Factors affecting the solubility of calcium pyrophosphate dihydrate crystals.

R M Bennett, J R Lehr, D J McCarty

J Clin Invest. 1975;56(6):1571-1579. https://doi.org/10.1172/JCI108239.

Research Article

The solubility of triclinic calcium pyrophosphate dihydrate (CPPD) crystals was measured under varying conditions using 45Ca-labeled crystals, expressing solubility as micromoles per liter of 45Ca in solution. In a 0.1-M Tris-HC1 buffer pH 7.4, the solubility of accurately sized CPPD crystals (37-20mum) was 60muM with maximal solubility being attained after about 8 h incubation at 37degreeC. Reduction in crystal size, decrease in pH, increase in ionic strength, Mg++, citrate, and albumin all increased solubility. The most marked effects on solubility occurred when changing the calcium concentration or by enzymatic hydrolysis of inoganic pyrophosphate to orthophosphate. It was found that decreasing the ionized calcium level below 5 mg/100 ml resulted in a progressive enhancement of solubility. The observed solubility-enhancing effects of albumin could be explained solely on its calcium-binding ability and thereby, altered ionized calcium level. Diffusible calcium in synovial fluid was only 40% of the total calcium concentration, which means most joint fluids are normally near the critical concentration of 5 mg/100 ml of ionized calcium, below which solubility is enhanced. During surgery, especially parathyroidectomy, calcium levels fall, favoring dissolution of CPPD crystals. We speculate that the slight decrease in crystal size during dissolution frees them from their cartilaginous mold, resulting in a dose-dependent inflammatory reaction as they are "shed" into the joint space. Crystal shedding may be reinforced by […]

Factors Affecting the Solubility of Calcium Pyrophosphate Dihydrate Crystals

ROBERT M. BENNETT, JAMES R. LEHR, and DANIEL J. MCCARTY

From the Section of Arthritis and Metabolism, Department of Medicine, University of Chicago, Chicago, Illinois 60637

ABSTRACT The solubility of triclinic calcium pyrophosphate dihydrate (CPPD) crystals was measured under varying conditions using ^{45}Ca-labeled crystals, expressing solubility as micromoles per liter of ^{45}Ca in solution. In a 0.1-M Tris-HCl buffer pH 7.4, the solubility of accurately sized CPPD crystals (37-20 μm) was 60 μM with maximal solubility being attained after about 8 h incubation at 37°C. Reduction in crystal size, decrease in pH, increase in ionic strength, Mg^{++}, citrate, and albumin all increased solubility. The most marked effects on solubility occurred when changing the calcium concentration or by enzymatic hydrolysis of inorganic pyrophosphate to orthophosphate. It was found that decreasing the ionized calcium level below 5 mg/100 ml resulted in a progressive enhancement of solubility. The observed solubility-enhancing effects of albumin could be explained solely on its calcium-binding ability and thereby, altered ionized calcium level. Diffusible calcium in synovial fluid was only 40% of the total calcium concentration, which means most joint fluids are normally near the critical concentration of 5 mg/100 ml of ionized calcium, below which solubility is enhanced. During surgery, especially parathyroidectomy, calcium levels fall, favoring dissolution of CPPD crystals. We speculate that the slight decrease in crystal size during dissolution frees them from their cartilaginous mold, resulting in a dose-dependent inflammatory reaction as they are "shed" into the joint space. Crystal shedding may be reinforced by the modest fall in joint fluid pH accompanying the inflammatory response.

Dr. Lehr's present address is the Fundamental Research Branch, Division of Chemical Development, Tennessee Valley Authority, Muscle Shoals, Ala. 35660. Dr. McCarty is presently with the Department of Medicine, The Medical College of Wisconsin, Milwaukee, Wisc. 53226.

Received for publication 13 February 1975 and in revised form 4 August 1975.

INTRODUCTION

Over a decade has passed since the initial association of calcium pyrophosphate dihydrate (CPPD)[1] crystals ($Ca_2P_2O_7$) with a gout-like arthritis (1). The articular inflammatory response includes the phagocytosis of intra-articular CPPD crystals by polymorphonuclear leukocytes with the subsequent release of lysosomal enzymes (1-3). In many patients developing pseudogout, CPPD deposits are seen radiologically in both hyaline and fibrocartilage, and we have assumed that the crystals found in pseudogout joint fluid originate from these preformed deposits, but there is no definitive evidence on this point. The mechanism of this cartilaginous crystal deposition remains obscure, but is found more commonly in patients with hyperparathyroidism (4) and hemochromatosis (5). More recently, elevated levels of inorganic pyrophosphate (PP_i) have been found in the synovial fluid of patients with pseudogout (6-9); during an acute episode, the PP_i level is paradoxically reduced (9).

As synovial fluid levels of PP_i are higher than in plasma (6, 8, 9), it is likely that PP_i originates from within the joint. It could arise either from the dissolution of CPPD crystals or as a metabolic by-product of joint tissue metabolism. A knowledge of the solubility of CPPD crystals under various conditions and the rate of attaining equilibrium is of importance in resolving this issue. Moreover, some recent clinical observations (10) have indicated that factors increasing the solubility of CPPD crystals may be of importance as a "trigger" to the acute episode of pseudogout.

This present investigation is an extension of our previous work on CPPD crystal solubility (7). The use of

[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; CPPD, calcium pyrophosphate dihydrate; SP, simulated plasma.

^{45}Ca-labeled CPPD crystals permitted precise measurement of even small increments in solubility.

METHODS

General. Acid-washed glassware or disposable plastic ware was used. All solutions were prepared in doubly distilled, deionized water. Salts used to prepare standard solutions were stored over anhydrous $CaSO_4$ in a vacuum desicator. All solutions were made up in either 0.1 M Tris-HCl buffer, pH 7.4, or a protein and calcium-free simulated plasma, pH 7.4, (100 ml containing NaCl, 14 mg; K_2HPO_4, 13.4 mg; and NaH_2PO_4, 2.7 mg) hereafter designated SP.

Incubation was performed at 37°C in a Dubnoff metabolic shaker. All centrifugation was done at 37°C in a Sorvall RC-2 centrifuge (Ivan Sorvall, Inc. Norwalk, Conn.) unless otherwise specified.

The following antimicrobial and antifungal agents were added to each 30 ml of solution: penicillin 10,000 U, streptomycin 100 mg, amphotericin B 2.5 mg.

Radioactive labeled CPPD crystals. Triclinic CPPD crystals were prepared in vitro by a modification of the method of Brown et al. (11) with the addition of ^{45}Ca (New England Nuclear, Boston, Mass.) to give a specific activity of approximately 0.1 μCi/mg. After synthesis, the crystals were washed once in 0.1 M Tris-HCl buffer pH 7.4 for 2 h in a mass/volume ratio of 100 mg/2 ml.

After washing, the suspension was gently centrifuged, washed twice with a small volume of distilled water, and the crystal dried at 180°C overnight before storing over anhydrous Ca_2SO_4 in a vacuum desiccator. This washing step effectively removed some highly soluble ^{45}Ca surface contaminates that were present in small amounts.

The crystals were examined using compensated polarizing light microscopy to determine the uniformity of morphological and optical properties (12). Previous X-ray diffraction studies had shown that crystals prepared in this way were biaxial, triclinic forms of CPPD (11), and similar to natural crystals (13).

Three accurately weighed samples of (^{45}Ca) CPPD crystals were dissolved in 0.5 ml of 0.5 N HCl and counted in 10 ml of Bray's solution. The mean specific activity of these standards was used throughout the study to translate counts per minute ^{45}Ca in the supernate into moles of Ca (and PP_i) dissolved. Solubility was expressed as micromolar concentration of crystal-derived calcium.

Experimental design. The radioactive crystals were sieved to obtain crystals of three sizes: < 20 μm, between 20 and 37 μm and > 37 μm, (Endecotts Filters, London SW19).

In each experiment, a constant mass/volume (5 mg CPPD to 30 ml solution) was maintained, using a 100-ml conical flask containing a magnetic stirrer.

After the addition of the ^{45}Ca CPPD, the flask was removed at intervals timed with a stopwatch and the contents magnetically stirred to a uniform suspension. 1 ml of this suspension was passed through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) to remove the crystals; 0.5 ml of the filtrate was then counted.

The effects of crystal size, pH, ionic strength, Ca^{++}, PP_i, P_i, albumin, citrate, Mg^{++}, and synovial fluid on solubility were determined.

Verification of methodology. Three major concerns were: (*a*) Do the radioactive CPPD crystals behave in the same way as nonradioactive CPPD crystals, or does the insertion of a ^{45}Ca atom affect the solubility? (*b*) Does the 0.45-μm filter completely eliminate all microcrystals? (*c*) In experiments in which there is calcium in the bathing medium, is there a significant exchange of Ca for ^{45}Ca calcium at the crystal surface?

The first problem was tested by comparing the solubility of the radioactive crystals (20–37 μm), using ^{45}Ca going into solution, to the solubility of similarly sized nonradioactive crystals, using a modification of the method of Chen et al. (14) to measure P_i produced by hydrolysis of PP_i. In this modification, 50 μl of 4.4 N HCl was added to 150 μl of the filtrate (from the crystal suspension) and boiled in a tightly sealed screw-capped tube for 60 min. After cooling, 10 μl of 6.5% wt/vol ammonium molybdate was added, and during continuous mixing on a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.), 50 μl of 6.5% (wt/vol) ascorbic acid was slowly added. After incubation at 45°C for 50 min the OD was measured in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a Gilford model 222 absorbance indicator (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Standard P_i solutions, 5–50 μM, and a reagent blank were subjected to the same procedure.

The second problem was tested by differential density centrifugation (Fig. 1). 800 μl of the filtrate from the crystal suspension was divided into two portions: (*a*) 200 μl was counted directly (*b*), 600 μl was divided into two equal portions; 300 μl of carbon tetrachloride (CCl_4) was added to one portion. Both were centrifuged (20,000 *g* for 2 h) and 200 μl of each supernate counted. A further 200 μl was carefully pipetted off, and the remaining CCl_4 evaporated to dryness; 0.5 ml of 0.5 N HCl was added and counted as before. A control experiment was done without prior filtration of the crystal suspension and the results are shown in parentheses in Fig. 1.

The third problem was probed by an isotopic exchange experiment. Intermediate size nonradioactive CPPD crystals were washed and heated as previously described: 13 mg (44.82 μmol) of crystals was incubated with 5 ml of 0.1 M Tris-HCl buffer pH 7.2, containing 0.2 μmol of carrier-free ^{45}Ca (sp act 26 μCi/μmol of calcium), over 48 h at 37°C in a Dubnoff metabolic shaker. A control omitting the nonradioactive CPPD crystals was treated similarly. At timed intervals, the container was removed and the contents stirred magnetically to a uniform suspension. 1 ml of this suspension was passed through a 0.45-μm Millipore filter and the crystals washed with 2-ml aliquots of the Tris-HCl buffer until no radioactivity was detected in the filtrate. The Millipore filter was then carefully removed from its holder and dried in an oven at 37°C. Sequential weighing determined that after 24 h it was free of all moisture. Its weight after 24 h was compared to its original dry weight and the difference was taken to represent the mass of filtered CPPD crystals. The control was similarly treated. The dried filters were transferred to counting vials and 0.5 ml of 0.5 N HCl added and counted in 10 ml of Bray's solution. The specific activity of the filtered crystals and hence the exchange of ^{45}Ca with nonradioactive CPPD was calculated from these results.

Treatment of protein-containing solutions. Human serum albumin was dialyzed against SP for 3 days to remove most of the bound calcium. The protein concentration of the dialysate was measured by the biuret method (15); and calcium determined by flame photometry, using a Perkin-Elmer model 303 atomic absorption spectrophotometer as described in the Perkin-Elmer manual (Perkin-Elmer Corp., Norwalk, Conn.). Appropriate additions of Ca^{++} (in the form of anhydrous $CaCl_2$) were added as indicated. Synovial fluids were similarly treated. After 48 h of a solubility experiment, equilibrium dialysis was performed at 37°C

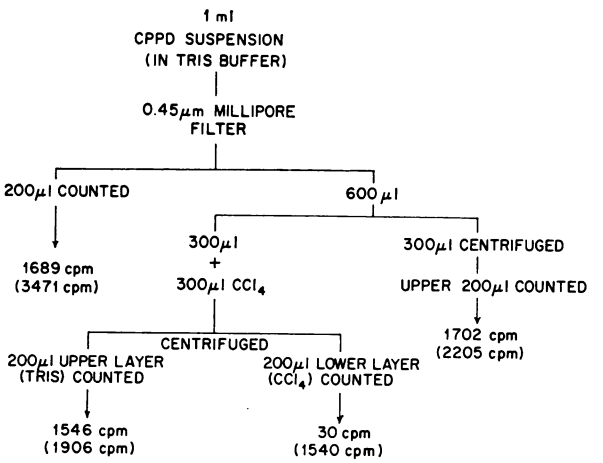

FIGURE 1 Verification of the 0.45-μm Millipore filter technique. The counts in the supernate after centrifugation approximate those of the unspun fluid, and after differential centrifugation employing carbon tetrachloride negligible counts are registered in the nonaqueous phase. Bracketed numbers are counts per minute in a control experiment without prior filtration.

for 1 h in a plexiglas chamber of 1 ml capacity (Chemical Rubber Co. Apparatus, Cleveland, Ohio), separated by dialysis membrane (pore diameter 4.8 nm, Fisher Scientific Co., Pittsburgh, Pa.), and prepared according to the method of Brewer et al. (16). 1-ml aliquot of the filtrate was dialyzed against 1 ml of SP for 1 h at 37°C. Prior ^{45}Ca tracer experiments had shown free calcium equilibrated within 20 min.

The total calcium content in each chamber was measured by atomic absorption flame spectrophotometry and a 100-μl aliquot from each was counted for ^{45}Ca.

10 synovial fluids (pH adjusted to 7.4) were similarly dialyzed, with the difference that 10 μl of a ^{45}Ca solution (20,000 cpm) was introduced into the SP side of the dialysis chamber. Again the distribution of bound and free calcium was determined both by atomic absorption and by radioactivity distribution.

Estimation of hydrolysis of PP_i. This was routinely estimated in all experiments at 24 and 48 h. A 2-ml aliquot of the original solution was incubated separately with the addition of 100 μl of $^{32}P^{32}P_i$ (New England Nuclear) with a specific activity of approximately 800 μCi/μmol. Nonradioactive synthetic crystals of the same size, were added to provide an identical mass/volume ratio to that in the test flask.

At specified times, 100 μl was taken from the "hydrolysis tube," put into ice, and thereafter, all procedures were performed in the cold. 100 μl of 6 N perchloric acid was added to the 100-μl aliquot, mixed, and centrifuged at 20,000 *g* for 5 min at 4°C. To 100 μl of the supernate, 400 μl of cold P_i solution (144.2 mg anhydrous Na_2HPO_4/100 ml distilled water) and 1.0 ml of cold PP_i solution (12.8 mg $Na_4P_2O_7 \cdot 10\ H_2O$/100 ml distilled water) were added. Next 1.5 ml of reagent A (13.4 ml 5 g/100 ml ammonium molybdate + 3.6 ml 15 N H_2SO_4 + 3.0 ml H_2O) was added and thoroughly mixed. 1 ml of this was added to 10 ml of 0.5 N HCl for counting (total counts T) using Čerenkov emission and a Packard Tri-Carb liquid scintillation counter, model 3320 (Packard Instrument Co., Inc., Downers Grove, Ill.). The efficiency of this counting technique was approximately 50%. 2 ml of reagent B (four parts isobutanol + one part petroleum ether) was added to the remaining solution and mixed for exactly 1 min.

After centrifugation at 1,000 g for 5 min, 1 ml from the upper layer (U) was counted in 10 ml of isobutanol, and 1 ml from the lower layer (L) was counted in 10 ml of 0.5 N HCl. By this method, P_i was extracted into the isobutanol layer and the percentage of hydrolysis of PP_i to P_i was calculated thus: percent hydrolysis $PP_i = (\text{cpm/ml}\ U \times 0.8 \times 100)/(\text{cpm/ml}\ U + L)$. The factor 0.8 was used to correct for the increased efficiency of counting in isobutanol.

In one experiment, the effect of hydrolysis was examined by adding approximately 2 U of inorganic pyrophosphatase (type III, Sigma Chemical Co., St. Louis, Mo.) to the incubation solution containing 2 mg/100 ml of Mg^{++}.

Microbial contamination. As many bacteria and fungi possess pyrophosphatases, it was essential to eliminate such contamination. Hence, penicillin, streptomycin, and amphotericin were added to all incubation solutions routinely as described above. At the end of each experiment, a small aliquot of the incubation material was transferred under full sterile conditions to a nutrient agar plate. Contamination occurred in about 5% of experiments and such results were discarded.

RESULTS

Verification of methods. Fig. 1 shows the result of the experiment designed to ascertain whether any microcrystals were escaping through the 0.45-μm Millipore filter. There were 1,689 cpm in 200 μl of the unprocessed filtrate. After centrifugation of another 300 μl of the same filtrate, there were 1,702 cpm in the upper 200 μl layers, making it unlikely that any crysals had leaked through. There were 1,546 cpm in 200 μl of the upper layer at the CCl_4 (aqueous phase). The miscibility of CCl_4 in H_2O is only 0.7%.[2] There were only 30 cpm in the denser carbon tetrachloride. If microcrystals had escaped through the filter, they would have been deposited in the CCl_4 layer after centrifugation, as shown in the control experiment, when without prior filtration there were 2,205 cpm in the CCl_4.

A comparison of the solubility in Tris buffer of radioactive and nonradioactive CPPD crystals is illustrated in Fig. 2. Both crystals achieved a similar final solubility, although the data for the nonradioactive CPPD crystals showed greater variability than did the corresponding values derived by counting ^{45}Ca. The inset graph demonstrates the correlation between the two methods. The calculated specific activities were virtually constant, showing that the crystals were uniformly labeled with ^{45}Ca.

In the isotopic exchange experiment in which 0.2 μmol of ^{45}Ca was allowed to "equilibrate" with 44.82 μmol of CPPD crystals in 5 ml of Tris-HCl buffer for 48 h, the respective specific activities of the filtered CPPD crystals at 4, 9, 25, and 48 h were: 0.19, 0.26,

[2] Personal unpublished observation.

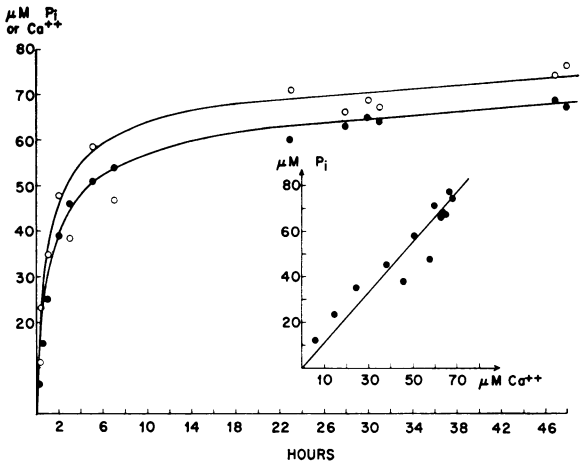

FIGURE 2 Comparison of solubility of CPPD crystals size 20–37 μm in Tris-HCl 0.1 M pH 7.4, 37°C measured both colorimetrically as P_i (●) and isotopically as Ca (○), expressed as micromolar concentration. The inset shows the correlation between solubilities of the two preparations measured at different times.

0.25, and 0.27 μCi/μmol. Thus, it appeared that after 9 h no measurable isotope exchange occurred. As the specific activity of the added carrier-free ^{45}Ca was 26 μCi/μmol, the exchange of Ca for ^{45}Ca on a molar for molar basis was 0.5% at an equilibrium time of 9 h.

Effect of crystal size. Smaller crystals with a larger surface area to volume ratio dissolved more quickly than large crystals (Fig. 3). As expected, the final solubility approached a uniform value. As this is an important variable, all subsequent experiments were performed with the crystals sized 20-37 μm, as these are representative of the majority of cartilaginous CPPD crystals.

Effect of pH and ionic strength. Final solubilities reached after 48 h incubation in 0.2 M glycine buffer of varying pH are shown in Fig. 4. Between pH 8 and 6, there is an almost linear rise in solubility, with a value of 38 μM at pH 7.4, which is a similar value to that found in SP at pH 9.0, after which it again increases with increasing pH.

Increasing the ionic strength by the addition of sodium chloride caused a moderate increase in solubility. This experiment was performed in 0.05 M phosphate buffer pH 7.4 which itself has an ionic strength of 0.15, and this must be added for absolute values.

Common ion effect. Ionized calcium concentration has a marked effect on solubility (Fig. 5). This experiment was done in SP and it is noted that the final solubility with no added calcium is 20 μM lower than in 0.1 M Tris-HCl buffer. In the range between 14 and 5 mg/100 ml, there is little change, but at lower concentrations there was a progressive increase in solubility.

The other common ion, PP_i (Fig. 6), had unexpected effects on solubility; on an equimolar basis, up to a concentration of about 500 μM, it had a more marked depressant effect on solubility than does Ca^{++} although, in

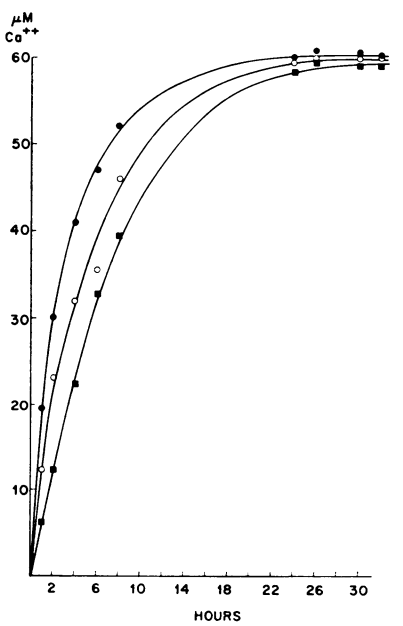

FIGURE 3 Effect of crystal size on the rate of dissolution in Tris-HCl buffer, pH 7.4, 37°C. (●) < 20 μm (○) < 37 μm to > 20 μm (■) > 37 μm.

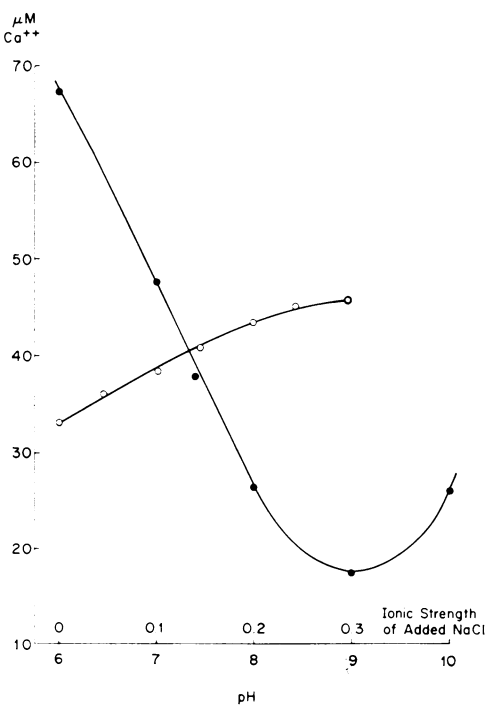

FIGURE 4 Effect of pH (●) on solubility in a glycine HCl/NaOH buffer, 0.2 M, and effect of ionic strength (○) in phosphate 0.05 M, pH 7.4. Both experiments performed at 37°C.

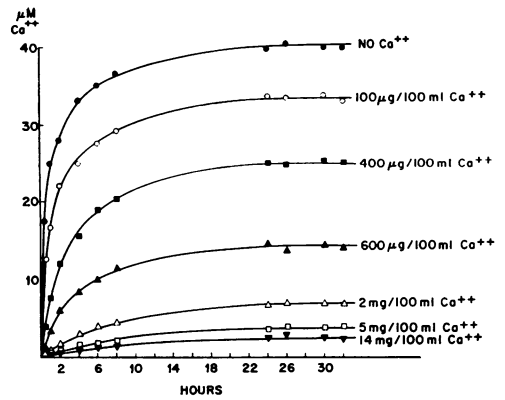

FIGURE 5 Effect of ionized calcium on solubility of CPPD crystals. Performed in a protein-free SP, pH 7.4, 37°C.

the usual concentrations found in synovial fluid (2-25 μM), it had little effect on solubility. As its concentration increased above 1,000 μM, it had a progressive solubility-enhancing effect, possibly due to Ca^{++} chelation.

In a similar experiment, a modest increase in solubility was found with increasing concentration of P_i; namely, a solubility of 44 μM at a P_i concentration of 1 mM, increasing to 50 μM at a P_i concentration of 50 mM.

Effect of hydrolysis. The hydrolysis of PP_i lead to increased solubility. When yeast pyrophosphatase was added to Tris-HCl buffer containing 2 mg/100 ml of Mg^{++}, there was 100% hydrolysis in less than 1 h and a corresponding fivefold rise in solubility over the course of 30 h as compared to control (Fig. 7). No unhydrolyzed PP_i was found at any subsequent time in the flask containing the enzyme.

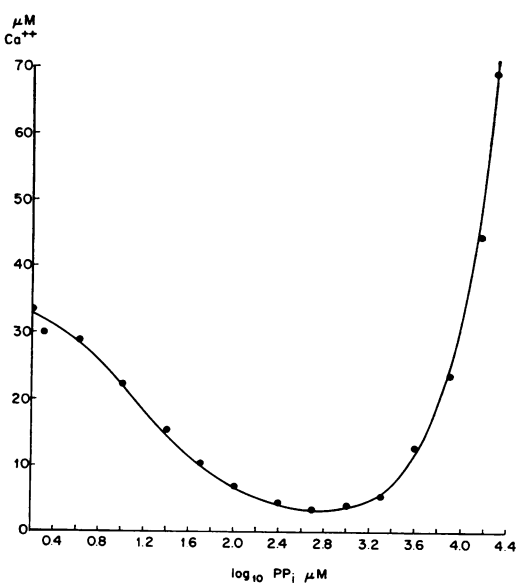

FIGURE 6 Effect of PP_i on solubility of CPPD crystals performed in a protein/calcium-free SP pH 7.4, 37°C.

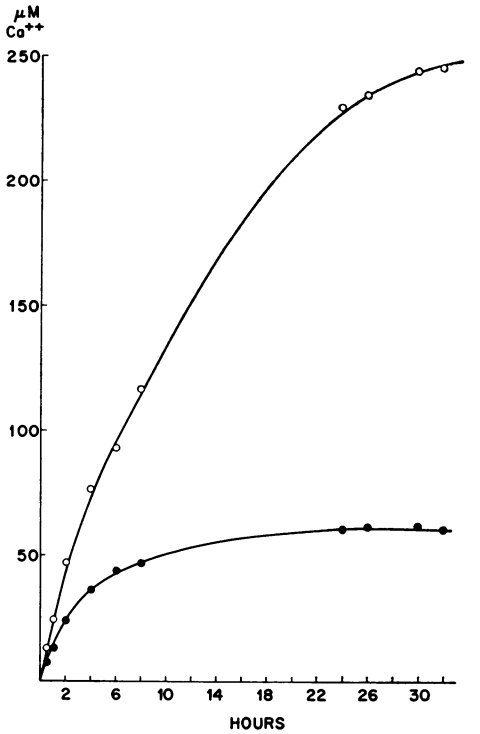

FIGURE 7 Effect of hydrolysis induced by 2 U of yeast inorganic pyrophosphatase (○) in a Tris-HCl buffer, 0.1 M, pH 7.4 plus 2 mg/100 ml Mg^{++}, 37°C; 100% hydrolysis occurred within 1 h while in the control experiment, without pyrophosphatase (●) 20% hydrolysis was achieved after 30 h in the control.

Equilibrium had not been achieved even after 30 h incubation, and it is probable that the slight upward slope, noted in all of our figures is a result of continuing hydrolysis. Measurements of hydrolysis made in all experiments at 24 and 48 h, showed no values greater than 30% at 48 h, the mean value being 24%. Based on the results of this experiment, it is unlikely that this degree of hydrolysis significantly influenced the observed final solubility.

Effect of protein. When dialyzed human serum albumin having a calcium concentration of 0.3 mg/100 ml was added to SP (final protein concentration 5 g/100 ml) and Ca^{++} added to a final concentration of 3, 1, and 0.8 mg/100 ml (Fig. 8), it is seen that there was an apparent increase in solubility when compared to the same concentration of Ca^{++} in the absence of protein (Fig. 5). When equilibrium dialysis was performed after 48 h incubation, the diffusible[3] calcium values are seen to be 1.98, 1.6, and 0.26 mg/100 ml, respectively. Thus the

[3] Diffusible calcium levels are assumed to approximate the ionized calcium concentrations here and elsewhere in this paper.

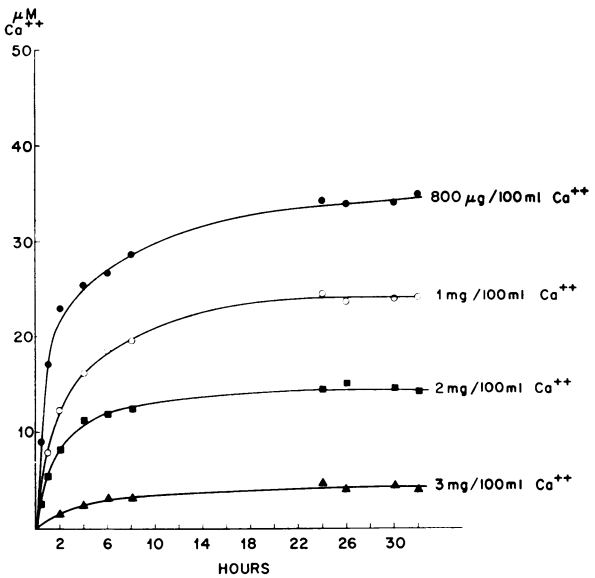

FIGURE 8 Effect of CPPD crystal solubility of adding human serum albumin (dialyzed to remove most of its bound calcium) in a final concentration of 5% to protein-free SP and then returning known amounts of calcium to the solution. Performed at pH 7.4, 37°C. Equilibrium dialysis against the SP was then performed on aliquots of each solution at 48 h, and the distribution of calcium measured. Percent ionization was : (●) 32, (○) 46, (■) 53, and (▲) 66.

solubility-enhancing effect of albumin is entirely due to calcium binding according to the equilibrium:

$$n\ Ca^{++} + m\ Alb \rightleftharpoons x(Alb - Ca) + (m - x)Alb + (n - x)Ca^{++},$$

where n Ca^{++} and m Alb represent the original molar concentration of free calcium and albumin, an "x" represents the molar concentration of the albumin-calcium complex.

Two randomly selected synovial fluids, from patients with pseudogout and rheumatoid arthritis, respectively, displayed a similar dependence of solubility on ionized calcium. The calcium contents before and after dialysis, solubilities at 48 h of incubation, and diffusible calcium values at 48 h of incubation (derived from equilibrium dialysis) are shown in Table I. When the calcium is removed, the solubility increased markedly; the individual levels were those predicted from the data Fig. 5, suggesting ionized calcium is the major determinant of CPPD crystal solubility. A similar phenomenon was seen when calcium was returned to the system, the final solubility was of the order of magnitude predicted from the data shown in Fig. 5. Actual measurements were of diffusible calcium; ionized calcium levels are probably somewhat lower.

Effect of magnesium and citrate. Magnesium is selectively concentrated within cells to a concentration of 45 meq/liter (reference 17, p. 3) and hence Mg^{++} levels are likely to be elevated in stored blood. The finding of an enhanced solubility on the addition of Mg^{++} ions is therefore of some interest (Fig. 9). Likewise citrate, a constituent of ACD (acid citarte dextrose) solution used in anticoagulating stored blood has an appreciable effect on solubility (Fig. 10) depending on its concentration. In this particular example, the concentrations of sodium citrate expected when 5 U of blood stored in ACD solution is distributed in volumes of 5 liters (intravascular), 15 liter (extravascular), and 45 liters (total body water), were used arbitrarily.

Diffusible calcium in synovial fluid. In view of the demonstrated effect of increased solubility at ionized calcium values below 5 mg/100 ml, it was of some importance to know what percentage of total calcium in synovial fluid is diffusible. Similar results were obtained by both the chemical and radioactive methods in 10 assorted synovial fluids; the mean value being 39.5% (Table II). This compares with a value of approximately 60% in plasma (reference 17, p. 311).

DISCUSSION

In this study we have equated the solubility of CPPD crystals with the molar concentration of ^{45}Ca in solution. This is not a true solubility in the terms of ionic activities, or a true solubility product, as only the Ca^{++} concentration and not both Ca^{++} and PP_i was being measured. In our "verification of methodology," it is seen that PP_i values (derived from P_i measurements) closely correspond to Ca^{++} values (Fig. 2) as would be expected from the law of mass action:

$$Ca_2P_2O_7 \rightleftharpoons 2\ Ca^{++}P_2O_7 \text{.----}$$

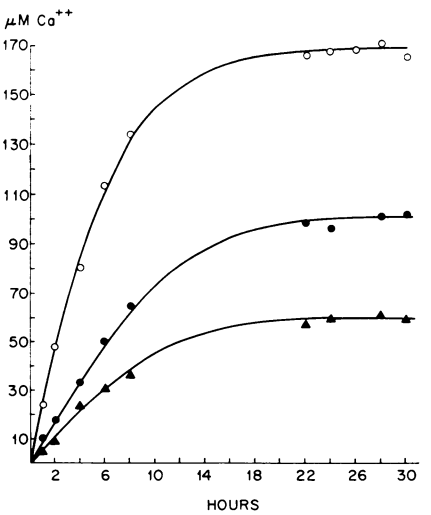

FIGURE 9 Effect of Mg^{++} on the solubility of CPPD crystals. Performed in Tris HCl 0.1 M pH 7.4, 37°C. (○) 5 mg/100 ml Mg^{++}, (●) 50 mg/100 ml Mg^{++}, (▲) Zero.

TABLE I

CPPD Solubility in Synovial Fluid, Effects of Calcium

Measured and derived values	PG* Synovial fluid			RA* Synovial fluid		
	Undialyzed	Dialyzed	Dialyzed plus 4.4 mg/100 ml Ca	Undialyzed	Dialyzed	Dialyzed plus 4.4 mg/100 ml Ca
Total calcium, *mg/100 ml*	7.2	0.56	5.0	8.6	0.48	5.0
Solubility at 48 h *μM* ^{45}Ca	7.5	42.9	8.9	5.0	31.3	3.7
Diffusible Ca at 48 h, %	21	24	44	24	26	47
Calculated diffusible Ca, *mg/100 ml*	1.52	0.134	2.2	2.06	0.125	2.35

* PG, pseudogout; RA, rheumatoid arthritis.

As our measured values behave in a way predicted by the law of mass action and as the crystals were uniformly labeled, it is reasonable to assume the validity of PP_i concentration as derived from ^{45}Ca measurements. The solubility of a slightly soluble salt (K_{sp}) such as $Ca_2P_2O_7 \cdot 2H_2O$ is given by:

$$K_{sp} = \frac{[Ca^{++}]2[P_2O_7]}{[Ca_2P_2O_7]}.$$

As the active mass of the minimally soluble $Ca_2P_2O_7$ is very small and constant the solubility product is given by the product (18):

$$K_{sp} = [Ca]^2 \cdot [P_2O_7].$$

As we are measuring only the calcium concentration and by the law of mass action the PP_i concentration is directly related: in our system $K_{sp} = [Ca]^3$. We have chosen to use the concentration of crystal-derived calcium as an index of solubility. This is obviously directly proportional to a derived K_{sp}, but avoids making any of the above assumptions.

In those experiments in which there was added calcium in the incubation medium, the isoionic exchange of cold calcium with the ^{45}Ca-labeled CPPD crystals will lead to some error in our calculated solubilities. In our isotope-exchange experiment, approximately 0.5% of calcium in the incubation medium exchanged with crystal-derived calcium. This implies that the true solubilities, in experiments containing exogenous calcium,

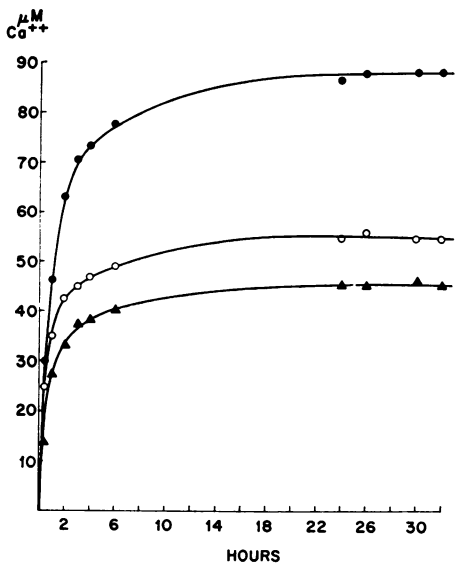

FIGURE 10 Solubility-enhancing effect of citrate on CPPD crystals in protein and calcium-free SP, pH 7.4, 37°C. Citrate concentration was adjusted to that found in 5 liters of blood stored in ACD solution distributed (●) intravascularly (citric acid 20.6 mg + sodium citrate 160 mg/100 ml SP). (○) extracellularly (citric acid 6.9 mg + sodium citrate 53.3 mg/100 ml SP) (▲) total body water (citric acid 0.52 mg + sodium citrate 20 mg/100 ml SP).

TABLE II

*Diffusible Calcium in Synovial Fluids**

Synovial fluid source	Atomic absorption	Radioactive
	%	%
Osteoarthritis	45	43
Osteoarthritis	42	41
Osteoarthritis	27	29
Rheumatoid arthritis	42	41
Rheumatoid arthritis	43	43
Pseudogout	44	44
Pseudogout	43	43
Gout	35	35
Traumatic	35	34
Traumatic	40	41

* Equilibrium dialysis against calcium-free SP ultrafiltrate, 37°C, pH 7.4.

are probably somewhat higher than the observed values; but with an isoionic exchange of only 0.5% after 9 h of incubation, this error is negligible.

Our results are generally in agreement with the previous preliminary report from this laboratory on the solubility of synthetic CPPD crystals (7), namely, the solubility-enhancing effects of protein and magnesium ions and depressant effect of inorganic orthophosphate (P_i): in fact, P_i increased solubility to the degree expected by an increase in ionic strength. Our data indicate that the solubility-enhancing effect of protein results from calcium-ion binding with a fall in the concentration of free calcium.

The effects of altering the concentrations of PP_i and Ca^{++} on the solubility of CPPD crystals were of interest. On a molar basis at low concentrations, PP_i had a greater solubility-depressant effect than did Ca^{++}. With decreasing concentrations of Ca^{++} from levels actually found in synovial fluid, solubility was markedly enhanced while a decrease in PP_i concentration had relatively little effect. Such effects are probably a function of their physiologic concentrations, as the PP_i level in synovial fluids is only 2-25 μM (6-9), whereas, the ionized calcium level has a mean value of 3.5 mg/100 ml, this is 17-370 times greater (7). Moreover, much of the PP_i in solution is in the form of MeP_2O_7 rather than as $P_2O_7^{----}$, where Me^{++} refers to divalent cations (chiefly Mg).

We have shown that factors which increase the solubility of CPPD crystals lead to an acute inflammatory episode of pseudogout (10). We have termed this hypothetical process "crystal shedding" as CPPD crystals embedded in the articular cartilage become smaller until, loosened from their mold of matrix, they float freely into the joint cavity. Thus, the level of ionized calcium appears to be the most important regulator of CPPD dissolution. Within the range of 14-5 mg/100 ml there is relatively little effect on solubility, but with levels below 5 mg/100 ml, a rapid increase in solubility occurs.

It is tempting to speculate that postoperative attacks of pseudogout (19), especially those after parathyroidectomy (20), result from the depression of ionized calcium actually attained in synovial fluid; Is the depression of Ca^{++} during surgery of sufficient magnitude to induce crystal shedding? The diffusible calcium in 10 randomly selected synovial fluids was about 40% of the total, as compared to an ionized plasma level of 60% (reference 17, p. 311), and the total calcium concentration of synovial fluid is lower than in plasma. Calculated synovial fluid-ionized calcium levels (obtained with a calcium electrode), from the published figures of McCarty et al. (7), give a mean of 3.5 mg/100 ml, or 41% of the total calcium concentration in synovial fluid.

These findings indicate there is a gradient in the level of ionized calcium between plasma (60% ionized) and synovial fluid (40% ionized). The level of diffusible calcium will be somewhat higher due to complexing with ultrafiltrable substances such as citrate, but this only accounts for approximately 0.5 mg/100 ml (14). We assume this ionized calcium gradient is due to "additional binding" by, as yet, unidentified substances in synovial fluid. This property of synovial fluid may be of critical importance in the etiology of the postoperative attack of pseudogout, bearing in mind the rapid increase in CPPD solubility below an ionized calcium level of 5 mg/100 ml, as is seen in the following example:

For a patient undergoing a major surgical procedure, it has been shown the mean fall in ionized calcium is 73% (21) towards the end of the operation, returning slowly towards normal by the 4th postoperative day. Hence, a patient with a total plasma calcium of 10 mg/100 ml would have an ionized level of 6 mg/100 ml (assuming 60% of total is ionized in plasma) and this would be reflected by a synovial fluid-ionized calcium not greater than 4 mg/100 ml. If the plasma-ionized Ca^{++} level were reduced 73%, it would fall to 4.38 mg/100 ml and the corresponding synovial fluid level would drop to 2.92 mg/100 ml.

The increased CPPD crystal solubility during surgery will be augmented by blood transfusion as shown by the experiments with citrate (the main constituent of anticoagulation mixtures used in stored blood) and magnesium (which is elevated in stored blood).

CPPD crystals take 12 h or more to reach equilibrium with common ions in the surrounding liquid. This is of importance relative to the origin of synovial fluid PP_i, which is higher than plasma PP_i (8, 9), suggesting local production. PP_i could result from metabolism within cartilage, subchondral bone, or synovial membrane cells, or from dissolution of CPPD crystals embedded in the cartilage or free in the synovial fluid. Recent work has shown a mean turnover of 102%/h for the intra-articular PP_i pool in chronic pseudogout and even higher values in more acute inflammation and in rheumatoid joints (22). Therefore, CPPD crystal dissolution does not contribute significantly to the PP_i levels found in synovial fluid.

It has been suggested that a lower than normal activity of inorganic pyrophosphatase in joint tissue could account for the higher PP_i levels in the synovial fluid in pseudogout than in controls (23). The case reported by O'Duffy (24) of coincident hypophosphatasia and pseudogout supports such a hypothesis, as there is good evidence that alkaline phosphatase is a pyrophosphatase (25-27). A reduced activity of glucose-6-phosphate pyrophosphate phosphotransferase was found in the joint fluid of patients with both gout and pseudogout, as compared to rheumatoid arthritis (28), while Russell and coworkers found a significantly reduced level of alkaline phosphatase activity in pseudogout synovial fluid com-

pared to fluids from other patients with different types of arthritis, but we found no such difference in synovial fluid alkaline phosphatase levels (6). Pyrophosphatase activity markedly augments the solubility of CPPD crystals. Theoretically, in the acute attack of pseudogout, neutrophil leukocyte phosphatases may be released and accelerate the shedding of crystals. However, actual synovial fluid PP_i hydrolysis rates in acute and chronic pseudogout showed no significant difference (22).

The decrease in joint fluid pH which accompanies inflammatory arthritis (29) may augment CPPD crystal dissolution and shedding. Crystal size may also be important, influencing crystal shedding as the smaller the crystals, the more rapid its dissolution rate (Fig. 3).

ACKNOWLEDGMENTS

The authors are indebted to Susan Loresco and Young Park for their excellent technical assistance.

This work was supported by U. S. Public Health Service grant AM 13069 and by a grant from the Illinois Chapter of the Arthritis Foundation.

REFERENCES

1. McCarty, D. J., Jr., N. N. Kohn, and J. S. Faires. 1962. The significance of calcium phosphate crystals in the synovial fluid of arthritic patients: the "pseudogout syndrome." I. Clinical aspects. *Ann. Intern. Med.* **56**: 711–737.
2. Schumacher, H. R., and P. Phelps. 1971. Sequential changes in human polymorphonuclear leukocytes after urate crystal phagocytosis. An electron microscopic study. *Arthritis Rheum.* **14**: 513–526.
3. Andrews, R., and P. Phelps. 1971. Release of lysosomal enzymes from polymorphonuclear leukocytes (PMN) after phagocytosis of monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) crystals: effect of colchicine and indomethacin. *Arthritis Rheum.* **14**: 368. (Abstr.)
4. Bywaters, E. G. L., A. St. J. Dixon, and J. T. Scott. 1963. Joint lesions of hyperparathyroidism. *Ann. Rheum. Dis.* **22**: 171–187.
5. Hamilton, E., R. Williams, K. A. Barlow, and P. M. Smith. 1968. The arthropathy of idopathic haemochromatosis. *Q. J. Med.* **37**: 171–182 + plates 12–15.
6. Russell, R. G. G., S. Bisaz, H. Fleisch, H. L. F. Currey, H. M. Rubinstein, A. A. Dietz, I. Boussina, A. Micheli, and G. Fallett. 1970. Inorganic pyrophosphate in plasma, urine, and synovial fluid of patients with pyrophosphate arthropathy (chondrocalcinosis or pseudogout). *Lancet.* **2**: 899–902.
7. McCarty, D. J., S. D. Solomon, M. L. Warnock, and E. Paloyan. 1971. Inorganic pyrophosphate concentrations in the synovial fluid of arthritic patients. *J. Lab. Clin. Med.* **78**: 216–229.
8. Altman, R. D., O. E. Muniz, J. C. Pita, and D. S. Howell. 1973. Articular chondrocalcinosis. Microanalysis of pyrophosphate (PPi) in synovial fluid and plasma. *Arthritis Rheum.* **16**: 171–178.
9. Silcox, D. C., and D. J. McCarty. 1974. Elevated inorganic pyrophosphate concentrations in synovial fluids in osteoarthritis and pseudogout. *J. Lab. Clin. Med.* **83**: 518–531.
10. Bennett, R. M., J. R. Lehr, and D. J. McCarty. 1976. Crystal shedding and pseudogout—a hypothesis based on a therapeutic failure. *Arthritis Rheum.* In press.
11. Brown, E. H., J. R. Lehr, J. P. Smith, and A. W. Frasier. 1963. Preparation and characterization of some calcium pyrophosphates. *J. Agric. Food Chem.* **11**: 214–222.
12. Phelps, P., A. D. Steele, and D. J. McCarty, Jr. 1968. Compensated polarized light microscopy. Identification of crystals in synovial fluid from gout and pseudogout. *J. Am. Med. Assoc.* **203**: 508–512.
13. Gatter, R. A., and D. J. McCarty. 1967. Pathological tissue calcifications in man. *Arch. Pathol.* **84**: 346–353.
14. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**: 1756–1758.
15. Dittebrandt, M. 1948. Application of Weichselbaum biuret reagent to the determination of spinal fluid protein. *Am. J. Clin. Pathol.* **18**: 439–441.
16. Brewer, J. M., A. J. Pesee, and R. R. Ashworth, editors. 1974. Experimental Techniques in Biochemistry. Prentice-Hall, Inc., Englewood Cliffs, N. J.
17. Campbell, E. J. M., C. J. Dickinson, and J. D. H. Slater. 1968. Clinical Physiology, Blackwell Scientific Publications, Ltd., Oxford. 3rd edition. 3, 311.
18. Angus, L. H. 1954. Physical Chemistry. University Tutorial Press Ltd., Cambridge. 242 pp.
19. O'Duffy, J. D. 1973. Pseudogout syndrome in hospital patients. *J. Am. Med. Assoc.* **226**: 42–44.
20. Bilezikian, J. P., G. D. Aurbach, T. B. Connor, W. N. Pachas, A. Aptekar, S. A. Wells, J. Freijanes, and J. L. Decker. 1973. Pseudogout after parathyroidectomy. *Lancet.* **1**: 445–447.
21. Clowes, G. H. A., Jr., and F. A. Simeone. 1957. Acute hypocalcemia in surgical patients. *Ann. Surg.* **146**: 530–541.
22. Camerlain, M., D. J. McCarty, D. C. Silcox, and A. Jung. 1975. Inorganic pyrophosphate pool size and turnover rate in arthritic joints. *J. Clin. Invest.* **55**: 1373–1381.
23. Russell, R. G. G. 1972. Pyrophosphate metabolism and pseudogout. (Calcium pyrophosphate crystal deposition disease) 8th Symposium on Advanced Medicine. G. Neale, editor. Pitman Medical Publishing Co., Ltd., London. 380–396.
24. O'Duffy, J. D. 1970. Hypophosphatasia associated with calcium pyrophosphate dihydrate deposits in cartilage. Report of a case. *Arthritis Rheum.* **13**: 381–388.
25. Cox, R. P., and M. J. Griffin. 1965. Pyrophosphatase activity of mammalian alkaline phosphatase. *Lancet.* **2**: 1018–1019.
26. Cox, R. P., P. Gilbert, Jr., and M. J. Griffin. 1967. Alkaline inorganic pyrophosphatase activity of mammalian-cell alkaline phosphatase. *Biochem. J.* **105**: 155–161.
27. Eaton, R. H., and D. W. Moss. 1967. Alkaline *ortho*-phosphatase and inorganic *pyro*phosphatase activities in human serum. *Nature (Lond.).* **214**: 842–843.
28. Good, A. E., and W. H. Starkweather. 1969. Synovial fluid pyrophosphate phosphohydrolase (PPPH) in pseudogout, gout and rheumatoid arthritis. *Arthritis Rheum.* **12**: 298. (Abstr.)
29. McCarty, D. J. 1974. Selected aspects of synovial membrane psysiology. *Arthritis Rheum.* **17**: 289–296.