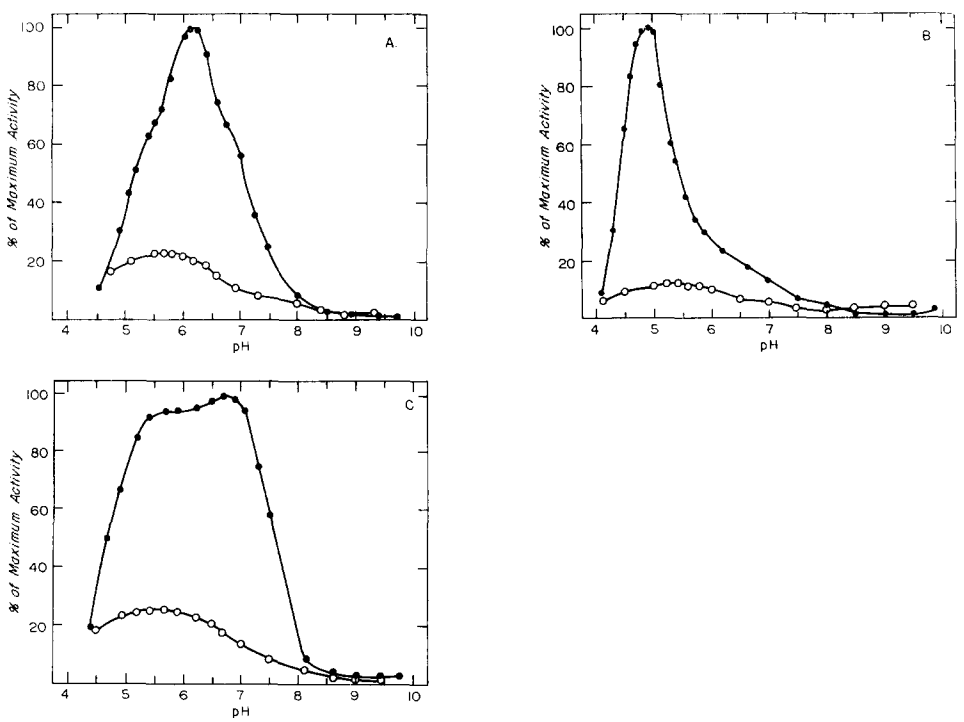


Fig. 1. Effect of pH on $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activities. The activities of the soluble-precipitated fractions (SP) from 4-day chick liver, duodenum and metanephros were determined at various pH values by the *p*-nitrophenol assay described under Methods, in the presence or absence of 10 mM $ZnSO_4$, and with 3 mM *p*-nitrophenyl phosphate as substrate. Buffers used were 50 mM acetate for pH range 4.0–6.0, 50 mM Tris–maleate for pH range 5.5–8.5, and 100 mM 2-amino-2-methyl-1-propanol for the pH range 8.5–10. In the regions of pH overlap, the activities with two sets of buffers were essentially the same under the assay conditions used. A, liver; B, duodenum; C, metanephros. ●—●, activity with added $Zn^{2+}$, plotted as percentage of the activity observed at optimal pH. Specific activities (nmoles *p*-nitrophenol formed/min per mg protein) at optimum pH were, for liver SP, 310; for duodenum SP, 275; for metanephros SP, 160. ○—○, activity without added $Zn^{2+}$. For duodenum and metanephros, activity without $Zn^{2+}$ is plotted on the same scale as activity with $Zn^{2+}$; for liver (A), activity without added $Zn^{2+}$ has been multiplied by 4 for better visualization.

peak in the pH range 5.4–5.8. Profiles obtained for each preparation over the pH range 4.0–8.0 with 10 mM $Mg^{2+}$ added (not plotted) were very similar to those obtained without added metal, except that the activities were 10–25% higher over the pH range studied. The pH profiles of the total particulate fractions (P) from liver and duodenum (not plotted), obtained by the same procedures as for the profiles of Fig. 1 over the pH range 4.5 to 7.0 with and without 10 mM added $MgCl_2$, were similar to those obtained for the analogous SP preparations; peak activity was at pH 5.5–5.8 for both tissues, with or without added $Mg^{2+}$.

*Effects of metal ions on activity*

As is illustrated in Fig. 2, the stimulatory effects of $Zn^{2+}$ vary with buffer and pH. However, 10 mM $Zn^{2+}$ gave maximum activities with the SP fractions from both liver and duodenum with the standard assays used in this study, and higher zinc concentrations were generally inhibitory. Lineweaver–Burk plots from data obtained over the range 0.05 to 10 mM $Zn^{2+}$, all with 3 mM *p*-nitrophenyl phosphate, gave the following apparent $K_M$ values for stimulation by $Zn^{2+}$: for the liver SP fraction in 50 mM Tris–maleate (pH 6.2), 1.7 mM $Zn^{2+}$; for liver SP fraction in 50 mM MES (pH 6.2), 0.5 mM $Zn^{2+}$; for liver SP fraction in 5 mM acetate (pH 5.0), 3.4 mM $Zn^{2+}$; and for duodenum SP fraction in 50 mM acetate (pH 5.0), 2.3 mM $Zn^{2+}$.

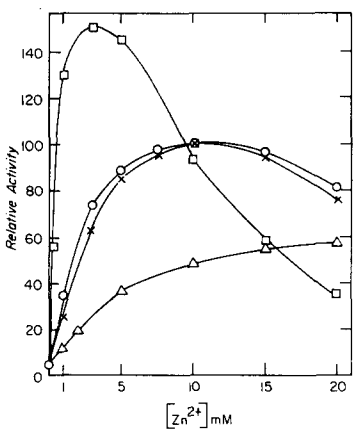


Fig. 2. Effect of $Zn^{2+}$ concentration on activity. Activity of the liver SP and duodenum SP fractions were assayed by the *p*-nitrophenol procedure described under Methods, in the presence of various concentrations of $ZnSO_4$, without addition of EDTA. The curves represent relative activities obtained with various buffers: ○—○, liver SP in 50 mM Tris–maleate (pH 6.2); □—□, liver SP in 50 mM MES (pH 6.2); △—△, liver SP in 50 mM acetate (pH 5.0); ×—×, duodenum SP in 50 mM acetate (pH 5.0). Activity in 50 mM Tris–maleate (pH 6.2) with 10 mM $Zn^{2+}$ is taken as the point of reference for liver SP (specific activity = 290 nmoles *p*-nitrophenol formed/min per mg protein). For duodenum SP, the point of reference is the activity in 50 mM acetate (pH 5.0) with 10 mM $Zn^{2+}$ (specific activity = 260 nmoles *p*-nitrophenol formed/min per mg protein).

That the optimal concentration of $Zn^{2+}$ at pH 6.2 was lower with MES than with Tris–maleate (Fig. 2) may at least in part be due to binding of $Zn^{2+}$ by the Tris–maleate buffer. When the SP fraction of liver was assayed with 3 mM *p*-nitrophenyl phosphate in 25, 50 or 75 mM Tris–maleate (pH 6.2) and with varying concentrations

of $Zn^{2+}$, maximum activity was obtained with 5 mM, 10 mM and 13 mM $Zn^{2+}$, respectively, for the three buffer concentrations. In analogous experiments using MES (pH 6.2) as buffer, maximum activity was obtained with 3–3.5 mM $Zn^{2+}$ at all three buffer concentrations.

Other metal ions in 1 mM or 10 mM concentrations did not effectively substitute for $Zn^{2+}$ in stimulating the *p*-nitrophenyl phosphatase activities of the SP preparations, assayed at optimal pH (liver SP in Tris–maleate buffer, pH 6.2, and duodenum SP in acetate buffer, pH 5.0). Maximum activities obtained, expressed as percentages of the activity of a given preparation with 10 mM $Zn^{2+}$, were 8% without added metal ion, 12% with $Mg^{2+}$, 21% with $Mn^{2+}$, and 17% with $Co^{2+}$; $Pb^{2+}$ and $Ca^{2+}$ were inhibitory, and $(NH_4)^+$ or $K^+$ produced no change. The activity with combinations of 10 mM $Zn^{2+}$ *plus* $Mg^{2+}$, $Mn^{2+}$ or $Co^{2+}$ was always less than with 10 mM $Zn^{2+}$ alone.

Preincubation of SP preparations (4 mg protein/ml) from liver and duodenum for 1 h at 2 °C in 20 mM Tris–maleate (pH 6.2) with 10 mM $Zn^{2+}$ did not produce any significant change in the activity of the preparations in subsequent assays with or without added $Zn^{2+}$, as compared to preparations that had not been preincubated.

*Variation of activity with substrate concentration*

The activities of SP preparations from liver and duodenum were determined by the *p*-nitrophenol assay, with *p*-nitrophenyl phosphate concentrations varied over the range 0.02 mM to 10 mM, both without added $Zn^{2+}$ and with 10 mM $Zn^{2+}$. Lineweaver–Burk plots of the data gave the following apparent $K_M$ values for *p*-nitrophenyl phosphate: for liver SP in 50 mM Tris–maleate (pH 6.2), 0.50 mM *p*-nitrophenyl phosphate without $Zn^{2+}$ and 1.3 mM *p*-nitrophenyl phosphate with $Zn^{2+}$; for duodenum SP in 50 mM acetate buffer (pH 5.0), 0.15 mM *p*-nitrophenyl phosphate without $Zn^{2+}$ and 0.71 mM *p*-nitrophenyl phosphate with $Zn^{2+}$. When liver SP was assayed in 50 mM acetate (pH 5.0), the apparent $K_M$ values were similar to those found for duodenum SP: 0.13 mM *p*-nitrophenyl phosphate without $Zn^{2+}$ and 0.40 mM *p*-nitrophenyl phosphate with 10 mM $Zn^{2+}$.

*Substrate specificity*

The hydrolytic activities of SP preparations from 4-day chick liver and duodenum on various substances in 3 mM concentration were determined at both pH 6.2 in 50 mM Tris–maleate buffer and at pH 5.0 in 50 mM acetate buffer, without added metal and with 3 mM $Mg^{2+}$ or 3 mM $Zn^{2+}$. Table III lists those phosphorylated substances which gave activities at either pH 5.0 or pH 6.2 of at least 5% of the activity obtained with *p*-nitrophenyl phosphate as substrate in the presence of 3 mM $Zn^{2+}$ at the optimal pH for that tissue. The activities with the following substances were less than 5% of that seen for *p*-nitrophenyl phosphate with preparations from either tissue, under all of the assay conditions defined above: α-glycerophosphate, β-glycerophosphate, glucose-6-phosphate, glucose-1-phosphate, galactose-6-phosphate, galactose-1-phosphate, AMP, GMP, UMP, casein (2 mg/ml), and phosvitin (2 mg/ml). Cyclic 3',5'-AMP phosphodiesterase activity, assayed by the procedure of Cheung[18], was negligible. In assays with the *p*-nitrophenol procedure, the following substances also showed less than 5% of the activity seen with *p*-nitrophenyl phosphate: bis (*p*-nitrophenyl) phosphate, *p*-nitrophenyl acetate, *o*-nitrophenyl acetate and *p*-nitrophenyl sulfate.

TABLE III

RELATIVE RATES OF HYDROLYSIS OF VARIOUS SUBSTRATES BY THE SOLUBLE-PRECIPITATED FRACTIONS (SP) FROM LIVER AND DUODENUM

SP fractions from 4-day chick liver and duodenum were assayed by the inorganic phosphate procedure as described under Methods, in 50 mM Tris–maleate buffer (pH 6.2) and 50 mM acetate buffer (pH 5.0) with 3 mM substrate, without added metal ion and with 3 mM $MgCl_2$ or 3 mM $ZnSO_4$. The activities are expressed relative to the activity observed with *p*-nitrophenyl phosphate as substrate in the presence of $Zn^{2+}$ at the preferred pH of the tissue, *i.e.* pH 6.2 for liver SP (Specific activity = 230–250 nmoles/min per mg protein), and pH 5.0 for duodenum SP (specific activity = 240–280).

| *Substrate* | *pH* | *Relative activity in SP fraction from* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Liver* | | | *Duodenum* | | |
| | | — | $Mg^{2+}$ | $Zn^{2+}$ | — | $Mg^{2+}$ | $Zn^{2+}$ |
| *p* Nitrophenyl phosphate | 6.2 | 6 | 9 | 100 | 6 | 11 | 44 |
| | 5.0 | 10 | 11 | 62 | 7 | 11 | 100 |
| Phenyl phosphate | 6.2 | 4 | 4 | 3 | 2 | 2 | 2 |
| | 5.0 | 5 | 5 | 4 | 2 | 2 | 2 |
| ATP | 6.2 | 3 | 11 | 9 | 3 | 8 | 6 |
| | 5.0 | 2 | 6 | 4 | 1 | 3 | 2 |
| ADP | 6.2 | 1 | 10 | 1 | 0 | 5 | 0 |
| $PP_i$ | 6.2 | 2 | 430 | 3 | 3 | 560 | 4 |
| Fructose-1,6-$P_2$ | 6.2 | 1 | 41 | 2 | 2 | 11 | 2 |
| DL-Phosphoserine | 6.2 | 1 | 9 | 1 | 0 | 0 | 0 |

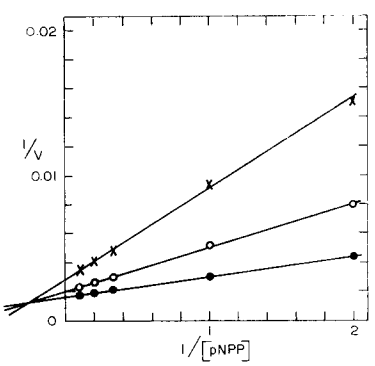


Fig. 3. Inhibition of $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase by ATP. Aliquots of SP fraction from liver containing 0.01 mg protein were assayed by the *p*-nitrophenol procedure described under Methods in 50 mM Tris–maleate (pH 6.2) with 10 mM $Zn^{2+}$ and with concentrations of ATP and *p*-nitrophenyl phosphate varied as indicated. The reciprocal of the activity is plotted against reciprocal of *p*-nitrophenyl phosphate concentration. Less than 10% of the substrate was consumed with the lowest *p*-nitrophenyl phosphate concentration used. ●—●, no ATP; ○—○, 1 mM ATP; ×—×, 3 mM ATP. Substrate concentration in mmoles/l, velocity in nmoles *p*-nitrophenol formed/min per mg protein. Specific activity of preparation with 3 mM *p*-nitrophenyl phosphate and no ATP = 295 nmoles *p*-nitrophenol formed/min per mg protein.

With the compounds tested, only the hydrolysis of *p*-nitrophenyl phosphate was markedly stimulated by $Zn^{2+}$. The hydrolysis of ATP by SP preparations from both liver and duodenum was moderately stimulated by $Zn^{2+}$, but in each case the stimulation was less than that elicited by $Mg^{2+}$ (Table III); these stimulatory effects of $Zn^{2+}$ and $Mg^{2+}$ were not additive (data not tabulated). ATP was found to inhibit

the $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activity of liver SP preparations, and this inhibition could be reduced by increasing the concentration of *p*-nitrophenyl phosphate or $Zn^{2+}$, relative to ATP, over certain concentration ranges. However, as is illustrated in Fig. 3 for such a set of conditions, the inhibition by ATP appeared to be of a non-competitive or mixed type with respect to *p*-nitrophenyl phosphate, rather than typical competitive inhibition.

## DISCUSSION

The soluble cytoplasm fractions of chick liver, duodenum and metanephros contain substantial $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activities whose pH optima are in the region of pH 5–7 but are different in the three tissues. These activities are distinct from the particulate *p*-nitrophenyl phosphatases of these tissues, which are most active at pH 5.5–5.8 and are inhibited by concentrations of $Zn^{2+}$ that are optimal for the soluble activities[12]. The pH optimum of 6.2 obtained for the SP fraction of liver is similar to the value of 5.8 found by Wang[13] for the total soluble fraction of chick liver. It is not known whether the low activity of the SP fraction without added $Zn^{2+}$ is a property of the enzyme(s) responsible for the high activity with added $Zn^{2+}$. In each of the three tissues, the pH optimum of this activity without added $Zn^{2+}$ in the SP preparation was similar to that of the particulate *p*-nitrophenyl phosphatase activity in the acid and neutral pH range.

The studies on substrate specificity suggest that the enzyme(s) in liver and duodenum responsible for $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activity at neutral or acid pH are phosphoric monoester hydrolases of restricted substrate specificity, whose probable physiologic substrate(s) cannot be identified at present. It cannot be ruled out that this activity is a property of some other phosphatase known to be present in the crude SP preparations, or that several enzymes contribute to the activity. It appears unlikely that the $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activity is catalyzed by the fructose-1,6-diphosphatase (fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) or inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) present at high level in the SP preparations: both fructose-1,6-diphosphatase and inorganic pyrophosphatase require $Mg^{2+}$ for activity and are markedly inhibited by the concomitant addition of $Zn^{2+}$ [12], and the patterns of change in the activity of these enzymes in liver and duodenum during development are different from the changes in $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activity[12]. The inhibition of $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase activity by ATP suggested a possible relation between this enzyme and the ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity in the SP preparation. However, this inhibition by ATP did not appear to represent typical competition of substrates for the same enzyme site(s) (Fig. 3), and may in part be due to binding of $Zn^{2+}$ by ATP[19].

The low activity of the soluble $Zn^{2+}$-stimulated *p*-nitrophenyl phosphatase preparations from the chick tissues toward most other substrates tested suggests that these enzymes are different from previously described acid or neutral phosphatases of microbial cells or mammalian tissues that are active with various physiological and non-physiological substrates[6,8,20–26]. The marked stimulation by $Zn^{2+}$ of the soluble *p*-nitrophenyl phosphatases described in this paper also appears to distinguish them from various acid or neutral phosphatases of mammalian cells that are

highly active with *p*-nitrophenyl phosphate as substrate[22,25,27–31], but the effects of $Zn^{2+}$ were not always examined in those earlier studies.

The role(s) of $Zn^{2+}$ in the stimulation of soluble *p*-nitrophenyl phosphatase activity remains unclear. $Zn^{2+}$ is the most effective if not the specific ion for this stimulatory effect. There appears to be a direct and rapid interaction of $Zn^{2+}$ with some component of the catalytic system, since no lag period is observed in the assays. There is no evidence of an additional requirement for firmly-bound $Zn^{2+}$ or other metal as an essential component of the enzyme. The high concentration of $Zn^{2+}$ required for maximum activity is more typical of a metal activator than of a metalloprotein component[32], and the apoenzymes of some metalloprotein phosphatases are reactivated by preincubation with 0.01–0.03 mM $Zn^{2+}$ (refs 8, 9, 33). Stimulation of the soluble *p*-nitrophenyl phosphatases by $Zn^{2+}$ may involve formation of a metal complex with substrate rather than with enzyme. Formation of such complexes was proposed for the $Mg^{2+}$-stimulated enzymatic hydrolysis of inorganic pyrophposhate[34,35] and for the $Zn^{2+}$-stimulated hydrolysis of ATP by inorganic pyrophosphatase[36]. Since $Zn^{2+}$ did not stimulate the hydrolysis of phenyl phosphate by the soluble *p*-nitrophenyl phosphatases (Table III), the formation of a complex involving the electronegative nitro group in *p*-nitrophenyl phosphate and the $Zn^{2+}$ and/or a charged group on the enzyme may be a factor in enhancing either binding of *p*-nitrophenyl phosphate to the enzyme or catalytic action on this substrate.

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