Transphosphorylation from Nucleoside Di- and Triphosphates by Apatite Crystals*

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In a previous study (1), the authors showed that mineralized bone from a wide variety of sources possessed adenosine triphosphatase-like activity at neutral pH which was stable to heating at 100° for 30 minutes. A part of the activity was associated with the collagen matrix, since residual nucleotide phosphatase was present in bone particles which had been heated prior to complete demineralization, as well as in native unmineralized collagenous tissues and in highly purified reconstituted collagens. However, a significant part was unaccounted for unless it was assumed that the mineral portion of the bone also possessed this catalytic activity.

Since it was not possible to rule out contamination of the collagen preparations by adsorption or coprecipitation of cellular ATPases, we have examined the acellular bone (operculum) of codfish. Fresh, sterile, and fully mineralized codfish, operculi were also shown to possess ATPase-like activity which was resistant to heating at 100° for 30 minutes (1). The characteristics of the reaction were atypical compared to other ATPases since it was not possible to inhibit the reaction by reagents such as fluoride, p-chloromercuribenzoate, and pentachlorophenol.

A possible explanation for these findings was that apatite crystals were participating in the reaction. This reasoning was based in part on the reports of Lowenstein, which described a nonenzymatic transfer of the terminal phosphorus of nucleoside triphosphates and diphosphates to crystal surface phosphate groups at physiological temperature and pH. This reasoning was confirmed when residual ATPase-like activity was observed in the apatite crystals of bone particles which had been ashed at 600° for 12 to 18 hours.

In the present study, synthetic apatite crystals were also found to form inorganic pyrophosphate by the transfer of the terminal phosphorus of nucleotide triphosphates and diphosphates to crystal surface phosphate groups at physiological temperature and pH. The reaction was similar to that described by Lowenstein (2, 3), but differed in several important respects. Furthermore, the transphosphorylation proceeded more readily with those inorganic crystals possessing the general

apatite crystal structure as well as containing specific cations of the group which crystallize in the form of apatites.

EXPERIMENTAL PROCEDURE

Materials

Nucleotides were obtained from Pabst Laboratories. p-Nitrophenylphosphate and DPNH were obtained from Sigma Chemical Company. The trietylhexylammonium salt of phosphoenolpyruvate (Boehringer) was obtained from the California Corporation for Biochemical Research. Fructose 1-phosphate was a gift of Dr. Robert K. Crane. Lactic dehydrogenase, twice recrystallized, was obtained from the Worthington Chemical Company. Adenylate kinase was partially purified from rabbit muscle according to Colowick (4). Crystalline inorganic pyrophosphatase from yeast (5) was a gift of Dr. M. L. Stephen¬son.

Preparation of Crystals

Calcium Apatites—(a) A 0.032 M solution of KH$_2$PO$_4$ buffered with Tris to pH 8.5 was slowly added with constant stirring to a 0.025 M CaCl$_2$ solution, previously prepared by dissolving primary standard CaCO$_3$ in HCl and then adjusting the pH with Tris to pH 8.5. The temperature was approximately 25°. The pH was continuously monitored with the outside electrodes of a Radiometer model 22 pH meter. As precipitation occurred, the pH was maintained between pH 7.5 and 8.0 by the addition of dilute KOH. The final pH was adjusted to pH 7.5. The precipitates were centrifuged, redispersed, and washed several times with a 0.02 M Tris buffer, pH 7.5, brought to ionic strength 0.165 with KCl. They were then dispersed into this buffered solution with constant mixing at approximately 4° until needed. Just before use, the precipitates were again washed five times by suspension and centrifugation.

(b) An aliquot of this dispersion was refluxed at 100° for varying periods up to 4 weeks.

(c) Highly crystalline calcium hydroxyapatite was prepared by the slow hydrolysis of brushite to octacalcium phosphate in 0.5 M acetate buffer at pH 5.9 to 6.2 (6) followed by refluxing for 28 days in frequent changes of distilled water at 100° (7). Needlelike crystals, the largest of which were approximately 0.5 x 3.0 µ, resulted. After drying over P$_2$O$_5$ in a vacuum at room temperature, the powder was ground in a porcelain mortar and pestle and passed through 325- and 400-mesh sieves. By this method, a sample of crystals that passed through 325-mesh but were retained on 400-mesh was separated.

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† Established Investigator, Helen Hay Whitney Foundation.
(d) Calcium apatites were prepared in the absence of added metal cations as follows. A solution of phosphoric acid, about 0.04 M, was buffered to pH 8.0 by the addition of Tris (1.0 M), and slowly titrated into the CaCl₂ solution which had been adjusted as above to pH 8.5 with Tris. Precipitation was carried out by maintaining the pH at approximately 7.5 with Tris only.

**Strontium Apatite, Manganese, Magnesium, and Barium Phosphates**—These were prepared in essentially the same fashion as described under "Calcium Apatites (a)" with the exception of the manganese and magnesium salts. In the preparation of the latter two crystals, higher concentrations of both cations were employed. Lower concentrations of the manganese and magnesium salts. In the preparation of the latter two crystals, higher concentrations of both cations and the phosphates were employed. Lower concentrations of BaCl₂ were used to prevent precipitation of Ba(OH)₂ at alkaline pH values.

**Lead Apatite**—A 0.032 M K₂HPO₄ solution adjusted to pH 3.5 was slowly titrated into a 0.010 M solution of Pb(NO₃)₂ also adjusted to pH 3.5. Precipitation was carried out below pH 4.0 to 5.0 because of the coprecipitation of hydrated lead oxides above this pH. After precipitation had occurred, the solid was separated by centrifugation, washed, and resuspended in K₂HPO₄ solution adjusted to pH 7.5. After the washing procedure was repeated, the precipitate was equilibrated for 24 hours in the phosphate solution with rapid stirring at room temperature.

**Zinc Phosphate**—An 0.08 M solution of K₂HPO₄ adjusted to pH 4.0 was added in excess to a 0.015 M ZnCl₂ solution adjusted to pH 3.0. After precipitation had occurred, the pH was raised by adding an excess of 0.08 M K₂HPO₄ (at pH 9.4) to a final pH 7.5, and the crystals were equilibrated for 24 hours at room temperature with constant stirring.

**Calcite (CaCO₃)**—Samples of primary standard CaCO₃ were ground by hand in a porcelain mortar and pestle for several minutes and used without further preparation. One sample was powdered for 40 hours in a ball mill and passed through a series of graded sieves. A sample that passed through a 325-mesh but was retained in the 400-mesh screen was thus separated.

**Brushite (CaHPO₄.2H₂O; Secondary Calcium Phosphate (a))**—Molar solutions of CaCl₂ and (NH₄)₂HPO₄.2H₂O were mixed together in a beaker and allowed to stand overnight undisturbed at 25°C. The fine crystals that formed were collected by filtration on a Buchner funnel, washed thoroughly with distilled water, and dried at room temperature.

(b) Other samples were prepared as above except that the crystals were washed with repeated changes of 0.5 M ammonium acetate, pH 5.1, for 4 days. Half the crystals were ground wet in the ball mill and then passed through a 325-mesh sieve. The suspension that passed through the sieve was then centrifuged, and the precipitate was suspended in 0.03% acetic acid and dried at room temperature. From this precipitate, a sample was passed through 325- and 400-mesh sieves. Those crystals which did not pass through the 400-mesh were separated and dried at room temperature until use.

**Identification of Crystals by X-ray Diffraction**

Samples of the wet crystals were introduced into 0.2-mm (diameter) thin-walled glass capillaries and packed down by centrifugation. The capillaries were sealed and powder patterns obtained with a Debye-Scherrer type camera with a 114.7-mm lens (diameter). The copper Kα radiation, obtained from a copper target tube operated at 35 kv and equipped with a nickel filter, was used. The Bragg spacings were determined and the crystalline phases identified by means of the A.S.T.M. powder data file (8).

**Inorganic orthophosphate-P₃₂**, of high specific activity (greater than 90,000 me per g) was obtained from the Oak Ridge National Laboratories. ATP₃₂ labeled predominantly in the terminal phosphate was synthesized according to the method of Lowenstein (9) and purified by chromatography on columns of Dowex 1-bicarbonate (10). The ATP₃₂, also of high specific activity, was diluted by the addition of nonradioactive ATP before use. ATP₃₂ free of metal cations was prepared by equilibration at 2°C with excess Dowex 50-H⁺, the resin was removed by centrifugation, and the supernatant was neutralized to pH 7.4 with Tris base. Inorganic pyrophosphate labeled with P₃₂ was prepared by heating 30 μmoles of P₃₂-labeled K₂HPO₄ in an oxygen flame for 2 to 3 minutes and then purified on Dowex 1-bicarbonate.

Calcium apatite crystals labeled with P₃₂ were prepared by two different methods as follows.

**Method 1. Predominantly Surface-labeled Apatite Crystals**—Apatite crystals (1.0 to 2.0 ml, packed volume) were washed five times in 40 ml volumes of 0.05 M Tris buffer, pH 7.4, by repeated suspension and centrifugation. A 10% suspension in 0.05 M Tris, pH 7.4, was incubated with orthophosphate-P₃₂ (high specific activity) at 2°C for 1 hour with continuous agitation. Excess solution was removed by washing and centrifuging five more times with 0.05 M Tris, pH 7.4.

**Method 2. Uniformly Labeled Apatite Crystals**—These were prepared as described under "Calcium Apatites (a)" except that the 0.032 M solution of K₂HPO₄ contained 200 to 300 μCi of P₃₂ per 100 ml.

All determinations of radioactivity were carried out by drying aliquots in stainless steel planchets and counting in a Nuclear-Chicago end window Geiger counter.

**Incubation and Assay Procedures**

**Binding of Inorganic Pyrophosphate and Nucleotides**—Binding of inorganic pyrophosphate and nucleotides was determined at 2°C to eliminate pyrophosphate formation from the nucleotides. Fine suspensions of apatite crystals containing 55 to 70 μmoles of crystal phosphorus were incubated with inorganic pyrophosphate or nucleotides in a total volume of 3.0 to 4.0 ml of 0.03 M Tris buffer, pH 7.4, in 25-ml Erlenmeyer flasks with constant shaking in a Dubnoff metabolic incubator. At the end of the incubation period, the apatite crystals were separated by centrifugation at 2°C and 2500 r.p.m. for 5 minutes, and the difference in initial and final concentration in the supernatant solution was determined. Inorganic pyrophosphate and ATP binding was computed with P₃₂-labeled compounds. The binding of ADP and AMP was determined on the basis of their absorption at 260 μm with the use of a millimolar extinction coefficient of 15.4 at pH 7.0.

**Formation of Pyrophosphate**—A known weight of crystal suspension was incubated with ATP₃₂ at the indicated concentration, temperature, and pH in 0.03 M buffer, total volume 3.0 to 4.0 ml, in 25-ml Erlenmeyer flasks with constant shaking in a Dubnoff metabolic incubator. At the end of the incubation period, flasks were removed and chilled on ice for 5 minutes and the contents were centrifuged at 2°C at 2500 r.p.m. for 5 minutes.
The supernatant was decanted, and the sediment was dissolved in 4.0 ml of cold 5% trichloroacetic acid or cold 0.5 N HCl. At each step in the procedure to be described, aliquots were removed for determination of radioactivity. The solution was then placed on columns of Dowex 50-H+ (3 x 1 cm) to remove metal ions and washed twice with 2.0 ml portions of water, collecting the eluates in iced tubes. This was followed by the addition of 25 to 60 mg of acid-washed charcoal (Nuclai), the contents of the tubes were mixed and allowed to stand on ice for 10 minutes, and then centrifuged at 2500 r.p.m. for 10 minutes. An aliquot of the supernatant after charcoal treatment (free of nucleotide-P32) was allowed to react with molybdic acid and extracted with isobutanol-benzene (1:1, volume for volume) according to the Nielsen and Lehninger modification (11) of the method of Martin and Doty (12). In the latter procedure, inorganic orthophosphate-P32 is quantitatively extracted into the organic phase whereas inorganic pyrophosphate-P32 remains in the aqueous phase.

Binding of the ATP32 to the crystal was determined by the difference in supernatant counts and optical density at 260 μg between the experimental and control sample, which contained no crystal. Pyrophosphate (μmoles) was calculated from the crystal-bound P32 remaining in the aqueous phase of the Martin-Doty procedure, and the specific activity of the ATP32 added. In some experiments, ADP formed and released into the supernatant after centrifugation was measured enzymatically by coupling the pyruvate kinase reaction with lactic dehydrogenase and measuring oxidation of DPNH spectrophotometrically at 340 μg. AMP was also assayed by the same procedure after the addition of adenylate kinase in the presence of excess ATP. Total adenosine nucleotides was determined by absorption at 260 μg at pH 7.0. Total crystal inorganic phosphorus was determined on the trichloroacetic acid solution by the method of Lowry and Lopez (13).

In several experiments, binding was carried out at 2° for 15 minutes as described above. Crystals were then separated by centrifugation at 2° and washed three times with 15 ml portions of 0.05 M Tris buffer, pH 7.4. The crystals were then resuspended in 3.0 ml of the same buffer and incubated for varying periods of time at 37°, and the pyrophosphate formed was determined.

Nonradioactive nucleotides as well as other phosphate compounds were tested as substrates in the crystal-transphosphorylation system with calcium apatite labeled with P32 and proceeding as described for ATP32. When predominantly surface P32-labeled apatite was used, the absolute rate of pyrophosphate formation was not determined since a variable number of surface phosphates were labeled. In each of these experiments, the rates of pyrophosphate formation with nonradioactive substrates were calculated as percentage of the rate obtained with ATP as substrate.

**Determination of Specific Activity of Pyrophosphate Formed—** Predominantly surface-labeled and uniformly labeled apatite crystals were incubated with nonradioactive ATP at 37°. The crystals were separated by centrifugation, dissolved in 0.2 N HCl, neutralized with KOH, and chromatographed on Dowex 1-bicarbonate. The fractions containing orthophosphate-P32 and pyrophosphate-P32 were collected, and radioactivity was determined. Total phosphate was measured by the method of Fiske and SubbaRow (14) after hydrolysis for 15 minutes in 2.5 N H2SO4 at 100°, and the specific activities of orthophosphate and pyrophosphate phosphorus were calculated.

**Effect of Yeast Inorganic Pyrophosphatase on Crystal Pyrophosphate—** Apatite crystals, which had been incubated with ATP32 or inorganic pyrophosphate-P32 at 37° and thoroughly washed with 0.05 M Tris buffer, pH 7.4, were suspended in 2.0 ml of the same buffer to which had been added 10 μmoles of MgCl2 and 0.05 mg of yeast inorganic pyrophosphatase. Preparations containing no enzyme were used as controls. The flasks were incubated for an additional 15 minutes, 2.0 ml of 0.2 N HCl were added, and the orthophosphate-P32 liberated was determined by the modified Martin-Doty procedure (11, 12).

In other experiments, the inorganic pyrophosphate formed after incubation of apatite crystals with ATP32 was separated after dissolving the crystals and isolating the inorganic pyrophosphate by column chromatography. The activity of inorganic pyrophosphatase was assayed on this fraction as described above.

**RESULTS**

**Binding of Inorganic Pyrophosphate and Nucleotides by Calcium Apatite Crystals at 2°—** The binding of inorganic pyrophosphate and nucleotides by aliquots of apatite crystals from the same preparation was determined as a function of time. After 15 minutes of incubation with well dispersed crystals, maximal binding was observed. At this temperature, essentially no inorganic pyrophosphate was formed from the nucleotides. At the constant solid to solution ratio used, saturation of binding sites was observed at concentrations greater than 0.004 M (Fig. 1). At saturation, almost twice as much pyrophosphate was bound as ATP or ADP. No binding of 5′-AMP was detected.

The binding of ATP was inhibited by inorganic pyrophosphate when both compounds were added simultaneously (Fig. 2, Table 1). In addition, pyrophosphate displaced previously bound ATP, when the pyrophosphate was added after incubation of the crystals and the nucleotide. From the data shown in Fig. 2, the inhibition of ATP binding by pyrophosphate appears to be

![Fig. 1. Binding of nucleotides and inorganic pyrophosphate to calcium apatite as a function of initial concentration. All flasks contained calcium apatite (50 to 60 μmoles of crystal Pi) in 3.5 ml of Tris buffer, 0.05 M, pH 7.4, containing a compound at the concentration indicated. Flasks were incubated at 2° for 15 minutes.](http://www.jbc.org/)

![Fig. 2. Effect of Yeast Inorganic Pyrophosphatase on Crystal Pyrophosphate—Apitate crystals, which had been incubated with ATP32 or inorganic pyrophosphate-P32 at 37° and thoroughly washed with 0.05 M Tris buffer, pH 7.4, were suspended in 2.0 ml of the same buffer to which had been added 10 μmoles of MgCl2 and 0.05 mg of yeast inorganic pyrophosphatase. Preparations containing no enzyme were used as controls. The flasks were incubated for an additional 15 minutes, 2.0 ml of 0.2 N HCl were added, and the orthophosphate-P32 liberated was determined by the modified Martin-Doty procedure (11, 12). In other experiments, the inorganic pyrophosphate formed after incubation of apatite crystals with ATP32 was separated after dissolving the crystals and isolating the inorganic pyrophosphate by column chromatography. The activity of inorganic pyrophosphatase was assayed on this fraction as described above.](http://www.jbc.org/)
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3.3

\[ 1 \times 10^{-3} \text{M PPI} \]

\[ 0.5 \times 10^{-3} \text{M PPI} \]

\[ 1.6 \times 10^{-3} \text{M PPI} \]

FIG. 2. Inhibition of ATP binding to calcium apatite by inorganic pyrophosphate. All flasks contained calcium apatite (57 pmoles of crystal P$_i$) in 3.5 ml of Tris buffer, 0.03 M, pH 7.4, containing ATP and pyrophosphate at concentrations indicated. Flasks were incubated at 2°C for 15 minutes. Lower figure is a reciprocal plot of the data in the upper figure. $B$, micromoles bound per 100 pmoles of crystal P$_i$; $A$, final concentration of ATP.

TABLE I
Competitive inhibition of nucleotide and inorganic pyrophosphate binding to calcium apatite

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial concentration</th>
<th>$\mu$Moles bound per 100 pmoles crystal P$_i$</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP $\times 10^{-3}$ M</td>
<td>ATP $\times 10^{-3}$ M</td>
<td>Inorganic pyrophosphate</td>
</tr>
<tr>
<td>1</td>
<td>0 0 4.7 4.7 11.4 11.1</td>
<td>3 2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0 4.6 0 6.8 1.4</td>
<td>79 18</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0 4.6 0.16 0.93 0.81 2.8 2.4</td>
<td>13 14</td>
<td></td>
</tr>
</tbody>
</table>

The results showed that the C$^{14}$ was completely absorbed on the charcoal and only P$^{32}$ remained in the aqueous phase in the modified Martin-Doty procedure. The presence of small amounts of inorganic orthophosphate-P$^{32}$ on the crystal was accounted for by exchange of crystal phosphate with the 1 to 2% orthophosphate-P$^{32}$ initially present in the ATP$^{32}$ solutions. Similar results were obtained when the crystals were dissolved in 0.5 N HCl instead of trichloroacetic acid.

Transphosphorylation was also observed when the crystals, and the binding of pyrophosphate was measured, no significant inhibition was detected (Table I). When lower than saturation concentrations of pyrophosphate were incubated in the presence of saturation concentrations of ATP, 13 to 14% inhibition of pyrophosphate binding was observed (Table I). ADP in equimolar concentration to ATP inhibited the binding of ATP by only 18% (Table I).

When the data (from Fig. 1) for the binding of inorganic pyrophosphate and ATP are plotted in the manner suggested by Scatchard (15), deviation from linearity is noted (Fig. 3).

Pyrophosphate Formation—At pH 7.4 and 37°C, all preparations of calcium apatites bound ATP with the formation of inorganic pyrophosphate associated with the crystal phase. ADP and, to a lesser extent, AMP were liberated into the supernatant. The results of a typical experiment are shown in Table II. After the crystals were dissolved in trichloroacetic acid, and charcoal was added to remove nucleotide-P$^{32}$, the major part (77 to 90%) of the remaining P$^{32}$ did not behave as inorganic orthophosphate in the modified Martin-Doty procedure. It was identified as inorganic pyrophosphate by ascending paper chromatography in an isopropyl ether-formic acid system and by column chromatography on Dowex 1-bicarbonate.

The validity of the analytical procedure was checked in experiments where ATP labeled in carbon 8 with C$^{14}$ and in the terminal phosphorus with P$^{32}$ was incubated with apatite crystals. The results showed that the C$^{14}$ was completely absorbed on the charcoal and only P$^{32}$ remained in the aqueous phase in the modified Martin-Doty procedure. The presence of small amounts of inorganic orthophosphate-P$^{32}$ on the crystal was accounted for by exchange of crystal phosphate with the 1 to 2% orthophosphate-P$^{32}$ initially present in the ATP$^{32}$ solutions. Similar results were obtained when the crystals were dissolved in 0.5 N HCl instead of trichloroacetic acid.
End of the incubation period, 90% of the crystal-bound P32 was recovered as inorganic pyrophosphate-P32. The proportion was essentially constant over the entire range of ATP percentage of pyrophosphate formed, slowly increased until at pyrophosphate formed to ATP bound, after 30 minutes of incubation where the crystals were separated and washed after incubation with ATP at 2°C and then placed at 37°C for up to 17.5 hours, the observations that indicated that the pyrophosphate was dissolved the crystals (Table II).

**Table II**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystal phosphorus</th>
<th>ATP bound</th>
<th>Inorganic pyrophosphate formed</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium apatite</td>
<td>170</td>
<td>0.50</td>
<td>0.20</td>
<td>0.50</td>
</tr>
<tr>
<td>Control (no apatite)</td>
<td>0</td>
<td>1.30</td>
<td>0.02</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Calcium apatite crystals prepared at 25°C and refluxed for 30 days. A total of 267,300 c.p.m. of ATP32 with a specific activity of 65,000 c.p.m. per pmole was incubated with each sample for 60 minutes at indicated temperatures, and pH 7.4. The total volume of the reaction mixture was 3.0 ml.

Effect of temperature on reaction of calcium apatite crystals with ATP32

Calcium apatite crystals prepared at 25°C and refluxed for 30 days. A total of 267,300 c.p.m. of ATP32 with a specific activity of 66,000 c.p.m. per pmole was incubated with each sample for 60 minutes at indicated temperatures, and pH 7.4. The total volume of the reaction mixture was 3.0 ml.

In all experiments, the rate of pyrophosphate formation decreased with time of incubation. The results of a typical experiment are shown in Fig. 5 in which a saturating concentration of ATP was used. Similar results were obtained in other experiments where the concentration of ATP was much lower than that producing saturation of binding sites. In an experiment where the crystals were separated and washed after incubation with ATP at 2°C and then placed at 37°C for up to 17.5 hours, the percentage of pyrophosphate formed slowly increased until at the end of the incubation period, 90% of the crystal-bound P32 was recovered as inorganic pyrophosphate-P32. The proportion, pyrophosphate formed to ATP bound, after 30 minutes of incubation, was essentially constant over the entire range of ATP concentration from well below to slightly above saturation of binding sites (Fig. 4). Increasing the concentration of ATP in solution did not increase the amount of pyrophosphate formed, that is the amount of pyrophosphate formed was proportional to the nucleotide bound to the crystal, and not to the concentration of ATP in solution after saturation of binding sites was reached. Moreover, at constant initial concentrations of ATP when inorganic pyrophosphate was added to inhibit ATP binding, the amount of pyrophosphate formed was also proportional to the ATP bound and not to the amount of ATP in solution (Table IV). Essentially similar results were obtained in experiments not shown, in which the initial concentration of ATP was constant and increasing amounts of apatite added. The ratio of pyrophosphate formed to ATP bound was constant over a 5-fold increase in the amount of apatite added.

No magnesium or other free cations were required. Furthermore, no change in the rate of reaction was observed when apatite prepared in the absence of metal ions was incubated with metal ion-free ATP32. No stimulation was observed with the addition of either sodium or potassium ions at concentrations of 0.1 M.

When apatite uniformly labeled with P32 was incubated with...
FIG. 5. Rate of inorganic pyrophosphate formation from ATP by calcium apatite crystals. All flasks contained uniformly P32,labeled calcium apatite crystals (79 nmole of crystal Pi) in 3.5 ml of Tris buffer, 0.03 M, pH 7.4, and ATP at an initial concentration of 0.0063 M. Reaction was stopped by addition of 2.0 ml of 0.2 M HCl and pyrophosphate-P32 determined by the modified Martin-Doty procedure.

TABLE IV

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>ATP bound</th>
<th>Inorganic pyrophosphate formed</th>
<th>Ratio of inorganic pyrophosphate formed to ATP bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flask</td>
<td>ATP (mM)</td>
<td>Inorganic pyrophosphate (mM)</td>
<td>Inorganic pyrophosphate (mM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATP bound</td>
</tr>
<tr>
<td>1</td>
<td>0.0063</td>
<td>0.0004</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>0.0063</td>
<td>0.0004</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>0.0034</td>
<td>0.0004</td>
<td>4.4</td>
</tr>
<tr>
<td>4</td>
<td>0.0034</td>
<td>0.0004</td>
<td>0.8</td>
</tr>
</tbody>
</table>

nonradioactive ATP and the crystal inorganic orthophosphate and pyrophosphate fractions were isolated by column chromatography, the specific activities of the pyrophosphate and orthophosphate were compared (Table V). In two experiments, the ratio of the specific activities of pyrophosphate-phosphorus to orthophosphate-phosphorus was 0.50 and 0.51. The results with predominantly surface P32-labeled apatite showed a specific activity ratio of 1.40. With the two preparations, similar amounts of pyrophosphate were formed.

Apatite crystals which bound inorganic pyrophosphate-P32 from solution and apatite crystals which formed pyrophosphate-P32 by reaction with ATP32 were incubated as suspensions with yeast inorganic pyrophosphatase. The orthophosphate-P32 liberated was determined by the modified Martin-Doty procedure. In Experiment 1, P32-calcium apatite (113 nmole of crystal Pi) was incubated with ATP (0.0032 mM) in 3.7 ml of Tris buffer, pH 7.4, 0.03 M, for 30 minutes at 37º. Conditions were the same in Experiment 2, with the use of another preparation of uniformly labeled apatite (124 nmole of crystal Pi) and surface-labeled apatite (124 nmole of crystal Pi).

TABLE V

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Calcium apatite-P32</th>
<th>Inorganic pyrophosphate formed</th>
<th>Specific activity</th>
<th>Ratio of ( A ) to ( B )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{mole} )</td>
<td>( \text{c.p.m./}\mu\text{mole} )</td>
<td>Inorganic pyrophosphate-phosphorus (A)</td>
<td>Orthophosphate-phosphorus (B)</td>
</tr>
<tr>
<td>1</td>
<td>Uniformly labeled</td>
<td>2.02</td>
<td>37,400</td>
<td>73,800</td>
</tr>
<tr>
<td>2</td>
<td>Uniformly labeled</td>
<td>2.38</td>
<td>8,400</td>
<td>16,850</td>
</tr>
<tr>
<td>3</td>
<td>Surface-labeled</td>
<td>2.58</td>
<td>23,300</td>
<td>16,650</td>
</tr>
</tbody>
</table>
Phosphates. The lead phosphate crystals were insoluble in 10% trichloroacetic acid and could not be further analyzed. The supernatant after the reaction than with manganese or magnesium phosphates. The lead phosphate crystals were insoluble in 10% trichloroacetic acid and could not be further analyzed.

Comparison of calcium apatite, calcite, and brushite in reaction with ATP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystal weight</th>
<th>Reaction</th>
<th>Incubation time</th>
<th>ATP bound</th>
<th>Inorganic pyrophosphate formed</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium apatite</td>
<td>40</td>
<td>5.6</td>
<td>90</td>
<td>97</td>
<td>1.91</td>
<td>0.02</td>
</tr>
<tr>
<td>(small crystals)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium apatite</td>
<td>40</td>
<td>5.6</td>
<td>90</td>
<td>15</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>(large crystals)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Calcium apatite</td>
<td>40</td>
<td>7.8</td>
<td>30</td>
<td>28</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>(all crystals)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium apatite</td>
<td>40</td>
<td>7.8</td>
<td>34</td>
<td>15</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>(all crystals)</td>
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</tr>
</tbody>
</table>

Comparison of calcium apatite, other metal apatites and phosphate crystals in reaction with ATP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystal phosphates</th>
<th>Incubation time</th>
<th>ATP bound</th>
<th>Inorganic pyrophosphate formed</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium apatite</td>
<td>176</td>
<td>90</td>
<td>42</td>
<td>1.80</td>
<td>0.00</td>
</tr>
<tr>
<td>(small crystals)</td>
<td></td>
<td></td>
<td></td>
<td>0.66</td>
<td>0.22</td>
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<tr>
<td>Lead apatite</td>
<td>216</td>
<td>90</td>
<td>44</td>
<td>1.04</td>
<td>0.67</td>
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<tr>
<td>Manganese phosphate</td>
<td>252</td>
<td>84</td>
<td>49</td>
<td>1.81</td>
<td>0.00</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>252</td>
<td>10</td>
<td>17</td>
<td>9.01</td>
<td>0.99</td>
</tr>
<tr>
<td>Magnesium phosphate</td>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
<td>0.07</td>
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<tr>
<td>Barium phosphate</td>
<td>1800</td>
<td>60</td>
<td>96</td>
<td>17</td>
<td>0.31</td>
</tr>
<tr>
<td>Zine phosphate</td>
<td>952</td>
<td>60</td>
<td>84</td>
<td>12</td>
<td>0.18</td>
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<td>Strontium phosphate</td>
<td>154</td>
<td>60</td>
<td>84</td>
<td>35</td>
<td>0.61</td>
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<tr>
<td>Barium phosphate</td>
<td>449</td>
<td>60</td>
<td>58</td>
<td>12</td>
<td>0.16</td>
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<tr>
<td>Calcium phosphate*</td>
<td>250</td>
<td>60</td>
<td>37</td>
<td>34</td>
<td>0.58</td>
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</table>

* Formed by hydrolysis of octacalcium phosphate.

Discussion

The binding of nucleotides and inorganic pyrophosphate by calcium apatite crystals probably involves formation of complexes of the polyphosphate chain with the calcium ions on the crystal surface, similar to that described for ATP and calcium ions in solution (16, 17). The lack of a linear relationship for the binding data (Fig. 3) indicates either that more than one type of binding site is involved, or that interaction between bound ions alters the successive binding of other ions. The possibility that more than one type of binding site is involved is supported by the known structural differences in the coordination of the calcium atoms in the apatite lattice (18).

The recovery of labeled inorganic pyrophosphate with a specific activity of the phosphorus half that of the orthophosphate of the uniformly labeled apatite crystals after incubation with ATP (Table V), indicates that one phosphorus is donated by the crystal and one by the nucleotide.

Although the mechanism of the apatite crystal-catalyzed transphosphorylation is not known, it seems likely that the formation of a complex between the polyphosphate chain of the nucleotide and crystal surface metal ions neutralizes the negative charge on the polyphosphate chain and induces an increased positive charge on the terminal phosphorus of the nucleotide making it susceptible to nucleophilic attack by a crystal phosphophase group properly located on the surface. These considerations are similar to those discussed by Westheimer (19), Lowenstein (2, 3), and Pullman and Pullman (20).

The phenomenon described here is similar to that reported by Lowenstein (2, 3) for ATP, metal ions, and inorganic phosphate in solution, a transphosphorylation in the absence of a protein catalyst. There are several differences between the reaction reported here and that described by Lowenstein. These differ-
The reaction with apatite crystals is faster at pH 7.4 to 8.0 than at more alkaline pH and still shows 80% of maximal activity at pH 5.6. Lowenstein reported a sharp optimum at pH 9 and no significant transphosphorylation at pH 7. (2) Polyvalent ions did not stimulate the transphosphorylation catalyzed by apatite crystals but did stimulate transphosphorylation in solution. (3) The apparent rate of transphosphorylation catalyzed by apatite crystals at saturation (0.12 amole per hour per amole of crystal phosphorus) is considerably faster than the maximal rate reported for metal ions (Mn(++) and phosphate ions in solution at the same pH (0.0008 amole per hour per amole of phosphorus) (2). Since the rate for the crystals is calculated on the basis of total crystal phosphate, the true rate is undoubtedly even higher since only surface phosphates of the crystals are involved in the reaction.

The use of the ratio pyrophosphate formed to ATP bound seems to be a valid method of comparing the effectiveness of transphosphorylation by the different crystals, although it was impossible to control all the characteristics of the crystals such as size, shape, surface area, etc. The differences are most evident in the experiments with the mixed barium phosphates. Despite the fact that barium phosphate crystals bound more ATP than either calcium or strontium apatites, less pyrophosphate was formed.

Furthermore, the strontium apatite preparation used in Table VII, in contrast to the calcium apatite, showed no line broadening by x-ray diffraction and many more reflections at equal exposure time, indicating greater crystallinity and larger crystal size. Although, as would be expected, these strontium apatite crystals bound less ATP than calcium apatite crystals, they were equally effective in transphosphorylation when the results were expressed relative to the amount of ATP bound.

The rate of transphosphorylation of the different crystals may be influenced both by steric factors related to different interatomic distances between the metallic ions and phosphate ions, and by differences in the electronic structure of the various ATP-metal complexes.

The differences in the susceptibility to inorganic pyrophosphate of the pyrophosphate bound to the crystals, depending on whether the pyrophosphate was bound as such from solution (82% hydrolyzed) or whether it was formed from the transphosphorylation reaction with ATP (16% hydrolyzed), is probably due to differences in the type of pyrophosphate-crystal surface bonds formed.

The observed reaction between apatite crystals and nucleotides may account for the formation of acid-soluble inorganic pyrophosphate in the experiments in vitro reported by Cartier and Picard (21). They found that pyrophosphate was formed from ATP in the mineralization of slices of endochondral sheep cartilage. The ability of apatite crystals to form bound pyrophosphate in this manner at physiological temperature and pH may also account for the presence of the small amounts of inorganic pyrophosphate found in bone mineral (22). As far as its role in mineralization is concerned, conceivably, the pyrophosphate bound to the apatite in some way alters the properties of the crystal surface and may therefore be important in the control of secondary nucleation and the regulation of crystal size and shape.
Transphosphorylation from Nucleoside Di- and Triphosphates by Apatite Crystals
Stephen M. Krane and Melvin J. Glimcher


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