BBA 66557

# PHYTASE AND ALKALINE PHOSPHATASE ACTIVITIES IN INTESTINAL MUCOSAE OF RAT, CHICKEN, CALF, AND MAN

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(Received September 24th, 1971)
(Revised manuscript received December 7th, 1971)

## SUMMARY

Phytate hydrolysis (phytase (*meso*-inositol-hexaphosphate phosphohydrolase, EC 3.1.3.8)) activity was demonstrated in the extracts of the mucosae of the small intestine of the rat, chicken, calf, and man. The four species differed markedly in pH–phytase activity profiles and these in turn differed from those of the intestinal alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) measured by release of phosphate from *p*-nitrophenyl phosphate or 3-glycerophosphate.

Partial separation of phytase from phosphatase activities was accomplished in the course of purification of the enzymes by treatment with *n*-butanol and $(NH_4)_2SO_4$, followed by chromatography on DEAE-cellulose. Differences in substrate–activity relationships, and inhibition by L-phenylalanine and NaF provided additional support for the hydrolysis of phytate as an activity distinct from that promoting hydrolysis of *p*-nitrophenyl or 3-glycerophosphates. Evidence to the contrary based on assays of the two activities in the presence of mixture of their substrates is invalidated by the finding that sodium phytate inhibits intestinal alkaline phosphatase activity.

Treatment of phytase concentrates with EDTA lowers their activity significantly. Alkaline phosphatase is affected in a similar manner. Partial restoration in chicken and enhancement in rat preparations of both activities follows addition of $Zn^{2+}$. Higher concentrations of $Zn^{2+}$ inhibit, although at different levels.

## INTRODUCTION

Recently it has been recognized that populations of developing countries subsisting mainly upon cereals may consume large quantities of phytic acid (inositol hexaphosphoric acid) as phytate[1]. Phytic acid is known to decrease absorption from the intestine of calcium, magnesium, iron and zinc. Its presence in high concentrations in food may be harmful because of this effect upon divalent metal uptake.

That the intestine has the ability to split phosphate from phytic acid was first shown by Patwardhan[2] who attributed this action to phytase (*meso*-inositol-hexaphosphate phosphohydrolase, EC 3.1.3.8). He believed this ability to be peculiar to the rat, but others have shown that phosphate is split from phytic acid in intestines of the chicken[3], pig and cow[4], and sheep[5]. The intestine is by far the most active among various tissues examined[6], and maximal activity occurs in the proximal 12 inches of rat intestine[7].

The importance of phytate-splitting activity in the human intestine, insofar as destruction of phytate is concerned, is unknown. That this activity exists in man is shown in this paper, and obviously, it would be advantageous if appreciable portions of the ingested phytate could be hydrolyzed. However, little information is available regarding the parameters defining phytate-splitting activity of the intestine, and indeed, its attribution to phytase has been questioned[6,8]. We have, therefore, examined certain aspects of its behavior. Our findings are described in the present paper.

## METHODS

### *Materials*

Sodium phytate was prepared from calcium phytate (Calbiochem) by the method of Peers[9]. The free inorganic phosphate concentration was less than 3% of the total phosphate. *p*-Nitrophenyl phosphate or disodium $\beta$-glycerophosphate substrates were used as purchased from the Sigma Chemical Co. *n*-Butanol was distilled through a fractionating column. Sucrose and $(NH_4)_2SO_4$ were purchased as specially purified preparations for enzyme analysis (Mann Research Laboratories). DEAE-cellulose (Sigma Chemical Co.) was conditioned by treatment with 0.1 M NaOH and then with deionized water. Sephadex G-200 (particle size 40–120 $\mu$m) was purchased from Pharmacia, Uppsala.

### *Animals*

The rats used weighed 200–350 g and were mainly of a Sprague–Dawley strain maintained in the Animal House of the Medical School of the American University of Beirut. These were at times supplemented with rats obtained from Animal Suppliers, Ltd, England. 10–20 rats were killed by exsanguination after etherization and the intestines were flushed with cold 0.14 M NaCl, and processed within 1 h. The mucosas were removed by stripping the unopened proximal two-thirds of the small gut. Calf intestines were obtained from the Beirut Slaughter House shortly after death. They were washed thoroughly with tap water and stored frozen. The mucosa of one calf was processed at a time. Intestines of white Leghorn chickens were chilled immediately after death by exsanguination and washed with tap water. The mucosas of 40–60 chickens were stripped and combined for processing. Five human intestines were obtained at necropsy by courtesy of the Department of Pathology of this University. All were normal in appearance. As in the rat, the mucosa was collected from the proximal two-thirds of the small intestine, the ileal portion being rejected.

### *Partial purification of phytase and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1)*

Both enzymes were treated according to procedures adapted from those of

Morton[10] and Behal and Center[11]. The chilled mucosae of calf and chicken were diluted 1:1 (v/v) with cold 0.14 M NaCl, the pH adjusted to 7.5 by 0.1 M NaOH, the suspension stirred mechanically for 30 min, with final maceration for 1 min in a Waring blendor. Calf and chicken homogenates were filtered through washed cotton wool after centrifugation at 1000 × *g* for 20 min. Rat and human homogenates were diluted 1:10 (v/v) and filtered directly. All operations were carried out at 5 °C unless otherwise mentioned.

The filtrates were adjusted to pH 5.0 by slow addition of 2 M sodium acetate buffer (pH 4.0). After 45 min, a gray precipitate was separated by centrifugation at 1800 × *g* for 30 min. The precipitate was dispersed in 0.14 M NaCl, adjusted to pH 7.50 with 0.5 M $Na_2CO_3$ and stirred slowly overnight. The treatment at pH 5.0 was repeated, and the resulting precipitate was washed into centrifuge cups with the aid of cold water and centrifuged. The precipitate was again dispersed in water and adjusted to pH 7.5 as before, 0.4 vol. of *n*-butanol was then added slowly at room temperature with stirring. After centrifugation at 1800 × *g* for 30 min, the lower aqueous layer was aspirated and dialyzed through cellophane against water overnight. The dialysate was filtered through a 4-cm layer of Hyflo Super Cel on a Büchner funnel to remove insoluble material. The pH was adjusted and maintained at 7.0 with $NH_4OH$ (1:5, v/v) while solid $(NH_4)_2SO_4$ was added slowly with stirring until a concentration of 45% satn was attained[12]. The solution was centrifuged and the precipitate discarded. Additional $(NH_4)_2SO_4$ was added to 70% satn without further adjustment of pH. Most of the phytase and alkaline phosphatase activities precipitated between these two concentrations.

*Chromatography on DEAE-cellulose*

A portion of the precipitate dissolved in a minimal volume of 0.05 M Tris–HCl at pH 7.8 was dialyzed exhaustively against this buffer. The sample was then applied to a DEAE-cellulose column (20 cm × 1 cm) packed by gravity and equilibrated with the same buffer. Elution was with successive 10-ml portions of increasing NaCl concentrations (0, 0.10, 0.15 and 0.20 M) in 0.05 M Tris–HCl buffer (pH 7.8). 2-ml samples of effluent were collected automatically and pressure was kept constant by use of a Mariotte bottle.

*Kinetic and inhibition studies*

Kinetic behavior was examined by assaying activities of partially purified enzymes at various concentrations of sodium phytate and *p*-nitrophenyl phosphate in the absence and presence of 0.0022 M L-phenylalanine or 0.02 M NaF. The effects of increasing concentrations of sodium phytate added to *p*-nitrophenyl phosphate containing assay mixtures at pH 9.5 and of *p*-nitrophenyl phosphate upon sodium phytate-containing mixtures also were examined.

*Measurement of phytase and alkaline phosphatase activities*

Sodium phytate, disodium *p*-nitrophenyl phosphate or disodium β-glycerophosphate were used as substrates in concentrations found to produce optimal activities. Tris–succinate was used as buffer for the pH range 6.4–8.3, Tris–HCl in the range 8.5–9.5 and glycine–NaOH in the range 9.5–10.5. Each buffer was 0.04 M. All reaction mixtures contained $Mg^{2+}$ in concentrations shown in the protocols. Final volumes of the reaction system were 5 ml unless otherwise stated

in the legends. Incubations at 37 °C for 15 min were stopped by the addition of 5 ml of 10% trichloroacetic acid. Liberated phosphate was measured by the method of Fiske and SubbaRow[13], and activity expressed as μmoles $P_i$/min per ml at 37 °C. In later experiments dealing with the effects of $Zn^{2+}$, alkaline phosphatase activities were determined by direct measurement of absorbance at 410 nm of the *p*-nitrophenol produced in 60 s.

*Specific activities*

Protein concentrations were measured by the biuret reaction[14] using bovine albumin (Fraction V, Sigma) as a standard or spectrophotometrically at 260 and 280 nm for colorless solutions. Protein concentration was calculated from the latter with the aid of Kalckar's formula[15], mg protein/ml = $(A_{280\text{ nm}} \times 1.45) - (A_{260\text{ nm}} \times 0.74)$. Specific activity is expressed as μmoles/min per mg of protein at 37 °C.

## RESULTS

*Phytase and alkaline phosphatase activities during purification*

Table I shows that accretions of phytase and phosphatase specific activities differed during the purification procedure. Gains were greater for alkaline phosphatase than phytase activity of rat and calf mucosas. The opposite was true of the chicken.

TABLE I

SPECIFIC ACTIVITIES OF INTESTINAL PHYTASE AND PHOSPHATASE IN THE COURSE OF PURIFICATION

Stage 1, homogenate in 0.14 M NaCl (rat) or water (calf, chicken) after centrifugation; Stage 2, the second pH 5.0 precipitate taken up in water at pH 7.5; Stage 3, aqueous phase after *n*-butanol treatment and centrifugation for 30 min at 18 000 × *g*; Stage 4, activities precipitated by $(NH_4)_2SO_4$ between 40 and 70% satn; Stage 5, eluate from DEAE-cellulose in 0.1 M NaCl buffered with 0.05 M Tris–HCl buffer at pH 7.8. Assay systems: Rat, 1.57 mM sodium phytate or disodium *p*-nitrophenyl phosphate, 0.04 M Tris–succinate buffer (pH 7.0). Calf, 0.44 mM sodium phytate or sodium β-glycerophosphate, 0.04 M Tris–HCl buffer (pH 8.6). Chicken, same as for calf except that the buffer was Tris–succinate (pH 8.3) and the phosphatase substrate was *p*-nitrophenyl phosphate. All systems contained 0.001 M $MgCl_2$, their final volumes were 5 ml.

| | *Stage* | *μmoles/min/mg protein* | | *Ratio phosphatase/phytase* |
|---|---|---|---|---|
| | | *Phytase* | *Phosphatase* | |
| Rat | 1 | 1.6 | 1.9 | 1.18 |
| | 3 | 24.1 | 37.8 | 1.57 |
| | 4 | 35.8 | 63.5 | 1.78 |
| | 5 | 81.0 | 153.0 | 1.89 |
| Calf | 1 | 0.33 | 0.74 | 2.22 |
| | 2 | 0.31 | 0.80 | 2.58 |
| | 3 | 1.80 | 6.00 | 3.33 |
| | 4 | 28.6 | 102.0 | 3.57 |
| Chicken | 1 | 1.08 | 2.28 | 2.11 |
| | 2 | 1.42 | 2.96 | 2.07 |
| | 3 | 4.35 | 7.70 | 1.72 |
| | 4 | 4.0 | 24.4 | 0.72 |

*Chromatography on DEAE-cellulose*

Elution of the partially purified preparations showed two well-defined zones of activity for both enzymes (Fig. 1). These differed, however, in the proportion of total

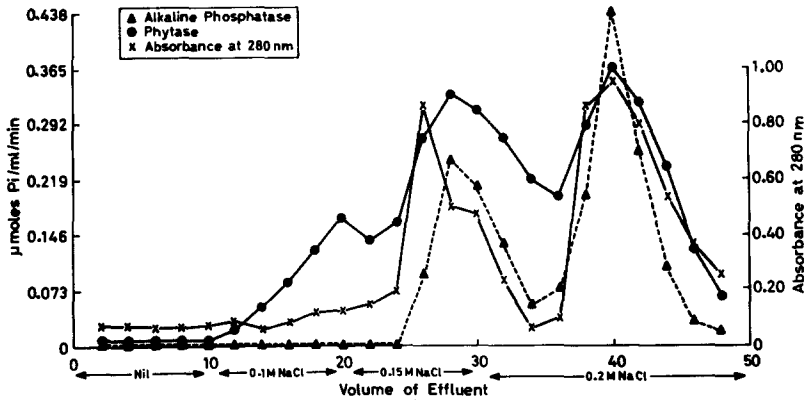


Fig. 1. Elution of phytase and phosphatase activities of intestinal mucosa of the chicken. Stage 5 preparations (Table I) were used in this and subsequent experiments. Both assay systems contained 0.44 mM sodium phytate or disodium *p*-nitrophenyl phosphate (pNPP), 1 mM $Mg^{2+}$, 0.04 M Tris–succinate buffer at pH 8.3 in a volume of 4.5 ml. Flow rate was 0.33 ml/min. NaCl concentrations of the eluant are shown along the abscissa. Volumes are in ml.

phytase and phosphatase activities appearing in the two zones. Most of the phytase activity of the rat was released into eluant of higher NaCl concentrations. The behavior was reversed when preparations from the chicken were subjected to similar treatment and substantial increments of phytase activity were eluted by 0.1 M NaCl before any phosphatase activity was detected (Fig. 1). On the other hand, most of the phosphatase required higher concentrations of NaCl for elution in the chicken. In both rat and chicken, phytase activities were less sharply resolved than those of phosphatase. No separation was effected on Sephadex G-200.

*pH–activity relationships*

The two activities differ markedly in pH optima (Figs 2 and 3) with striking

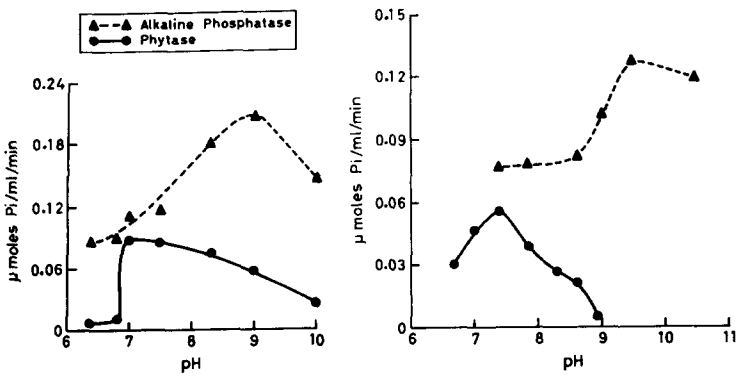


Fig. 2. pH–activity relationships of phytate compared with those of phosphatase in a partially purified preparations of rat intestinal mucosa (left) and homogenate of human mucosa (right). Activity was measured by means of the reagents described in Fig. 1 except that the buffers were varied. Tris–succinate buffer was used for the pH range 6.4–8.3; Tris–HCl buffer for pH 8.6–9.6 and glycine–NaOH buffer to obtain pH 10 and above. All buffers were 0.04 M. pH was adjusted to the desired value by titration.

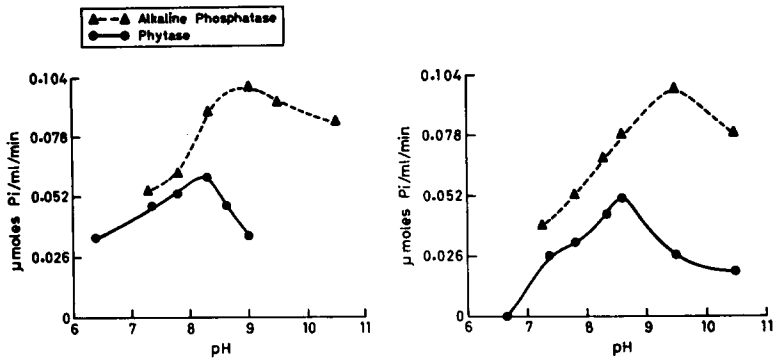


Fig. 3. pH–activity relationships of partially purified preparations of intestinal phytase and phosphatase of the chicken (left) and calf (right). The enzymes were assayed as described in Fig. 2 except that sodium $\beta$-glycerophosphate (0.44 mM) served as the substrate for phosphatase assay of the calf preparation. The buffers used were those listed under Fig. 2.

differences between the phytase activities of the four species. In the rat, optimal phytase activity of the partially purified enzyme extended from pH 7.0 to 7.5 with a gradual decrease between pH 7.5 and 8.4 (Fig. 2). Appreciable activity persisted at pH 10.0. Activity fell abruptly below pH 7.0. In homogenates of human mucosa, a sharply-defined maximum was located at pH 7.4 and activity approached zero at pH 9.0 (Fig. 2). The optimal pH for the partially purified phytase of the chicken was well-defined at pH 8.3, and that of the calf at pH 8.6 (Fig. 3). The activity of the chick phytase was high at pH 6.4; that of the calf nil at pH 6.6. Each phosphatase activity maximum was higher than that of the phytase of the same species by one to two pH units. The pH–activity curves of phosphatase also differed in profile from those of the phytases.

*Substrate–activity relationships*

The response of phytase activity of the rat preparation to increasing sodium phytate concentrations conformed to Michaelis–Menten kinetics (Fig. 4). Human

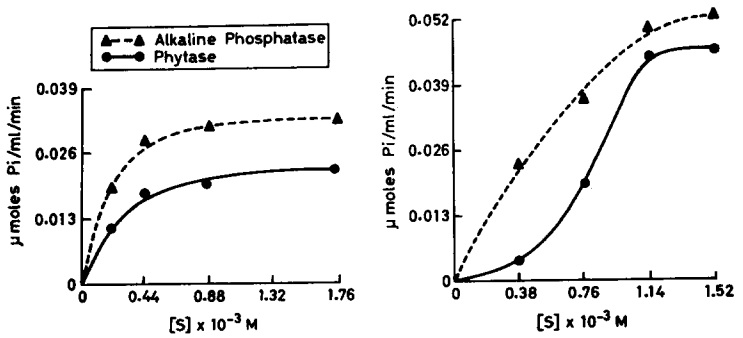


Fig. 4. The effect of increasing sodium phytate or *p*-nitrophenyl phosphate concentrations upon phytase or phosphatase activities of partially purified preparations of intestinal mucosa of the rat (left) and homogenate of the intestinal mucosa of man (right). Assays were made as described in Fig. 1 except that sodium phytate and *p*-nitrophenyl phosphate concentrations were 1.5 mM and pH 7.0 for the rat and for human material.

homogenates traced an S-shaped curve with rising substrate concentrations (Fig. 4). The preparations from chicken and calf showed inhibition by sodium phytate at relatively low concentrations. On the other hand, the activities of the phosphatases of each of the four species showed typical Michaelis–Menten curves (Fig. 4).

*L-Phenylalanine inhibition*

Both phytase and phosphatase of the rat were inhibited by L-phenylalanine. However, phytase was inhibited competitively (Fig. 5) and phosphatase uncompetitively.

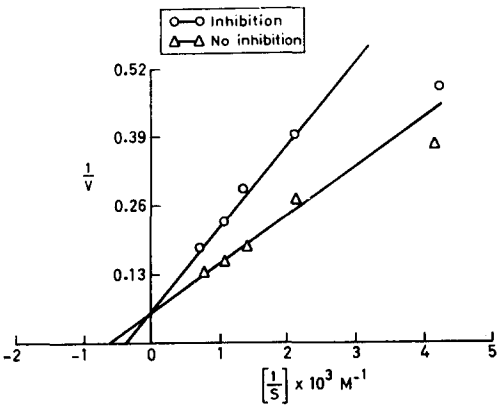


Fig. 5. Inhibition of intestinal phytase activity of the rat by L-phenylalanine. The reaction system contained sodium phytate, 1 mM $Mg^{2+}$, 0.044 M Tris–succinate buffer (pH 7.0). To this was added 0.022 M L-phenylalanine (○—○) or water (△—△). In this preparation, phytase activity of Stage 5 (Fig. 1) was increased 60-fold over the original homogenate. The eluate contained 0.027 mg of protein per ml.

*Other inhibitors*

Phytase activity in the rat was decreased by 36% in the presence of 0.02 M NaF. Phosphatase was unaffected at this concentration.

*Inhibition of alkaline phosphatase activity by phytate*

When enzyme assay systems either from rat or chicken included both *p*-nitrophenyl phosphate and sodium phytate, the yield of liberated phosphate was substantially less than that of the same enzyme preparations assayed with *p*-nitrophenyl phosphate alone. The cause of the lowered yield was a potent inhibition of alkaline phosphatase activity by phytate. Fig. 6 shows that the inhibition is uncompetitive for preparations from the rat, resembling that demonstrated for the action of L-phenylalanine. In preparations from the chicken, the inhibition differed in type from that in the rat, being noncompetitive (Fig. 7). Under similar conditions, disodium β-glycerophosphate had no effect on *p*-nitrophenyl phosphate hydrolysis by phosphatase preparations.

*$Zn^{2+}$ as a cofactor for phytase activity*

Addition of $Zn^{2+}$ to the rat phytase assay system (at pH 7.4) caused the activity to rise by about 40% as $Zn^{2+}$ concentration increased from 0.02 to 0.08 mM. At $Zn^{2+}$ concentrations exceeding 0.625 mM, zinc phytate precipitated.

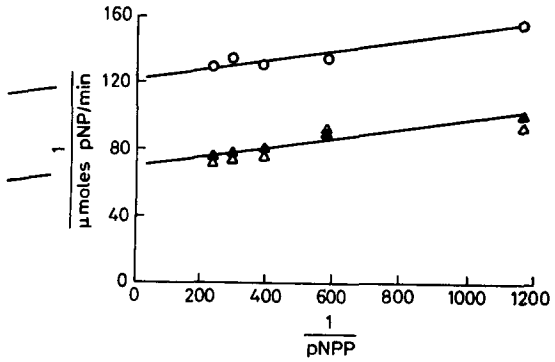


Fig. 6. Inhibition of rat intestinal phosphatase by sodium phytate. ▲—▲ describes the activity of a Stage 5 75-fold purified intestinal phosphatase in a system containing *p*-nitrophenyl phosphate; 0.04 M glycine–NaOH buffer (pH 9.5); 1 mM $Mg^{2+}$ in a volume of 1 ml. △—△, the activity of the same system in the presence of 1 mM sodium *β*-glycerophosphate; ○—○, the activities of the system in the presence of 1.32 mM sodium phytate.

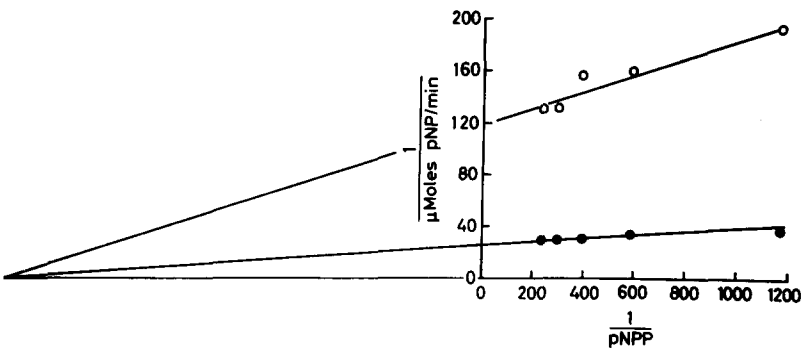


Fig. 7. Inhibition of intestinal phosphatase of the chicken by sodium phytate. The assay system was the same as that described in Fig. 9 except that the pH was adjusted to 10.0. ○—○, activities in the presence of 0.26 mM sodium phytate; ●—●, activities in its absence.

Addition of $Zn^{2+}$ to the phosphatase assay system (at pH 9.5) also caused the activity to rise to about the same extent as that of phytase. The maximum increase in activity occurred in 0.05 mM $Zn^{2+}$. Higher concentrations of $Zn^{2+}$ caused inhibition.

Removal of $Zn^{2+}$ by dialysis against 0.001 M disodium EDTA decreased phytase activity by one-third in the preparation of rat intestine but abolished it completely in that of the chicken (Fig. 8). In these experiments $Mg^{2+}$ was replaced before making assays. Addition of $Zn^{2+}$ to the EDTA-treated preparation not only restored but augmented phytase activity of the rat preparation to values some 40% higher than the original when the concentration rose to 0.375 mM. On the other hand, only a partial restoration of activity was brought about when $Zn^{2+}$ was added to preparations of chicken phytase.

Phosphatase subjected to similar treatment with EDTA and $Zn^{2+}$ responded in much the same way as the phytase (Fig. 8) but tolerated higher concentrations of $Zn^{2+}$. In the chicken, as with phytase, the restoration of activity was only partial.

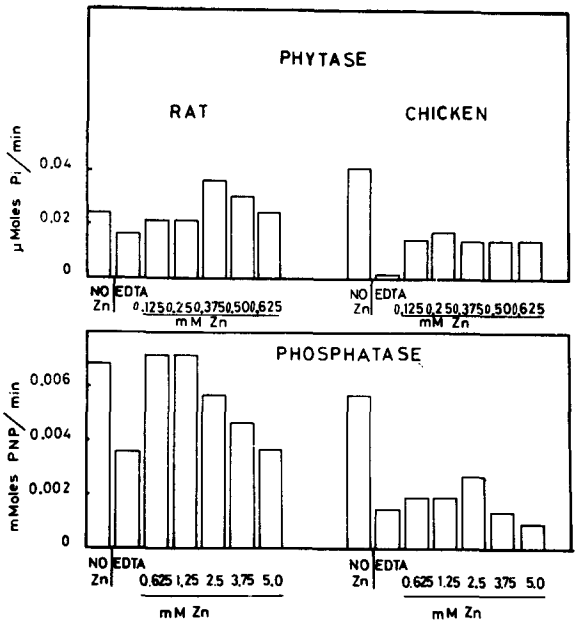


Fig. 8. The effect of removal and addition of $Zn^{2+}$ upon intestinal phytase and phosphatase activity. The activities of partially purified Stage 5 (Fig. 1) enzyme preparations of rat (left) and chicken (right) were assayed as described below. The dialysis for 24 h against 1 mM disodium EDTA brought about the decrease in activities shown. The addition of $Zn^{2+}$ to the EDTA-treated solutions partly restored phytase activity in the chick and enhanced it in the rat preparations. Phosphatase activity is similarly affected although optimal activity occurred at different $Zn^{2+}$ concentrations. Precipitation of zinc phytate at concentrations exceeding 0.625 mM limited the range of $Zn^{2+}$ concentrations available for examination. The phytase assay system included 2.6 mM sodium phytate, 0.04 M Tris–succinate buffer pH 7.4, 1 mM $Mg^{2+}$ and $Zn^{2+}$ at the concentrations indicated. The phosphatase assay system included 1.43 mM *p*-nitrophenyl phosphate, 0.04 M glycine–NaOH buffer (pH 9.5), 2 mM $Mg^{2+}$ and $Zn^{2+}$ as shown.

*Thermal inactivation*

A preparation of rat mucosa in which phosphatase and phytase activities were purified 50-fold containing 0.10 mg protein per ml was heated at 50 °C for 30 min in the presence of sodium phytate or *p*-nitrophenyl phosphate (1.87 mM), 1 mM $Mg^{2+}$, and 0.04 M Tris–succinate buffer (pH 7.0). Less than 10% inactivation occurred. This rose to 70% inactivation for phytase and 65% for phosphatase at 60 °C in 30 min. Inactivation of phytase was consistently somewhat greater (about 5%) than that of phosphatase.

*Phytase in human gut mucosa*

Homogenates of each of the mucosas of the five human small intestines examined showed phytase specific activities of the same order as those of the other species.

## DISCUSSION

Intestinal phytase activity has been attributed to the action of the non-specific alkaline phosphatases of the intestine[6,8]. The results of experiments in which addition of phytate to an alkaline phosphatase assay system failed to yield the increment of

phosphate expected if two enzymes were acting independently were offered as the principle support for this claim[8]. However, the inhibitory action of sodium phytate upon alkaline phosphatase activity demonstrated in this paper makes conclusions based on substrate combination invalid. Others have offered as evidence for a close association[16] the similarity of the pH optima of the two activities in the chick. However, this does not apply to the other three species examined and so loses force.

On the other hand, considerable evidence has been obtained indicating that the agent cleaving phosphate from phytate is not the same as the alkaline phosphatase that attacks *p*-nitrophenyl phosphate or *β*-glycerophosphate. This evidence includes the partial separation of phytate-splitting and *p*-nitrophenyl phosphate activities in the course of purification by the Morton[10] procedure and also by elution in an NaCl gradient from DEAE-cellulose. Among the eluates, were fractions that exhibited phytase but no phosphatase activity. Moreover, differences in pH optima of the phytases compared with those of the phosphatases suggest that more than one enzyme is involved. These differences are not explained by the peculiarities of phytate with its multiplicity of anionic groups as a substrate because the phytate-splitting activity of the four species studied ranged from an optimum of pH 7.0 in the rat to 8.6 in the calf, with human and chicken enzymes at intermediate pH values. In addition, the phytases differed in kinetic behaviour from alkaline phosphatases in three of the four species examined, the differences being greatest in the human preparations where velocity–substrate relationships followed a sigmoidal course for phytase in contrast to the typical Michaelis–Menten relationship exhibited by intestinal phosphatase.

The susceptibility of the two activities to L-phenylalanine inhibition differed in extent and type. Inhibition of phytase was competitive, that of phosphatase uncompetitive. The former was more sensitive to inhibition by $F^-$ than was alkaline phosphatase. On the other hand, the two activities were similar in that chromatography on DEAE-cellulose or Sephadex G-200 yielded two zones of isoenzyme activity which corresponded save for the exceptions noted. Thermal inactivation, subcellular distribution, and dependence of both upon $Mg^{2+}$ and $Zn^{2+}$ provide further evidence of similarity.

The ability of intestinal alkaline phosphatase to attack a variety of substrates makes reasonable the supposition that it would also split phosphate from phytic acid. Yet the differences observed are sufficient to prevent full acceptance of this view. The failure of phytase activity to parallel *p*-nitrophenyl phosphate or *β*-glycerophosphate activity in many circumstances recalls a similar lack of conformity of response by a number of other substrates found by Behal and Center[11]. These authors suggest that intestinal alkaline phosphatase is quite heterogeneous. Davies *et al.*[17] found that the ratios of phosphatase to phytase activities in chick intestinal mucosa varied with with sex, breed, and environmental factors. The source of phytate-splitting activity has more than academic significance. If phytase is a separate entity under independent genetic regulation, anomalies may be encountered. These may affect the ability of man to tolerate high phytate intakes.

Clues to a possible involvement of $Zn^{2+}$ in phytase activity were provided by the decreased activity of intestinal phytate-splitting activity observed in $Zn^{2+}$-depleted rats soon after $Zn^{2+}$ deprivation was initiated[18] often without a comparable change in alkaline phosphatase activity. The dependence of phytase upon $Zn^{2+}$ is clearly demon-

strated *in vitro* by the complete restoration of activity to rat mucosal preparations that occurs following replacement of zinc previously removed by dialysis against EDTA. In the chick, however, only partial restoration of activity was effected in similar experiments, probably because the enzyme was unstable in the absence of $Zn^{2+}$ and $Mg^{2+}$. Aders and Hill[16] found partial restoration of intestinal phytase activities in the chick after replacement of $Zn^{2+}$ previously removed by EDTA treatment. $Co^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ were without effect.

ACKNOWLEDGEMENTS

This work was supported by Grant AM-05622 from the National Institute of Arthritis and Metabolic Diseases.

The authors acknowledge the helpful suggestions offered by Dr Nabil Wakid.

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