The Role of Nucleoside Triphosphate Pyrophosphohydrolase in in Vitro Nucleoside Triphosphate-dependent Matrix Vesicle Calcification*  

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Nucleoside triphosphate pyrophosphohydrolase (EC 3.6.1.8) activity is associated with matrix vesicles purified from collagenase digests of fetal calf epiphysseal cartilage. This enzyme hydrolizes nucleoside triphosphates to nucleotides and PPi, the latter inducing precipitation in the presence of Ca2+ and Pi. An assay for matrix vesicle nucleoside triphosphate pyrophosphohydrolase is developed using β,γ-methylene ATP as substrate. The assay is effective in the presence of matrix vesicle-associated ATPase, pyrophosphatase, and alkaline phosphatase activities. A soluble nucleoside triphosphate pyrophosphohydrolase is obtained from matrix vesicles by treatment with 5 mM sodium deoxycholate. The solubilized enzyme induced the precipitation of calcium phosphate in the presence of ATP, Ca2+, and Pi. Extraction of deoxycholate-solubilized enzymes from matrix vesicles with 1-butanol produces a stable water-soluble enzyme which may be purified to homogeneity (4, 5). The ATPase, alkaline phosphatase, and pyrophosphatase activities are associated with a single molecular entity (4).

Matrix vesicles promote mineral deposition from metastable solutions of Ca2+ and Pi (6-8). A number of theories have been advanced to explain matrix vesicle-induced calcification. First, matrix vesicles may actively transport Ca2+ and Pi. Vesicle enzymes are believed to increase local Pi concentrations by their phosphatase activity and derive energy to pump Ca2+ from ATP cleavage (9). Secondly, matrix vesicles may initiate mineral precipitation by heterogeneous nucleation (10). Complexes of Ca2+, phospholipid, and Pi (11, 12) nucleate metastable calcium phosphate solutions. Phosphatidylserine and phosphatidylinositol are present in high concentration in matrix vesicle membranes (13). It is suggested that Ca2+-phospholipid-Pi complexes may invest these membranous surfaces and act as nucleation sites for mineral phase separation. Finally, matrix vesicles may initiate mineralization by removal of inhibitors (14). Pyrophosphate inhibits the transformation of amorphous calcium phosphate to crystalline hydroxyapatite (15). Hence, matrix vesicles may induce calcification through its pyrophosphatase activity.

Pyrophosphate is conspicuously present in mineralizing tissues. Calcification in tissue slices is stimulated by PPi (16). Successive zones of calcification in the epiphyseal growth plate show progressively increasing concentrations of PPi, proceeding from the resting zone to the zone of calcification (17). The initial precipitation of amorphous calcium phosphate from solution is enhanced by the presence of pyrophosphate (18, 19). Finally, elevated synovial fluid PPi concentrations are found in articular chondrocalcinosis, osteoarthritis, and gout, all conditions associated with pathological mineralization (20).

It is the purpose of this report to show that matrix vesicles are directly responsible for the production of PPi, in in vitro calcifying mixtures. Data are presented which support the presence in matrix vesicles of NTP-pyrophosphohydrolase which cleaves NTP to NMP and PPi. It is further demonstrated that the increase in PPi concentration induces in vitro calcification.

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1 The abbreviations used are: NTP, nucleoside triphosphate; NMP, nucleoside monophosphate; AMP-CP, α,β-methyleneadenosine-5’-triphosphate; AMP-PCP, α,β-methyleneadenosine-5’-triphosphate; GMP-PCP, β,γ-methyleneadenosine-5’-triphosphate; dTMP-PCP, β,γ-methyleneadenosine-5’-triphosphate; UMP-PCP, β,γ-methyleneuridine-5’-triphosphate; Hepes, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; Taps, 5-(tris(hydroxymethyl)methylaminopropanesulfonic acid; Caps, cyclohexylaminopropanesulfonic acid.
**EXPERIMENTAL PROCEDURES**

**Materials**—Alkaline phosphatase, Type III, from *Escherichia coli*, alkaline phosphatase, Type VII-S from bovine intestine, pyrophosphatase, AMP-PCP, GMP-PCP, and UMP-PCP were obtained from Sigma. DTMP-PCP was a product of P-L Laboratories, ATP was a Calbiochem product, and [4Ca]CaCl₂ was purchased from New England Nuclear. All other chemicals were reagent grade.

**Enzyme-Assays**—Alkaline phosphatase activity was measured using p-nitrophenyl phosphate as substrate at pH 10.5 and 25 °C by following the liberation of p-nitrophenolate ion spectrophotometrically at 405 nm (4, 21). A unit of activity is defined as the amount of enzyme required to hydrolyze 1 μmol of substrate/min. Enzymatic activity toward ATP was measured as previously described (5, 22).

NTP:pyrophosphohydrolase activity was measured using AMP-PCP to 0.25 mM. Duplicate aliquots were removed after 20 min and reactions were initiated by adding substrate. Duplicate aliquots of 100 μl were removed at 0, 15, 30, 60, 120, 180, and 240 min and assayed for P₇.

**Protein Concentration**—Protein concentration was measured by a modified Lowry method (23).

**Preparation of Matrix Vesicles**—Intact matrix vesicles were prepared from bovine fetal epiphyseal cartilage by the method of Ali et al. (24). The specific alkaline phosphatase activities of different preparations varied between 3.3 and 5.6 units/mg of protein. Preparations were stored frozen at 0 °C in 120 mM NaCl, 10 mM KCl, 2 mM Tes/NaOH buffer, pH 7.45, and generally lost 10% of their activity per month upon storage. Preparations were always used within 3 months of isolation. Matrix vesicle alkaline phosphatase was isolated and purified by the method of Fortuna et al. (4).

**Calcification Assay**—*In vitro* matrix vesicle calcification was measured at 25 °C using the sedimentation method of Hau and Anderson (25). Each reaction mixture contained approximately 6,000,000 cpm of [4Ca][CaCl₂]. Washed pellets of precipitated calcium phosphate and matrix vesicles were mixed with 0.1 ml of H₂O and 5 ml of Bray's solution and shaken overnight and the resuspended pellet was counted in a liquid scintillation counter. The extent of calcification is given as the percent of precipitated or unfiltered Ca²⁺ recovered in the precipitate and each point represents the average of three determinations. Errors are reported in the appropriate figures as the S.D.

**Solubilization of Matrix Vesicle NTP:pyrophosphohydrolase**—Matrix vesicles (1 mg of protein, 5.5 alkaline phosphatase units) were solubilized in 1.6 mM NaCl, 10 mM KCl, 2 mM Tes/NaOH, pH 7.45, containing 5 mM sodium deoxycholate. After 2.5 h, the solution was centrifuged at 6,000,000 X g-min and the supernatant was assayed for NTP:pyrophosphohydrolase and calcifying activity.

**Effect of Mg²⁺ and Ca²⁺ Ions on NTP:pyrophosphohydrolase Activity**—Matrix vesicles were decalcified by incubating 2.5 ml of vesicle suspension (15 alkaline phosphatase units) with an equal volume of 200 mM Na nitrate, pH 6.0, at 0 °C for 4 h. Vesicles were harvested by centrifugation and washed twice in 120 mM NaCl, 10 mM KCl, 2 mM Tes/NaOH, pH 7.45, and finally suspended in 2 ml of the pH 7.45 buffer. Assays were performed in 120 mM NaCl, 10 mM KCl, 25 mM Tris-HCl, pH 7.4, at 25 °C. Reaction mixtures contained 0.3 unit of *Escherichia coli* alkaline phosphatase and matrix vesicle equivalent to 0.24 alkaline phosphate unit/ml. Free Ca²⁺ and Mg²⁺ ion concentrations varied from 0.01 μM to 0.01 μM at 10-fold intervals. The fraction of total added metal ion bound to the substrate, AMP-PCP, was estimated from the conditional binding constants of AMP-PCP. Preparations were incubated at pH 7.4 and 25 °C for 20 min by adding AMP-PCP to 0.25 mM. Duplicate aliquots were removed after 0, 2, 4, and 6 h and the P₇ content was determined. All reactions were carried out in polyallomer tubes rinsed in 1 mM EDTA followed by deionized water.

**RESULTS**

The effect of matrix vesicle concentration on the progress-time curves for phase separation of calcium phosphate salts is shown in Fig. 1. Each curve is characterized by three distinct regions: an initial induction period in which no precipitate forms, an intermediate stage in which the rate of accumulation of insoluble calcium salts is roughly proportional to the matrix vesicle concentration, and a terminal phase in which no further precipitation occurs. The duration of the induction period is inversely proportional to the matrix vesicle concentration while the terminal phase is independent of matrix vesicle concentration. The effect of Ca²⁺ and P₇ concentrations on the time course of calcium phosphate precipitation is shown in Fig. 2. Increasing P₇ levels do not abolish or appreciably shorten the lag phase. The highest initial P₇ concentration used, 4.8 mM, represents one which would result if all the ATP were completely hydrolyzed to adenosine and 3 mol of P₇. In contrast, increasing the initial Ca²⁺ concentration decreases and eventually removes the lag while also accelerating the precipitation rate in the intermediate phase.

Preincubation of matrix vesicles in the presence of Ca²⁺ and P₇, for 2.5 h in the absence of ATP does not alter the kinetic course of calcium phosphate precipitation. When vesicles are preincubated with ATP and P₇, for 2.5 h in the absence of Ca²⁺, the induction period is abolished (cf. Fig. 3, X-X). When an ATP-regenerating system (27) composed of pyruvate kinase and phosphoenolpyruvate is added to the calcification mixture, the induction period is extended by 1 h (225% of control) in the standard calcification assay (initial Ca²⁺, 2.2 mM; initial P₇, 1.6 mM). When 1.6 mM P₇ plus 1 mM Ca²⁺ was added to the calcification mixture, the induction period is extended by 1 h (225% of control) in the standard calcification assay (initial Ca²⁺, 2.2 mM; initial P₇, 1.6 mM).
ATP are replaced by 3.2 mM P, and 1 mM ADP, conditions which represent the complete hydrolysis of ATP to ADP in the calcification mixture, no calcium phosphates precipitates. Addition of 1 mM ADP + 1 mM ATP to the calcification mixture reduces the relative extent of calcification after 3 h by over 90% compared to mixtures containing ATP alone.

The above experiments suggested that increased P production by matrix vesicles is not responsible for calcium precipitation, that ATP is specifically required, and that ATP must be consumed during the calcification process. To test whether matrix vesicles might support calcification by specifically binding a fraction of the ATP present without cleavage, ATP was replaced with AMP-PCP. Fig. 3 (○—○) shows that this ATP analog supports calcium phosphate precipitation, while the α,β-methylene analog, AMP-CPP (cf. Fig. 3, ●—●), does not. The analog, AMP-PCP, exhibits a lag phase similar to ATP and if preincubated with matrix vesicles in the absence of Ca2+ for 2.5 h, the induction period is destroyed (cf. Fig. 3, □—□).

It was next necessary to show whether the α,β- and β,γ-methylene analogs of ATP are cleaved by matrix vesicle enzymes. Fig. 4 demonstrates an initial linear release of P from ATP (●—●) and AMP-CPP (□—□). Inorganic phosphate is released in the enzymatic hydrolysis of AMP-PCP (cf. Fig. 4, ○—○) after a considerable lag phase. When reaction mixtures containing AMP-PCP are supplemented with Escherichia coli alkaline phosphatase, the lag phase is eliminated and the initial P release rises linearly in the supplemented reaction mixtures (cf. Fig. 4, △—△, ■—■) compared to the unsupplemented control (cf. Fig. 4, ○—○).

The data from Fig. 4 support a two-step sequential hydrolysis of AMP-PCP by matrix vesicles in which the initial site of attack is the α,β-phosphohydrylde linkage. In the second step, AMP is hydrolyzed to adenosine and P. The lag associated with P release when AMP-PCP is incubated with matrix vesicles alone indicates that in the initial phase of hydrolysis the second step is at least partially rate-determining. Addition of excess bacterial alkaline phosphatase ensures that the first step is rate-determining and provides a linear release of P throughout the entire time course of the reaction.

Table I illustrates that yeast pyrophosphatase acts as a calcification inhibitor by hydrolyzing the PPi, formed in the NTP:pyrophosphohydrolase reaction. A 95% inhibition is observed when ATP is substrate but no inhibition is seen when AMP-PCP is used consistent with the fact that yeast pyrophosphatase is capable of splitting methylene diphosphonate.

Support for the role of PPi in calcification comes from studies of Ca2+ precipitation in the absence of both ATP and matrix vesicles. Fig. 5 shows that after 24 h there is no Ca2+ precipitation in the absence of PPi, when the initial ion product of Ca2+ × P, varies between 3.5 and 7.9 mM2. On the other hand, addition of PPi to the same solutions at levels of 10, 50, and 100 μM induce Ca2+ precipitation. The extent of calcification is a function of both P, and PPi, concentration. Kinetic studies using 2.2 mM Ca2+ and 3.6 mM P, supplemented with 10 or 100 μM PPi, show that maximum precipitation occurs instantaneously. The extent of calcification observed after 24 h shows a 10% decrease compared to the zero time measurement.

Solubilization of matrix vesicles in aqueous buffers containing 5 mM sodium deoxycholate gives a nonsedimentable fraction which is capable of inducing calcium phosphate precipi-

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\text{Table I: Ca}^{2+} \text{ precipitation in the presence of added yeast pyrophosphatase}
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All reactions were run for 24 h at 25 °C and pH 7.6 in the standard calcification mixture containing initially 2.2 mM Ca2+, 1.6 mM P, and 1 mM substrate. Each sample contained matrix vesicles at a concentration equivalent to 0.39 alkaline phosphatase unit/ml of reaction mixture. Yeast pyrophosphatase (Sigma) was added to the appropriate reactions at a concentration of 1.4 units/ml of reaction mixture. Results are reported as the mean of triplicate determinations and errors are reported as the S.D.
Substrate specificity of the matrix vesicle NTP:pyrophosphohydrolase. Residual Ca\(^{++}\) present in the soluble phase at the plateau stage is independent of the structure of the substrate and is 8.00 \pm 0.2%.

The pH optimum for NTP:pyrophosphohydrolase using AMP-PCP as substrate is 10.0. Progress-time curves are linear from pH 6.0 to pH 8.0 and curvilinear at more alkaline pH values. All buffers used are zwitterionic in the acid form and monoanionic in the basic form. When a HCO\(_3\)/CO\(_2\) buffer was used at pH 10.5, inhibition was observed with increasing buffer concentration. At total HCO\(_3\) plus CO\(_2\) concentrations of 24 and 48 mM, the NTP:pyrophosphohydrolase activity is 61 and 36%, respectively, of the activity when the total buffer concentration is 12 mM.

Preincubation of matrix vesicles in buffers at pH 7.5 and 10.5 for up to 120 min results in no change in NTP:pyrophosphohydrolase activity, whereas preincubation in 12 mM sodium acetate buffer, pH 4.0, leads to irreversible loss of activity.

NTP:pyrophosphohydrolase is neither activated nor inhibited by concentrations of free Ca\(^{++}\) or Mg\(^{++}\) in the range of 0.1 to 2 mM.

![Graph]

**Fig. 5.** The effect of PPi on calcification in the absence of ATP and matrix vesicles. All reactions were carried out at pH 7.6 and 25 °C and contained 2.2 mM Ca\(^{++}\) initially. Initial P\(_i\) concentrations varied between 0 and 3.6 mM. Reactions were initiated by the addition of PPi, and incubations were carried out for 24 h.

**Table II**

| Sample                | Concentration | Time (min) | Ca\(^{++}\) precipitated (nmol/ml) |
|-----------------------|---------------|------------|-----------------------------------|
| Matrix vesicles       | 0.39          | 20         | 866 ± 76                          |
| Deoxycylate soluble extract\(^6\) | 0.39          | 20         | 957 ± 77                          |

\(^6\) The deoxycylate-soluble extract is the 6,000,000g precipitate. The final concentration of sodium deoxycholate in the calcification assay mixture is 0.75 mM.

![Graph]

**Fig. 6.** The effect of different \(\beta,\gamma\)-methylene analogs of nucleoside triphosphates on the time course of calcification. All reactions were carried out using 2.2 mM initial Ca\(^{++}\) and 1.6 mM initial P\(_i\). Reactions were initiated by the addition of analog to a concentration of 1 mM. The matrix vesicle concentration, expressed as alkaline phosphatase units per ml of reaction mixture, is 0.1 in all cases. The substrates used are: - - - - GMP-PCP; O - - O, AMP-PCP; \(\times\) - \(\times\), UMP-PCP; D - D, dTMP-PCP. Each point is the average of three determinations. The S.D. averaged 10.6% of the mean values and ranged from 1.1 to 22.7%.

**Table III**

| Substrate | NTP:pyrophosphohydrolase activity | Induction time (min) | Reciprocal induction time (min\(^{-1}\)) | Calcium precipitation rate (nmol Ca\(^{++}\)/ml precipitated/min) |
|-----------|----------------------------------|----------------------|----------------------------------------|-------------------------------------------------------------|
| dTMP-PCP  | 0.38 (1)                         | 34                   | 0.029 (1)                              | 9.5 (1)                                                     |
| UMP-PCP   | 0.18 (0.47)                      | 93                   | 0.011 (0.38)                           | 4.0 (0.42)                                                  |
| AMP-PCP   | 0.14 (0.27)                      | 113                  | 0.0088 (0.30)                          | 3.6 (0.38)                                                  |
| AMP-PCP   | 0.064 (0.14)                     | 139                  | 0.0072 (0.25)                          | 2.1 (0.22)                                                  |
The induction phase associated with the time course of calcium phosphate precipitation suggests a requirement for the build-up of a critical concentration of a component not present in the system initially. The rate of production of this component is proportional to matrix vesicle concentration since the induction time is inversely proportional to vesicle concentration. Elimination of the lag period by preincubation with ATP supports the concept that formation of the critical component results from an ATP-matrix vesicle interaction. The lengthy induction periods observed in Fig. 1 tend to rule out an ATP-matrix vesicle binding phenomenon. This idea is supported by the observation that AMP-CPD does not induce calcium phosphate precipitation.

Fig. 1 shows that after the lag period, the rate of precipitation of calcium increases with increasing matrix vesicle concentration. This could result from the requirement for a continued production of the critical component during the precipitation phase or from the effect of a second matrix vesicle component on the rate of precipitation. Active transport of Ca2+ and P, appears unlikely since the final amount of Ca2+ precipitated is independent of the vesicle concentration. In addition, vesicles solubilized in deoxycholate solution support calcification. Generally, transport against a concentration gradient in biological systems requires space enclosed by a membrane. Also, since exogenously added yeast pyrophosphatase may be expected to exert its action external to the intravesicular environment, the PPi, formed in the NTP:pyrophosphohydrolase reaction must be produced on and released from the outer surface of the vesicle membrane.

Initial Pi levels play no role in the induction phase; however, increasing initial Ca2+ levels ultimately eliminates the lag period suggesting that Ca2+ plays a role in the induction phase. The instantaneous precipitation observed upon ATP or AMP-CPD preincubation strongly suggests that the critical component accumulated during the induction phase is PPi and that a critical Ca2+ × PPi ion product may be necessary to induce calcium precipitation. When initial calcium levels are elevated, instantaneous precipitation is not observed because a finite time is required for the enzymatic formation of PPi from ATP. In previous studies have shown that PPi, stimulates the initial deposition of calcium phosphate salts (18, 19), in agreement with the results reported in this paper. This initial lag phase may also be viewed as a time when the enzymatic hydrolysis of ATP, which has a strong affinity for Ca2+, effectively increases the concentration of free Ca2+. Supportive evidence is derived from the observation that elevated ATP levels inhibit in vitro calcium (7) and phosphate deposition, and the addition of an ATP-regenerating system extends the induction time.

Experiments measuring Pi liberation from AMP-CPD show that matrix vesicles possess NTP:pyrophosphohydrolitic activity. An alternative mechanism in which ATP is cleaved to adenosine and trimetaphosphate, the latter undergoing cleavage to P, and PPi, is ruled out by the fact that AMP-CPD is hydrolyzed by matrix vesicles with an initial linear phosphate release, and this analog does not support matrix vesicle calcification. The liberation of P, from AMP-CPD, indicates that matrix vesicles also possess true ATPase activity. The presence of true ATPase activity is supported by the fact that the ATP-regenerating system, which depends on cleavage of ATP to ADP, is capable of increasing the induction time of calcification.

The results in Fig. 4 indicate that the Escherichia coli alkaline phosphatase modifies only the early phase of the time course of Pi, liberation from AMP-CPD in the presence of matrix vesicle NTP:pyrophosphohydrolase. The implied poor affinity of the matrix vesicle alkaline phosphatase for AMP makes 5'-AMPase activity rate-limiting at low AMP levels and, hence, early times. As AMP levels increase above the Km for matrix vesicle alkaline phosphatase, the NTP:pyrophosphohydrolase reaction becomes rate-limiting. The Escherichia coli alkaline phosphatase, which supposedly has a greater affinity for AMP than the matrix vesicle alkaline phosphatase (28), ensures that the NTP:pyrophosphohydrolitic reaction is rate-limiting at all AMP concentrations.

Fig. 3 and Table I show that the final concentration of calcium precipitated when vesicles are incubated with AMP-CPD exceeds that when ATP is present by a factor of 1.4 to 1.6. In the presence of vesicles, PPi concentrations may never be expected to reach methylene diphosphonate levels because of the competing ATPase and pyrophosphatase reactions. Differences in the final yields of precipitable Ca2+ when ATP and AMP-CPD are the substrates suggests that the extent of Ca2+ deposition depends on the magnitude of PPi formation. Support for this concept comes from Ca2+ precipitation studies in the absence of ATP and matrix vesicles (cf. Fig. 5). Since the extent of precipitation never becomes PPi-independent throughout the range of concentrations studied, it suggests that the sediments contain stoichiometric quantities of Ca2+, P, and PPi. The idea of a Ca2+-PPi, or Ca2+-PPi-P, nucleation complex which would induce further calcium phosphate deposition without the need for further PPi seems unlikely.

There is some evidence that this in vitro mechanism may be important physiologically. The presence of adenine nucleotides in the matrix has been confirmed by several investigators (29, 30) and the concentrations reported are in the range of ATP concentrations used in the present study. However, more recent evidence indicates that the 254 nm absorbing material in the matrix of chick epiphyseal cartilage is mainly adenosine with very little AMP and ADP and no ATP (31). Analytical studies reveal increasing PPi, levels progressing from the resting zone to the zone of calcification in epiphyseal cartilage (17). In addition, added PPi increases Ca2+ deposition 4-fold in slices of embryonic chicken femur that normally calcify (16). The role of PPi as an inhibitor of amorphous calcium phosphate → hydroxyapatite transformation and subsequent crystal growth is a well-established fact (15, 32). The matrix vesicles possess an active pyrophosphatase (33) and it may be responsible for removal of excess PPi, after initial precipitation of an amorphous Ca2+ form. Regulatory mechanisms may exist for both NTP:pyrophosphohydrolase and pyrophosphatase activities allowing for buildup of PPi prior to precipitation and hydrolysis of PPi, after phase separation occurs.

The matrix vesicle NTP:pyrophosphohydrolase hydrolyzes the β, γ-methylene analogs of pyrimidine nucleoside triphosphate more rapidly than the corresponding purine derivatives. NTP:pyrophosphohydrolase from the plasma membrane of

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2 R. Carty, C. Hummel, and S. Siegel, unpublished experiments.
the liver cell hydrolyzes ATP, CTP, GTP, UTP, and dATP at approximately equal rates (34). p-Nitrophenolphosphor- ylmethylenediphosphonate, wherein the p-nitrophenyl residue replaces the nucleoside moiety, is not a substrate for the matrix vesicle enzyme. Rate differences with different substrates are highly correlated with the reciprocal induction time of calcification as well as the ensuing intermediate stage calcification rate (cf. Table III). The high correlation between reciprocal induction time and intermediate stage calcification, illustrated in Table III, provides further evidence that the substance formed in each of these stages which is required for in vitro calcification is identical.

The pH optimum of 10.0 for matrix vesicle NTP:pyrophosphohydrolase is similar to that for the purified matrix vesicle alkaline phosphatase-associated ATPase (5) and the hepatocyte plasma-membrane NTP:pyrophosphohydrolase (34). The activity at pH 7.5 is 2% of the activity at the maximum, comparable to what is observed for the purified vesicle ATPase (5). Measurements of the rate of AMP-PCP hydrolysis at pH 7.5 and 10.0 at different substrate concentrations suggest that 0.234 mM is probably saturating over most of the pH range studied and that values recorded in Table IV are values of the maximum velocity. Substitution of the HCO3- / CO32- buffer system for the Caps/NaOH buffer at pH 10.5 indicates that bivalent anions may be effective inhibitors of the enzyme.

Matrix vesicle NTP:pyrophosphohydrolase is acid-labile and stable in mildly alkaline media, similar to vesicle alkaline phosphatase.

In contrast to these pH effects, the behavior of matrix vesicle NTP:pyrophosphohydrolase in the presence of Ca2+ and Mg2+ ions differs notably from that observed for the rat liver enzyme (34, 35). Matrix vesicle NTP:pyrophosphohydrolase activity is unaffected by either Mg2+ or Ca2+ ions. At 10 mM free Ca2+ and Mg2+, the fraction of total nucleotide existing as a 1:1 metal nucleotide complex is 0.98 and 0.99, respectively. These results suggest that either the enzyme is unaffected by either the enzyme.

Matrix vesicle NTP:pyrophosphohydrolase is fully saturated at 10 μM AMP-PCP at pH 7.5. Kinetic considerations in the coupled assay used in these experiments prevent an accurate determination of Km. However, the low Km is comparable to what is observed for the purified matrix vesicle ATPase for which Km is 2 μM at pH 7.5 (5).

All of the examined kinetic properties of matrix vesicle NTP:pyrophosphohydrolase are similar or identical with those of the purified ATPase, which by virtue of solubilization by deoxycholate followed by 1-butanol extraction is devoid of NTP:pyrophosphohydrolase activity. This suggests that the alkaline phosphatase may also possess, in addition to phosphatase, pyrophosphatase and ATPase activities, a pyrophosphohydrolase action. The expression of this activity may be related to the presence of an additional subunit which is sensitive to 1-butanol extraction. Attempts are under way at the present time to separate NTP:pyrophosphohydrolase activity from the associated activities of the alkaline phosphatase.

In conclusion, the present study demonstrates the existence of NTP:pyrophosphohydrolase activity in matrix vesicles isolated from bovine epiphyseal cartilage. This enzyme is directly responsible for the formation of PP, which in the presence of metastable Ca2+ > P, solutions acts as an inducing agent for calcium phosphate precipitation.

This report affirms the calcification theories of Cartier and Picard (36) and Perkins and Walker (37) who discovered matrix vesicle NTP:pyrophosphohydrolase in extracts from calcifying cartilage of sheep and rachitic rats, respectively.

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**Table IV**

The effect of pH on NTP:pyrophosphohydrolase activity

| Buffer    | pH  | Activity | µmol/min/mg protein x 10^4 |
|-----------|-----|----------|---------------------------|
| Mes/NaOH  | 6.05| 1.05     |
| Mes/NaOH  | 6.50| 2.23     |
| Heps/NaOH | 7.05| 6.83     |
| Heps/NaOH | 7.50| 12.4     |
| Heps/NaOH | 8.00| 44.6     |
| Taps/NaOH | 8.50| 141.0    |
| Taps/NaOH | 9.05| 218.0    |
| 6 mM Caps/NaOH | 9.55| 267.0    |
| Caps/NaOH | 9.45| 276.0    |
| Caps/NaOH | 10.02| 571.0   |
| Caps/NaOH | 10.43| 561.0   |
| Caps/NaOH | 10.83| 380.0   |
| Caps/NaOH | 11.05| 283.0   |
| Caps/NaOH | 11.31| 91.9    |
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