Chick Aorta Pyrophosphatase[1] (37249)

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(Introduced by R. Freedland)

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Aorta inorganic pyrophosphatase (Pyrophosphate pyrohydrolase, E.C. 3.6.1.1) may be important in the regulation of calcium metabolism in the aorta. This is primarily due to its role in maintaining homeostasis of inorganic pyrophosphate levels. Inorganic pyrophosphate at micromolar concentrations can inhibit the deposition of calcium in aorta tissue and on calcifying matrices (1–4). It has been proposed that the presence of calcium ion may also act as a self regulator in the process by inhibiting pyrophosphatase (1). The inhibition of pyrophosphatase would cause an increase in pyrophosphate concentration and thereby serve to promote the removal of calcium before deposition as calcium phosphate salts.

Reported here are some properties of chick aorta inorganic pyrophosphatase. The effect of calcium ion on activity was studied with respect to the mechanism by which it inhibits inorganic pyrophosphatase. Pyrophosphatase levels in tissue extracts obtained from magnesium-deficient and sufficient chicks were also investigated to determine the interrelationship between a condition which induces aorta calcification and pyrophosphatase activity. Like other inorganic pyrophosphatases (5–10), chick aorta pyrophosphatase was activated by magnesium. Of additional interest, the aorta enzyme appeared associated with fractions rich in collagen during partial purification and was inhibited when collagen was added to enzyme assays.

Materials and Methods. Preparations of partially purified aorta pyrophosphatase were obtained in the following manner. Approximately 20 g of chick aorta was homogenized in ice-cold 0.1 *M* Tris–HCl buffer (pH 7.6) containing 0.1 m*M* EDTA and 0.1 m*M* mercaptoethanol (5). The homogenate was stirred for 5 hr and the extract was obtained after centrifugation (25,000*g*; 60 min). To the extract, ammonium sulfate was added to give 0.5 saturation (fraction I). The resulting precipitate was separated by centrifugation and the procedure was repeated to yield protein precipitates at a 0.65 (fraction II) and a 0.85 (fraction III) saturation of ammonium sulfate, respectively. The precipitates (fractions I, II, III) were each suspended in 0.05 *M* Tris–HCl buffer, pH 7.6 (3–4 mg of protein/ml) and dialyzed overnight against the same buffer containing again 0.1 m*M* EDTA and 0.1 m*M* mercaptoethanol. One-third of the total activity in the fractions was usually found in fraction I and two-thirds of the total activity in fraction III. The activity in fraction III was further fractionated using phosphocellulose.

The phosphocellulose (capacity, 92 mEqiv/g) was first equilibrated with 0.05 *M* Tris–HCl buffer at pH 7.6. To the dialyzed protein solution one gram of phosphocellulose was added per 20 mg of protein. The phosphocellulose was then collected on a Buchner funnel and washed with 100 ml of the initial buffer. Care was taken to prevent air from being drawn into the cellulose cake. This procedure was repeated using stepwise additions (100 ml) of the initial buffer containing increasing amounts (0.1, 0.3, 0.5, and 1.0 *M*) of sodium chloride. When the sodium chloride concentration reached 0.5 *M* or in excess, most of the activity was eluted. The eluant containing the activity could then be concentrated by pressure dialysis (11) and was finally chromatographed on a column (1 × 30 cm) of Corning glass beads

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(CPG-10-370 Å) at a flow rate of 100 ml/hr using 0.05 *M* Tris buffer, pH 7.6. The glass beads separate proteins on the basis of size in the molecular weight range of 50,000–125,000. Throughout the purification the enzyme appeared to be quite unstable. The procedure as outlined, however, could usually be completed in 14–16 hr and generally resulted in fractions possessing a 25–50-fold increase in activity.

Pyrophosphatase activity was measured by the method of Wöltgens in the routine assay (6). The assay mixture (0.5 ml) contained 0.1 *M* Tris–HCl buffer (pH 8.0), 1 m*M* disodium pyrophosphate (PP_i), 2 m*M* $MgCl_2$, 0.1 m*M* EDTA and an appropriate amount of the pyrophosphatase preparation. The reaction was initiated by the addition of enzyme preparation and carried out at 37° for 30 min. The reaction was terminated by the addition of 1 ml of 10% TCA containing 10 m*M* of $CuSO_4 \cdot 5H_2O$ followed by the addition of 1.5 ml of iron–ammonium–molybdate complexing reagent (6). One unit of activity was defined as that amount of enzyme required to liberate 1 nmole of orthophosphate per minute. The initial velocities were linearly proportional with respect to the amount of enzyme added and time. Each assay was performed in duplicate with a reproducibility of 5%. Specific activity was defined as units/mg of protein.

In the kinetic studies, it was often necessary to define absolute concentrations of Mg^{2+}, $MgP_2O_7^{2-}$, and $Mg_2P_2O_7$. This was done by calculation of the ion concentrations from the stability constants given by Lambert and Watters (12). For immediate reference a table of values for the concentrations of Mg^{2+}, $MgP_2O_7^{2-}$, and $Mg_2P_2O_7$, has been given by Nayudu and Miles (9) for concentrations of $MgCl_2$ and PP_i ranging from 0.66 to 2.64 and 0.09 to 2.5 m*M*, respectively. From the stability constants ($K_{MgP_2O_7^{2-}}$, $10^{5.4}$; $K_{Mg_2P_2O_7}$, $10^{2.4}$), the amounts of $MgCl_2$ to be added to known amounts of PP_i could be obtained to yield varied concentrations of $MgP_2O_7^{2-}$ at fixed concentrations of free magnesium ion. When the effect of calcium ion addition was studied, preparations were first exhaustively dialyzed of EDTA.

The collagen used in the inhibition studies was obtained from rat skin and was purified as tropocollagen in the manner described by Piez *et al.* (13). In some of the studies, a fraction of tropocollagen usually defined as α_1-collagen was also used. The collagen was added to the reaction mixtures immediately after the addition of enzyme. The α_1-collagen (M.W., 95,000) was obtained after chromatography on columns of carboxymethylcellulose (13).

In the studies involving magnesium-deficient and sufficient chicks, day-old chicks were fed semipurified diets (14) containing 1.2% Ca and 0.6% P with 0% and 0.06% supplemental magnesium, respectively. Birds were killed at 7-day intervals and the aortas removed, cleansed of adhering tissue, and immediately homogenized in distilled H_2O to give a 3-ml volume of aorta homogenate. For the assays, 50 to 100 μl of the supernatant fraction obtained after centrifugation (25,000*g*, 30 min) was used.

Results and Discussion. The results of the partial purification are given in Table I. After ammonium sulfate addition, the preparation lost activity at the rate of 20% per 24 hr of storage. Losses of activity in the partially purified preparations were more rapid, if EDTA and mercaptoethanol were not included in buffers. The crude extract of activity, however, could be stored frozen for at least a month.

As mentioned in the methods, a portion of the activity (30–35%) was associated with proteins insoluble at concentrations of ammonium sulfate less than 0.5 saturation. Previous experience in the laboratory indicated that collagen precipitates from aorta extracts in this range of ammonium sulfate concentrations (15). Collagen and its relationship to pyrophosphatase was thus studied in terms of its possible effect on altering pyrophosphatase activity.

When tropocollagen or α_1-collagen was added to the reaction mixtures, inhibition was observed as shown in Fig. 1. The inhibition of pyrophosphatase by collagen did not appear readily reversible. For example, the inhibition was not markedly altered by NaCl addition (0.5 *M*) or immediate dilution of reaction mixtures after the addition of col-

TABLE I. Partial Purification of Chick Aorta Pyrophosphatase.

Step	Total volume (ml)	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)	Yield (%)
1. Extraction	500	1230	55,000	44.7	100
2. Ammonium sulfate (0.65 to 0.85 sat., III)[b]	100	940	25,670	75.7	47
3. Phosphocellulose (0.5 *M* NaCl)	100	56	12,000	213	22
4. Concentration and glass bead chromatography	15	7.5	8,670	1167	16

[a] One unit equals one mμmole orthophosphate liberated per minute.

[b] Fraction III represented two-thirds of the combined total activity of fraction I + fraction II + fraction III.

lagen. Furthermore, if the protein in the collagen-containing fraction (fraction I) was concentrated by pressure dialysis, inhibition of the pyrophosphatase activity occurred. This was not the case for the activity in fraction III.

The effect of collagen is of interest in that the collagen content of aorta has been shown to be inversely proportional to the calcium content of the tissue (16). Elastin and perhaps other ground substance proteins appear to comprise the calcifying substrate (17). It is proposed that an increase in aorta collagen

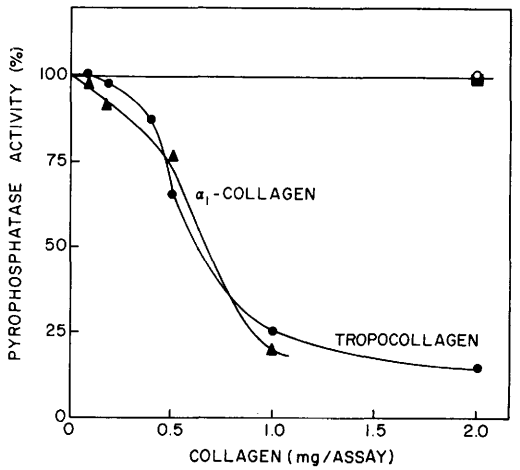

FIG. 1. Collagen and its relationship to the inhibition of aorta pyrophosphatase. Collagen in the amounts indicated was added immediately after the addition of enzyme (20 units). The reaction mixtures (0.5 ml) contained in addition: 1.0 m*M* PP_i 0.1 m*M* EDTA, 2 m*M* $MgCl_2$ and 0.1 *M* Tris buffer, pH 8.0. Hemoglobin (■) and albumin (○) served as controls.

may influence pyrophosphatase levels and concomitantly deposition of calcium salts. This observation is in contrast to the effect of collagen addition on pyrophosphatase activity in blood platelets (18). Collagen when added to platelet preparations stimulates the hydrolysis of PP_i which in turn affects purine metabolism in platelets.

The effects of pH and magnesium addition on aorta pyrophosphatase are given in Table II and Fig. 2. The pH optima was 7.8–8.1. All assays were performed at pH 8.0 unless otherwise indicated. As with other soft tissue pyrophosphatases, magnesium was required for maximal stimulation. Table II lists other metal ions which were tested. None of these had significant effect on the hydrolysis of pyrophosphate. All of the metals were tested at a 2:1 molar ratio of metal:PP_i. With respect to magnesium this ratio produced maximal stimulation. Increasing the ratio of metal:PP_i to greater than two had no additional stimulating or inhibiting effect as is the case for other inorganic pyrophosphatases (5–10). If concentrations of magnesium or PP_i were adjusted to give fixed concentrations of magnesium ion (0.1, 0.3 and 0.5 m*M*), no alteration in the apparent K_m or V_{max} was observed. The concentrations for the most probable substrate, $MgP_2O_7^{2-}$, were used in calculation of the kinetic parameters. The K_m apparent derived from reciprocal plots was found to be 7×10^{-4} *M*.

The hydrolysis of other phosphate containing substrates was also investigated (Table III). At pH 8.0, none of the compounds were significantly hydrolyzed. Alkaline phos-

TABLE II. The Relationship of Cation Addition on Pyrophosphatase Activity.[a]

Metal	Relative activity[b]
No addition	0.05
Mg^{2+}	1.00
Hg^{2+}	0.00
Ba^{2+}	ND
Co^{3+}	0.03
Mn^{2+}	0.02
Fe^{2+}	ND
Cd^{2+}	0.03
Zn^{2+}	0.12
Cr^{3+}	0.00
Ca^{2+}	0.00
Cu^{2+}	0.01
Pb^{2+}	0.00
Al^{3+}	0.00

[a] Metals were added as the acetate or sulfate salts at 2 m*M* concentration. The substrate was added at 1 m*M* in the presence of Tris–HCl buffer, pH 8.0, and 20 units of enzyme. The reaction was allowed to proceed 30 min before termination.

[b] Values are expressed relative to that for magnesium addition. The designation, ND, indicates no activity was detected. The reaction mixtures, however, were turbid. In each case the blank consisted of the reaction mixture without enzyme.

phatase activity (hydrolysis of *p*-nitrophenylphosphate), however, could be measured if the pH was increased in excess of pH 9.0 (pH optima, pH 10–11 at 2 m*M* magnesium ion and 1 m*M* *p*-nitrophenylphosphate).

The effect of calcium ion addition on pyrophosphatase activity is shown in Fig. 3. Calcium and PP_i were added to reaction mixtures to give predominantly $CaP_2O_7^{2-}$, and then magnesium and PP_i were added to give the concentrations of $MgP_2O_7^{2-}$ indicated. Calcium, apparently in the form of $CaP_2O_7^{2-}$, acted as a potent competitive inhibitor at physiological concentrations (K_i, 2.1 μM). The results were analogous to those previously reported by Nordlie *et al.* (1), who have investigated the effects of calcium on canine aorta pyrophosphatase.

In view of the strong evidence presented by Fleish and others (2–4) regarding the regulatory role of PP_i on deposition of calcium on calcifying substrates, calcium itself would appear to be an important feed-back regulator in controlling PP_i levels. The results from *in vivo* studies also offer some support for this concept (Table IV, Fig. 3). One sign of magnesium deficiency in various animal species is calcification of the aorta (19). Approximately 1.3–1.5 times as much calcium was extracted per 100 mg of aorta

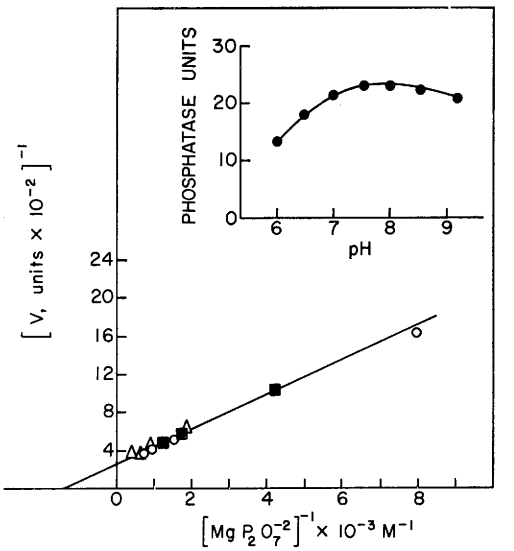

FIG. 2. The effect of altering pH on pyrophosphatase activity and Lineweaver–Burk plots for the hydrolysis of PP_i as $MgP_2O_7^{2-}$ at three fixed concentrations of magnesium ion (0.1 m*M*, ■; 0.3 m*M*, △; 0.5 m*M*, ○). Velocity is equal to nmoles P_i liberated per min. The concentrations of $MgP_2O_7^{2-}$ were calculated from data given in Ref. 9.

TABLE III. Effect of Pyrophosphatase on the Hydrolysis of Various Phosphate Esters and Anhydrides.

	Compound	Relative rate[a]
1.	Pyrophosphate	1.00
2.	Glucose-6-phosphate	0.04
3.	Glucose-1-phosphate	0.00
4.	Ribose-5-phosphate	0.00
5.	Phosphohydroxy pyruvate	0.00
6.	Pyridoxal-5′-phosphate	0.00
7.	Adenosine triphosphate	0.00
8.	3-phospho-glycerate	0.00
9.	*p*-nitrophenylphosphate	0.03

[a] Substrates (1 m*M*) and magnesium (2 m*M*) were incubated at pH 8.0 in the presence of 25 units of enzyme for 30 min at 37°. Values are expressed relative to that for PP_i. Blanks consisted of the reaction mixtures without enzyme.

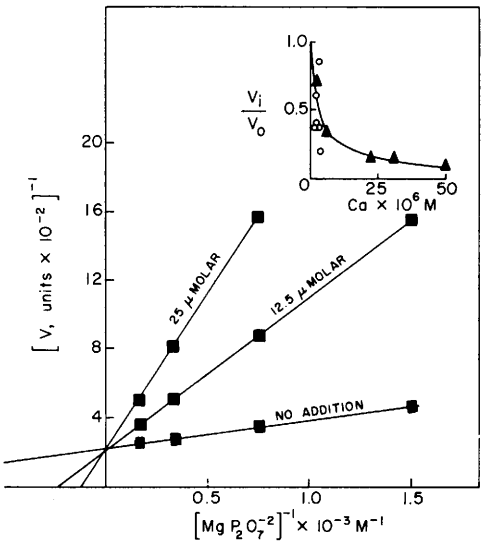

FIG. 3. Effect of calcium addition on aorta pyrophosphatase activity. The concentration of magnesium and PP_i were adjusted to give the amounts of $MgP_2O_7^{2-}$ indicated (■) with little or near zero concentrations of free magnesium in solution. Calcium chloride and disodium pyrophosphate were added to give concentrations of 12.5 and 25 μmolar of $Ca_2P_2O_7^{2-}$, respectively. Using the value of 7×10^{-4} *M* for the K_m apparent for $MgP_2O_7^{2-}$ and the observed K_i value of 2.1 μmolar for $CaP_2O_7^{2-}$, the theoretical inhibition curve (small figure) was obtained for varying amounts of calcium in the presence of 1 m*M* $MgP_2O_7^{2-}$. $Vi/Vo = \frac{S + K_m}{S + K_m(1 + i/K_i)}$, where S is equal to moles of substrate and i is equal to moles of calcium (see Ref. 20). The experimental values (▲) were obtained when calcium was added at the concentrations indicated. The small circles (○) indicate the average molar amounts of calcium added to assays when crude aorta extracts from the experimental groups given in Table IV were used. The term, Vi/Vo, in this case, is equal to the ratio of activity with and without 0.1 m*M* EDTA using the standard assay.

from magnesium-deficient chicks (21 days) as that from controls. If this calcium was not chelated in enzyme assays by the addition of EDTA, observed levels of pyrophosphatase activity were reduced from 20 to 60%. Upon chelation by EDTA (log $K_{Ca\text{-}EDTA}$, 10.6) in the presence of adequate magnesium (log $K_{mg\text{-}EDTA}$, 8.7), the levels of pyrophosphatase in extracts from magnesium-deficient chicks were elevated 2–4-fold and 1.2–2-fold in normal chicks (Table IV). The results point out the care that must be taken when assaying for pyrophosphatase activity in soft tissues. The total amounts of calcium in the crude tissue extracts ranged from 0.5 to 1.2 μg/ml. One-tenth of these amounts or the equivalent of 1–4 μmolar Ca were thus added to the reaction mixture. In addition, the results suggest that the lesion which results in aorta calcification in magnesium-deficient chicks is more complex than a mechanism involving only pyrophosphatase and regulation of PP_i levels. The aortas from magnesium-deficient chicks would presumably contain more PP_i than that in the controls and thereby inhibit calcification. The effects attributed to calcium in this regard, however, may be viewed as compensatory, *i.e.*, an attempt to partially inhibit potential calcification. The data suggest that magnesium-deficiency may provide an excellent model for the study of other alternative mechanisms related to calcium regulation in aorta tissue.

TABLE IV. Nutritional Magnesium Deficiency and the Relationship to Aorta Pyrophosphatase.

Treatment[a]	Age[b] (days)	Calcium[c,e] (μg/100 mg)	Pyrophosphatase units[d,e] —EDTA	+EDTA
—Mg	7	3.0 ± 0.2[a]	518[a]	1231[a]
+Mg	7	2.8 ± 0.2[a]	689[b]	1250[a]
—Mg	14	4.5 ± 0.7[b]	256[c]	1183[a]
+Mg	14	2.7 ± 0.5[a]	405[d]	956[b]
—Mg	21	2.4 ± 0.5[a]	266[c]	664[c]
+Mg	21	1.8 ± 0.2[c]	421[d]	502[d]

[a] Chicks were fed semipurified diets (14) containing 0% (—) and 0.06% (+) magnesium, respectively.

[b] Days after hatching.

[c] Total calcium extracted from 100 mg of aorta into 3 ml of H_2O. The tissue was homogenized (approximately 15 min) in an high speed blender and then centrifuged after 1 hour (25,000*g*; 30 min) to obtain the extract.

[d] EDTA was added to the reaction mixture at 0.1 m*M*. Values represent the total units extracted per 100 mg of aorta. Average of 10 values.

[e] Within a column letters associated with a given value designate significance ($p < 0.05$) with respect to the other values given in the column.

Summary. Chick aorta inorganic pyrophosphatase is inhibited by collagen and micromolar amounts of calcium. These effects may be important in the regulation of aorta calcification by controlling the degree to which PP_i in aorta tissue is hydrolyzed. The pH optima for the enzyme is near pH 8.0. The apparent binding constant for the substrate is 7×10^{-4} *M*, once it is associated with magnesium ($Mg_2P_2O_7^{2-}$). Magnesium was the only cation that markedly stimulated activity. At pH 8.0, inorganic pyrophosphate appears to be the primary substrate for the enzyme. The effect of magnesium-deficiency on pyrophosphate levels appeared most related to the elevation of calcium in tissue extracts which concomitantly resulted in partial inhibition of the extracted enzyme activity.

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