Measurement of Inorganic Pyrophosphate in Biological Fluids

ELEVATED LEVELS IN SOME PATIENTS WITH OSTEOARTHRITIS, PSEUDOGOUT, ACROMEGALY, AND UREMIA

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ABSTRACT A rapid and relatively simple method for measurement of inorganic pyrophosphate (PPi) in biological samples has been described. The mean ±SEM of plasma samples from 94 normal subjects was 1.8± 0.06 μM, giving a normal range (99% confidence limits) of 0.16 — 3.40 μmol/liter. Analysis of 17 plasma samples in duplicate showed a standard deviation of 0.18, giving a 99% probability that a single determination of plasma PPi would be ±0.68 μM of the true value.

The mean PPi levels in plasma from subjects with osteoarthritis, pseudogout, acromegaly, and uremia were significantly greater than the normal mean ($P < 0.01$). Samples from rheumatoid arthritis showed PPi levels distributed about a mean identical to the normal mean. Plasma inorganic orthophosphate levels correlated positively with PPi levels in samples from normal subjects and in samples from patients with osteoarthritis, pseudogout, and uremia, but not with acromegaly. This correlation was statistically significant only in the normal samples and in those from patients with osteoarthritis.

INTRODUCTION

It has become evident in recent years that further understanding of the pseudogout syndrome (1–3) (calcium pyrophosphate dihydrate crystal deposition disease) requires a better understanding of the metabolism of inorganic pyrophosphate (PPi).[1] Recently, Russell and coworkers reported that the PPi concentration in synovial fluid in pseudogout is elevated with reference to fluids from other types of joint disease (4). Plasma PPi levels in pseudogout fell within the normal range. Their results on joint fluid have been confirmed by McCarty, Solomon, Warnock, and Paloyan, although overlap of values from pseudogout and control fluids was found (5). More recently Altman, Muniz, Pita, and Howell reported elevated PPi levels in synovial fluid from osteoarthritic and gouty joints, as well as in pseudogout (6). The methods for measurement of PPi have been technically difficult and time-consuming. Most work has been performed using column chromatographic separation of PPi from other phosphates, a procedure which requires relatively large amounts of sample (5, 7). Ultramicro methods for PPi measurement in biologic material, based on adaptations of the uridine diphosphoglucose (UDPG) pyrophosphorylase method of Johnson, Shanoff, Bass, Boezi, and Hansen (8), have been described recently, but these require a capillary microcuvette (6) of long light path or very difficult isotopic procedures (9). A rapid, simple procedure to measure PPi specifically is described herein, together with data obtained on PPi concentrations in normal plasma and plasma obtained from patients with pseudogout, osteoarthritis, acromegaly, and uremia.

METHODS

General

Acid-washed glassware or disposable plastic ware was used throughout. All solutions were prepared in doubly distilled, deionized water. Phosphate salts used to prepare standard solutions were stored over anhydrous $CaCl_2$ in a

Received for publication 30 November 1972 and in revised form 2 April 1973.

[1] *Abbreviations used in this paper:* Pi, inorganic orthophosphate; PPi, inorganic pyrophosphate; $^{32}P^{32}Pi$, [^{32}P]inorganic pyrophosphate; UDPG, uridine diphosphoglucose.

vacuum desiccator. All centrifugation was performed at 4°C in a Sorvall RC-2 Centrifuge (Ivan Sorvall, Inc., Newtown, Conn.) unless otherwise specified.

Blood or other fluid was drawn into a plastic syringe containing a tracer amount of [^{32}P]inorganic pyrophosphate (^{32}P^{32}Pi) of high specific activity (770–6200 μCi/μmol), (New England Nuclear, Boston, Mass.) and sodium heparin (50 μl/10-ml sample as Liquemin Sodium "10," Organon Inc., West Orange, N. J.), and placed directly in ice. The entire procedure was then carried out in ice except where otherwise specified. It is important that venipuncture or other procedure used to obtain the specimen be performed as atraumatically as possible. Serum contains two to threefold as much PPi as does plasma prepared from the same blood. The increased PPi concentration of serum is due to its release by platelets. The identification of PPi in platelets and its release upon thrombin stimulation are described elsewhere (10). Platelet-poor plasma was prepared by centrifugation at 1110 *g* for 15 min; the supernate was then centrifuged at 49,500 *g* for 10 min, decanted, and centrifuged again at 49,500 *g* for 20 min.

Analysis for PPi

The procedure as used for plasma samples was as follows:

Initial $^{32}P^{32}Pi$ concentration. Two 100-μl aliquots of sample were counted in 10 ml of 0.5 N HCl in water using Cerenkov emission and a Packard Tri-Carb Liquid Scintillation Counter, Model 3320 (Packard Instrument Co., Inc., Downers Grove, Ill.). The efficiency of this technique approximated 50%.

Deproteinization. 0.6 ml of 80% (wt/vol) trichloroacetic acid was added to 5 ml of sample in a 15-ml Nalgene conical tube (Nalgene Piping Systems Div., Nagle/Sybron Corp., Rochester, N. Y.), and mixed in a vortex until homogeneous; after centrifugation at 28,000 *g* for 10 min, 3.5 ml of supernate was removed with a plastic pipette and transferred to a 15-ml plastic centrifuge tube.

Removal of inorganic orthophosphate (Pi) and residual protein. This was done by a modification of the method of Sugino and Miyoshi (11): 0.8 ml of a solution of four parts 1 N perchloric acid and one part 0.08 M ammonium molybdate were added, and after thorough mixing, 0.4 ml of 0.1 M triethylamine hydrochloride, pH 5.0. The final acid normality was 0.5. The resultant cloudy green precipitate was allowed to form for 5 min. After centrifugation at 28,000 *g* for 10 min, the clear supernate was poured into another tube and centrifuged again at 40,000 *g* for 15 min. (It is important that no precipitate be carried over into the next step.) 4 ml of supernate was transferred to a 50-ml glass-stoppered centrifuge tube with a blowout pipette.

Extraction of residual Pi and concentration of sample by removal of water. 1 ml of 1 N HCl was added to increase efficiency of isobutanol extraction, and to avoid the development of phosphomolybdenum blue. Then 30 ml of cold isobutanol was added, the tube inverted by hand repeatedly and rapidly for 30 s, after which the phases were separated by centrifugation at 425 *g* for 10 min. After removal of the isobutanol phase by suction, the sample was transferred to a 15-ml conical tube with a Pasteur pipette, 0.2 ml of 1 M Tris-HCl buffer, pH 7.2, was added, and the pH adjusted to 7.2±0.2 with 25–30 μl of 40% (wt/vol) KOH; 3–5 ml of isobutanol was then added to further reduce the volume to 0.4–0.5 ml. The organic phase was removed, and 1 ml of benzene was added under hood precautions. This solution was thoroughly mixed for 30 s and centrifuged at 1500 *g* for 10 min in an International Centrifuge, model PR-2 (International Equipment Co., Needham Heights, Mass.). The organic layer was again removed and 50 μl of 60 mM $MgCl_2$ was added.

Enzymatic hydrolysis of PPi. 100 μl of 0.9% NaCl containing 10 U of Type I (twice-crystallized) yeast inorganic pyrophosphatase (Sigma Chemical Co., S. Louis, Mo.) was added to 200 μl of sample and incubated at 20°C for 90 min; 100 μl of 0.9% NaCl was similarly incubated with 200 μl of sample. The reaction was terminated by adding 100 μl of 4.4 N $HClO_4$; after centrifugation at 29,000 *g* for 10 min, 200 μl of supernate was removed from each tube for colorimetric measurement of Pi and 50 μl for ^{32}P counting.

Completeness of enzyme action. 20 μl of 6.5% ammonium molybdate, 200 μl of 1 mM Pi, and 20 μl of 0.1 M triethylamine hydrochloride were added to 100 μl of sample supernate after enzyme incubation. After being mixed and centrifuged at 40,000 *g* for 10 min, two aliquots of 100 μl were removed for determination of residual ^{32}P^{32}Pi. The percent PPi that escaped hydrolysis was calculated according to the formula

$$\frac{\dfrac{\text{residual cpm}}{\text{unit volume}} \times 3.4\ \text{(dilution factor)}}{\dfrac{\text{initial cpm}}{\text{unit volume}}\ \text{enzyme-treated aliquot}} \times 100 = \%\ \text{PPi}.$$

Colorimetric measurement of Pi. The method used was adapted from that described by Chen, Toribara, and Warner (12). 10 μl of 6.5% (wt/vol) ammonium molybdate was first added and, during continuous mixing with a Vortex Genie (Vortex Mixers, Scientific Industries, Inc., Queens Village, N. Y.), 50 μl of 6.5% ascorbic acid was added slowly. After incubation at 45°C for 50 min all samples were again placed in ice and OD_{800} was measured in a Beckman DU spectrophotometer (Beckman Instruments, Fullerton, Calif.), equipped with a Gilford model 222 absorbance indicator (Gilford Instrument Laboratories, Oberlin, Ohio), and automatic cuvette positioner model 210; 600 μl cuvettes, 1-cm light path, centered to read 100–200 μl were used. Standard Pi solutions and a reagent blank were carried through the procedure.

Spontaneous hydrolysis in control sample during colorimetric step. An additional 100 μl of the control sample was carried through the colorimetric reaction, with water substituted for ascorbic acid. Triethylamine precipitation of phosphomolybdate was carried out as described above and the percent hydrolysis calculated.

Calculations. The PPi concentration in the original sample was calculated by isotopic dilution after correction of the ΔOD for possible incomplete enzyme action and for spontaneous hydrolysis in the control.

$$\mu\text{M PPi} = \frac{\Delta\text{OD } (\text{OD}_e - \text{OD}_b) \times \text{corrections}}{\mu\text{M extinction coefficient}} \times \frac{\text{cpm/unit volume of original sample}}{\text{cpm/unit volume of final sample}}.$$

Conditions of colorimetric assay

The optimal time of incubation was determined by plotting the absorbance obtained when standard Pi solutions were incubated at 45°C for varying lengths of time. The final acid normality was 0.86, obtained with $HClO_4$, instead of H_2SO_4 as originally described (12). This is well within the

range where color development is proportional to phosphomolybdate concentration. In our procedure, the final molybdate concentration varies because of its earlier use in removal of varying amounts of Pi. Therefore the effect on absorbance of molybdate concentration from 0 to 10 mM was determined with a 7.3-μM Pi standard solution. The effect of molybdate concentration was determined at various acid normalities in the range reported by Chen et al (12), i.e., from 0.43 to 1.05 N.

Color development has been reported as a result of the interaction of PPi and ammonium molybdate under certain conditions (13, 14). Therefore, standard solutions of Pi and of PPi were prepared in 10 mM Tris-Cl buffer (pH 7.2) and in simulated deproteinized plasma that had been carried through the preparative procedures described above. The final concentrations of both Pi and PPi was 5 μM. 100 ml of the simulated plasma contained 14 meq of NaCl, 8 mg of $MgCl_2 \cdot 6\ H_2O$, 13.4 mg K_2HPO_4, and 2.7 mg $NaH_2PO_4 \cdot H_2O$. Additional samples were prepared containing both Pi and PPi, each at final concentrations of 5 μM. After incubation, the solutions were scanned from 320 to 900 in a Carey model 14 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.); the light source was changed to tungsten at 800 nm.

Specificity of yeast inorganic pyrophosphatase (PPiase)

Although this enzyme is highly specific, Schlesinger and Coon (15), and Kunitz (16) have described activity against ATP using zinc as a cofactor. To determine possible activity against nucleotides in our system, $^{32}P^{32}Pi$ was diluted with cold PPi to a final concentration of 15 μM; ATP labeled in the gamma position with ^{32}P was diluted with cold ATP to a concentration of 20 μM; 0.6 ml of each were incubated for 25 min at 20°C with from 0 to 14 U/ml PPiase in the presence of 28 μM $MgCl_2$. 0.7 ml of Chen reagent (12) was added to 0.3 ml of each of the incubated solutions and OD_{800} measured. The Pi in the remaining 0.3 ml was precipitated as described by Sugino and Miyoshi (11) after 50 μl of 1 mM Pi had been added. The radioactivity remaining in the supernate was counted.

Recovery experiments and duplicate analyses

PPi was added to plasma and to simulated plasma to final concentrations of 0–5.0 μM. Duplicate analyses were performed on 17 consecutive plasma samples from normal subjects or patients with nonsystemic forms of arthritis, and on 18 consecutive synovial fluid samples obtained from the knees of patients with various types of arthritis.

Application of the method to urine

Urinary Pi levels were determined by the method of Fiske and SubbaRow (17) in seven sequential samples from a healthy 34-yr-old Caucasian man during varying degrees of hydration. Urine osmolality was obtained by measuring freezing point depression (Advanced Instruments, Inc., Needham Heights, Mass.). If necessary, the samples were diluted to reduce the Pi concentration below 1.5 mM, and the PPi determined in 5-ml samples as described above.

Plasma PPi levels in normal subjects and in patients with pseudogout, osteoarthritis, acromegaly, and uremia

Platelet-poor plasma was prepared from blood obtained from the antecubital vein of 94 normal subjects of both sexes ranging in age from newborn (cord blood) to 87 yr. PPi was measured as described above and Pi was measured by the method of Fiske and SubbaRow (17). Pi and PPi were also determined in 33 patients with definite pseudogout as diagnosed by our previously published criteria (3); in 30 patients with osteoarthritis of the knee or hip, or with the primary generalized form of this disease; and in 12 patients with definite rheumatoid arthritis by American Rheumatism Association criteria (18). All patients were attending the outpatient clinic or were hospitalized at the University of Chicago. Five samples from three patients with uremia, and four samples from three patients with active acromegaly were also analyzed.

RESULTS

Analysis for PPi. The method was reliable and relatively easy to perform. A single technician can analyze 30 samples a week for both Pi and PPi. With experience, the procedure was shortened by elimination of the checks for completeness of enzymatic hydrolysis and for the percent of PPi hydrolyzed by acid in the buffer portion during the colorimetric procedure; repeated determinations showed that these figures were virtually constant at 98–100% and 9–11%, respectively.

Conditions for colorimetric assay. The effect upon the OD_{800} of reduced phosphomolybdate of varying final concentrations of ammonium molybdate at different acid normalities is shown in Fig. 1. These effects were determined as molybdate was used to remove the endogenous Pi in the original sample. The original Chen colorimetric method for Pi uses 2.0 molybdate and 0.6 N acid concentrations (12). We added sufficient molybdate for the colorimetric reaction to achieve a final concentration of 1.2 mM, so that even if the initial sample contained only 0.5 mM Pi, the final molybdate concentration would be no greater than 7.0 mM.[2] Thus the OD at a final acid concentration of 0.86 N was proportional to the Pi present as reduced phosphomolybdate in the sample.

The μM absorbance for Pi using these conditions was 0.02, or 0.04 OD/μM PPi in the final solution. This color was developed maximally in 45 min and was stable for at least 24 h. In practice, the tubes were placed in ice after incubation and read promptly in order to prevent more than 11% hydrolysis of the PPi in the buffer portion. $HClO_4$ was substituted for H_2SO_4 used by Chen et al. (12) to precipitate the enzyme which, if not removed, interferes with color development.

The mean optical density of the buffer control portions ("background") of 30 consecutive plasma samples was 0.150 with a range of 0.077–0.385; the mean increase in optical density of the enzyme-treated aliquot was 0.135 with a range of 0.027–0.390.

PPi did not contribute to the absorbance at 800 nm

[2] Assumes 2 mol of Pi precipitates with 1 mol of molybdate (11), and a 10-fold concentration of ions by the isobutanol steps.

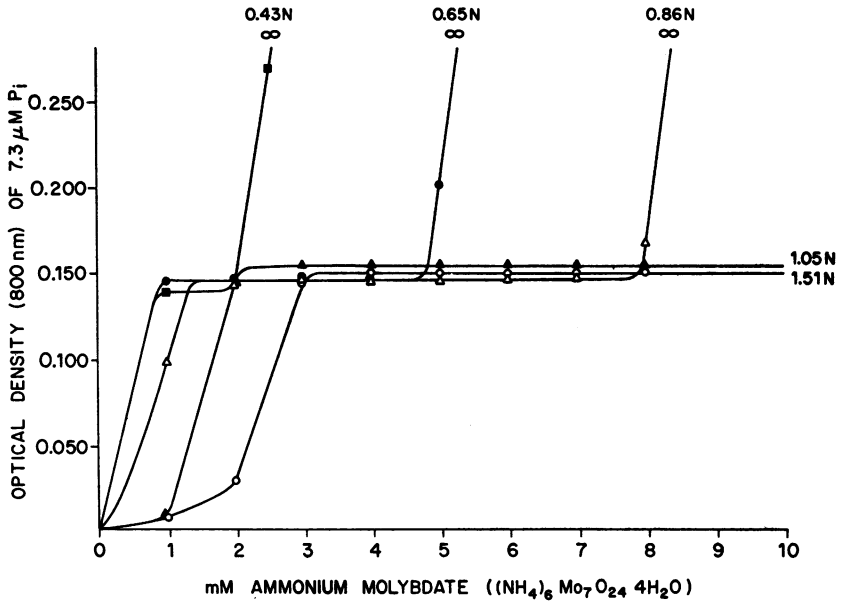

FIGURE 1 The optical density at 800 nm of reduced phosphomolybdate is shown at varying final concentrations of ammonium molybdate and acid normalities. See text for details.

under the conditions described (Table I). There was 8% hydrolysis of PPi in buffer, and in simulated plasma 22.5% of the added PPi was hydrolyzed. The only absorbance peak was 800 nm; no peak was seen at 575 nm as described for the PPi-molybdate chromophore (13).

Specificity of yeast inorganic pyrophosphatase. The hydrolysis of bovine ATP under the conditions described above was not measurable (Fig. 2). Even with the more sensitive radiochemical method (19), hydrolysis was negligible.

Recovery experiments and duplicate determinations. The mean recovery of PPi added to simulated plasma was 94.2% with a range of 87.3–112.0%. The mean recovery of PPi added to normal human plasma was 92% with a range of 88.8–111.0% (Table II).

Duplicate analyses of 17 consecutive plasma samples showed a standard deviation of ±0.18 μM (20). The values found are shown in Fig. 3A. A similar analysis of 18 consecutive joint fluid samples showed a standard deviation of ±0.85; the values are shown in Fig. 3B.

Application of the method to urine and plasma. Urinary Pi and PPi concentrations are given in Table III, together with the osmolality of each sample. Proportionality was found over a wide range of concentration.

The PPi concentrations found in plasma samples from 94 normal subjects ranging in age from newborn (cord

TABLE I
Absorbance at 575 nm and at 800 nm of Solutions of Pi and PPi

	Concentration	In buffer		In simulated plasma	
		OD_{575}	OD_{800}	OD_{575}	OD_{800}
	μM				
Pi	5	0.000	0.103	0.000	0.099
PPi	5	0.000	0.016	0.000	0.045
Pi + PPi	5 + 5	0.000	0.114	0.000	0.144

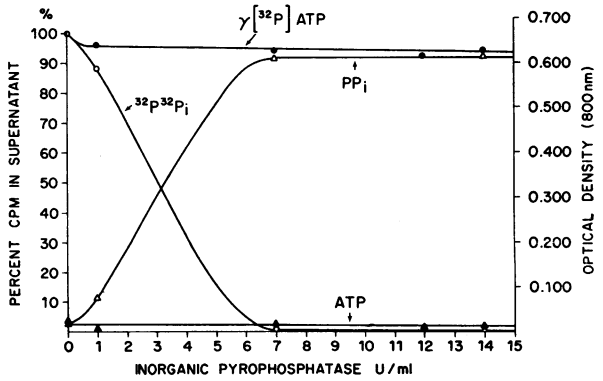

FIGURE 2 The absorbance at 800 nm of 15 μM pyrophosphate (PPi) and 20 μM equine ATP after each was incubated with crystalline Type I yeast PPiase for 45 min at 25°C is shown at varying concentrations of enzyme. In parallel experiments using gamma-labeled [^{32}P]ATP in 20 μM cold equine ATP and $^{32}P^{32}Pi$ in 50 μM cold PPi, the percent of radioactivity remaining in the supernate after precipitation of ^{32}P is shown.

TABLE II

Recovery of PPi Added to 5-ml Samples

PPi + simulated plasma*			PPi + plasma‡		
Added	Recovered	%	Added	Recovered	%
	nmol			*nmol*	
2.5	2.20	88.0			
2.5	2.80	112.0	5.0	5.55	111.0
5.0	4.90	98.0	5.0	5.20	104.0
10.0	9.75	97.5			
10.0	9.10	91.0	15.0	14.75	98.3
15.0	14.05	93.7	20.0	18.90	94.5
15.0	14.05	93.7	20.0	19.70	98.5
20.0	17.45	87.3			
20.0	18.35	91.8	25.0	22.20	88.8
25.0	23.15	92.6	25.0	22.75	91.0
25.0	22.75	91.0			

* See text for composition; the amounts of PPi added were calculated to produce final concentrations ranging from 0.5 to 6 μm in the sample.

‡ "Background" PPi subtracted.

TABLE III

Urinary Inorganic Pyrophosphate and Inorganic Orthophosphate during Varying Degrees of Hydration

Osmolality	PPi	Pi	Ratio
mosmol/kg	μM	*mM*	$(PPi/Pi) \times 10$
978	75.6	66.4	11.4
445	11.8	9.9	11.9
70	0.3	0.3	10.0
66	0.5	0.4	12.5
69	5.1	0.5	22.0
118	2.7	1.6	16.9
539	16.1	13.3	12.1

blood) to 87 yr are shown graphically in Fig. 4. The mean value was 1.8 μM±0.06 (SEM), giving a range (99% confidence limits) of 0.15–3.4 μM. The distribution, plotted for each 0.5 μM increment, appeared to be that of a single mode. This was tested by probit analysis. The probit points were distributed nearly in a straight line, suggesting normal distribution around a single mode (Fig. 5). The mean plasma PPi was 2.34±0.17 μM in pseudogout patients, which differed significantly from the normal mean ($P < 0.01$; Student's t test). The distribution of these values suggested more than one population; the mean value of the lower curve was 1.9 μM (Fig. 6A). Probit analysis suggested a bimodal distribution of values (Fig. 5). The mean PPi level in samples from osteoarthritis patients was 2.62±0.12 μM, also differing significantly from the normal mean ($P < 0.01$), but not from the mean of the pseudogout patients ($P =$ NS) (Fig. 6B). The mean values of plasma PPi in samples from patients with rheumatoid arthritis, acromegaly, and uremia was 1.85±0.14 μM, 5.9±2.2 μM, and 3.4± 1.14 μM, respectively. The distribution of probit points in rheumatoid arthritis closely approximated the normal distribution (Fig. 5).

Plasma Pi levels in the normal series ($n = 90$) were 1.03±0.02 mM. Pi values in samples from osteoarthritis patients ($n = 29$) showed a mean of 1.03±0.03, from pseudogout patients ($n = 33$) 0.96±0.04 mM, from rheumatoid arthritis patients ($n = 12$) 1.08±0.04 mM, from uremic subjects ($n = 5$) 3.39±0.98 mM, and from acromegalic patients ($n = 4$) 1.36±0.12 μM. With linear

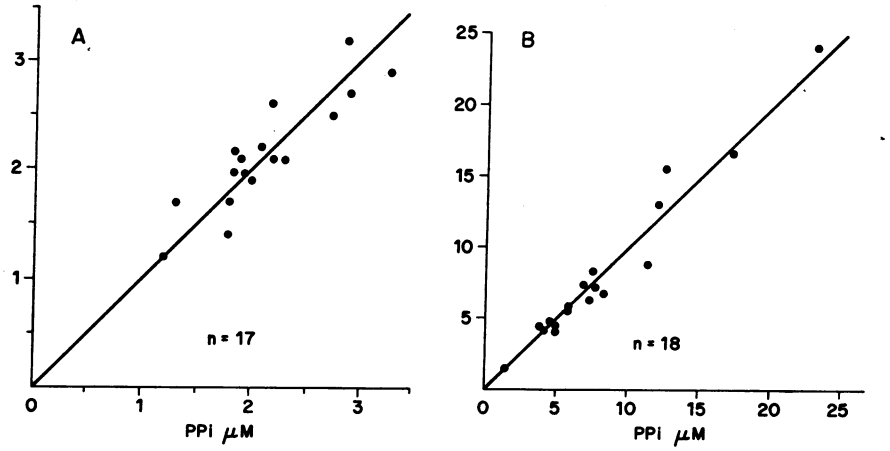

FIGURE 3 A. Duplicate measurements of PPi in 17 consecutive plasma samples are shown; the standard deviation was ±0.18 μM. B. Duplicate analysis of PPi in consecutive joint fluids are shown; the standard deviation was ±0.85. The ordinate and abscissa scales represent the values in micromoles per liter for each pair of determinations.

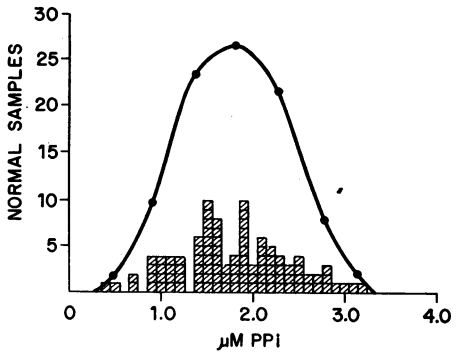

FIGURE 4 The results of PPi determinations in plasma samples from 94 normal subjects ranging in age from newborn (cord blood) to 87 yr are shown. The frequency distribution, plotted for each 0.5 μM increment, is that of a single population. The mean ±SEM is 1.8±0.06 μM, giving a normal range of 0.16–3.4 μM (99% confidence limits).

regression, plasma Pi and PPi correlated in the normal series ($r = \pm 0.49$; $P < 0.001$, assuming a two-tailed null hypothesis). A correlation was also evident using a nonparametric method (Spearman rank correlation), $r = 0.45$, $P < 0.001$. This correlation is shown graphically in Fig. 7. A correlation between Pi and PPi was also found in the samples from patients with osteoarthritis ($r = +0.5$; $P < 0.05$) but not in those from patients with pseudogout ($r = +0.25$; $P = \text{NS}$), rheumatoid arthritis ($r = +0.25$; $P = \text{NS}$), acromegaly ($r = -0.08$; $P = \text{NS}$), or uremia ($r = +0.25$; $P = \text{NS}$).

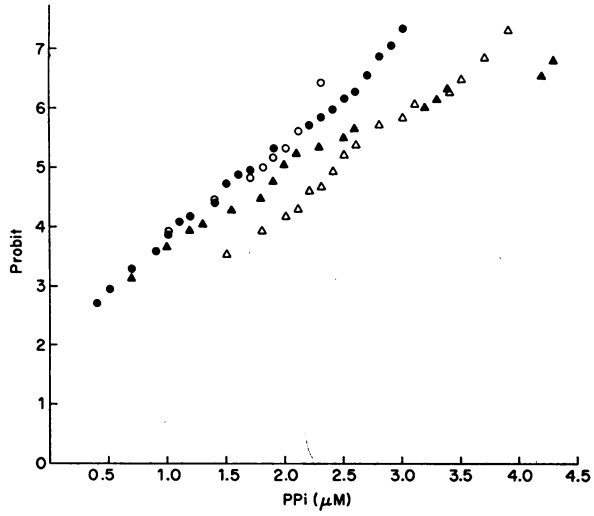

FIGURE 5 Probit points calculated from the distribution of PPi in normal subjects are distributed nearly in a straight line (●). The distribution of PPi values in pseudogout (▲) suggest a bimodal distribution with the lower values approximating the normal distribution. In osteoarthritis (△) the values were distributed as a single mode about a higher than normal mean. Values from rheumatoid arthritis subjects (○) were normally distributed.

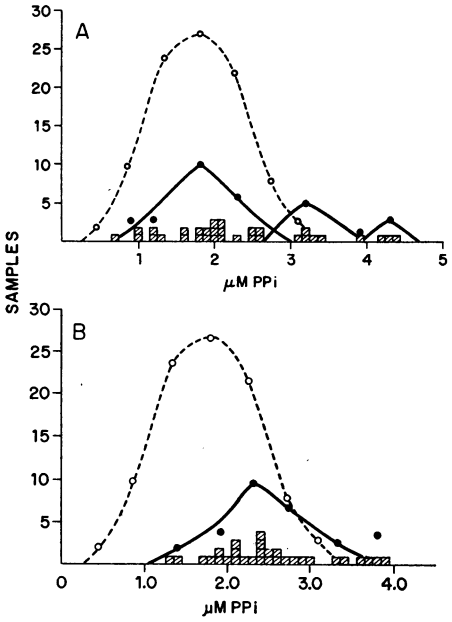

FIGURE 6 A. PPi concentration in samples from 33 patients with pseudogout are shown. The mean ±SEM is 2.34±0.17 μM. The distribution suggests more than one mode. The distribution in the normal population is shown (dashed curve) for comparison. The mean value of the lower curve is 1.9 μM. B. PPi concentration in 30 plasma samples from 30 patients with osteoarthritis showed a mean ±SEM of 2.62±0.12.

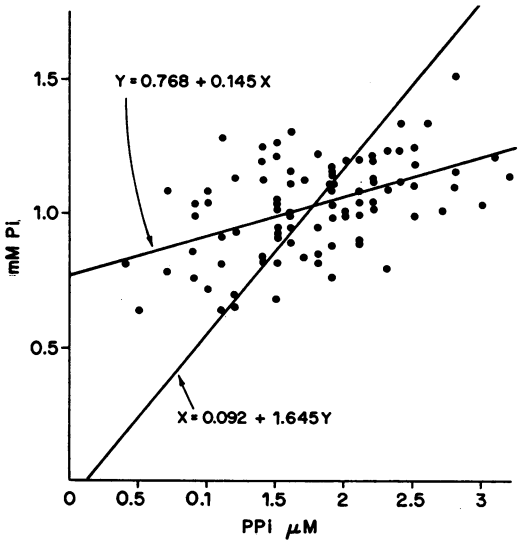

FIGURE 7 Scattergraph showing correlation of Pi and PPi in normal samples ($n = 90$). The correlation was significant ($P < 0.001$) using either linear regression or Spearman rank correlation.

DISCUSSION

A procedure for quantification of PPi in plasma and other biological material was developed based on spectrophotometric measurement of reduced phosphomolybdate in a portion of sample treated wtih yeast pyrophosphatase after deproteinization and removal of Pi. The background absorbance of a control portion not treated with enzyme was subtracted, and the micromolar concentration was related to that present in the original sample by isotope dilution. The variables in each step of the method were isolated and controlled. A single technician can analyze 30 samples a week for both Pi and PPi. The technique has been used by us to measure PPi in plasma, urine, synovial fluid, and tissue extracts.

Heretofore, only direct chemical analysis of PPi by acid hydrolysis after column chromatographic separation of phosphates has been used in clinical investigation. The method originally described by Fleisch and Bisaz for urinary PPi (21) has been used successfully after extensive preparative procedures for PPi determination in plasma and synovial fluid (4, 7). Russell, Bisaz, Donath, Morgan, and Fleisch found a mean ±SEM of 3.56±0.11 μM in plasma samples from 73 normal subjects, with a normal (99%) range of 1.19 — 5.65 μM (7). Plasma PPi in 94 normal subjects studied by our method showed a mean ±SE of 1.8±0.06 (range 0.4 — 3.2 μM), with a normal statistical (99%) range of 0.16 — 3.4 μM. The reason for these differences may lie in variation in sample preparation, i.e., completeness of platelet removal. The two methods are comparable with regard to precision. Analysis of 20 plasma samples in duplicate by Russell et al. (7) showed a standard deviation of 0.18 giving a 99% probability that a single determination would lie ±0.47 μM of the true value. Duplicate analysis of 17 plasma samples by our method also showed a standard deviation of 0.18, giving a 99% probability that a single determination would be ±0.68 μM of the true value.

Two adaptations of the UDPG pyrophosphorylase method for measurement of PPi described by Johnson et al. (8) have appeared which permit ultramicroanalysis. Flodgaard has measured concentrations of PPi as low as 1 μM in a sample volume of 10 μl, using tritrated UDPG of high specific activity and isolating [^{3}H]UTP after completion of the enzymatic reaction (9). Altman and colleagues have recently adapted the UDPG pyrophosphorylase method using spectrophotometry and cuvettes of long light path; an internal standard was added to a portion of original sample at the time of collection, and the original concentration calculated according to the proportional recovery of standard (6). The details of this adaptation have not yet been published.

A colorimetric procedure for microdetermination of PPi and Pi in the same sample has been described recently by Grindley and Nichol (13). The method is quantitative when both substances are present in concentrations of the same order of magnitude. Its possible use in quantifying PPi in biological material has not yet been explored.

Relatively little is known of PPi metabolism. Application of the method described in this paper and of the other procedures mentioned here should greatly expand our knowledge of the biological significance of this important metabolite.

The PPi levels in rheumatoid arthritis plasma samples showed a mean nearly identical to that found in normal controls (Fig. 5), whereas the mean PPi values in both osteoarthritis and pseudogout were significantly elevated. The increase in plasma PPi in some samples from patients with osteoarthritis and pseudogout was unexpected and cannot be readily explained. Many of these patients were elderly, but age per se does not seem to be a factor, as no significant difference in plasma PPi was noted in the aged normal subjects as compared to their younger counterparts. The synovial fluid PPi levels are often elevated in patients with pseudogout (4–6) and with osteoarthritis (6).[3] It is conceivable that diffusion from the joint into the systemic circulation could account for the observed increase. Indeed, measurement of the PPi concentrations in paired femoral arterial and femoral venous blood samples show a mean PPi gradient of 25%.[3] The samples analyzed are relatively few, and until something is known of the origin of plasma PPi and the factors influencing its turnover, we prefer to regard the findings reported here as tentative. In any event, the highest values found are far from those representing the apparent solubility of PPi in plasma equilibrated with crystalline calcium pyrophosphate dihydrate (5).[3]

Elevated levels of plasma PPi in uremic subjects have been reported previously by Russell, Bisaz, and Fleisch (22). Only one of five samples in our series was above the normal range, although the mean of the remaining four was 2.8 μM. Russell and coworkers speculate that such levels may be explained simply by a decreased glomerular filtration rate leading to retention of both Pi and PPi, or to increased inhibition by Pi of enzymatic hydrolysis of PPi by the kidney. Growth hormone is thought to increase plasma Pi by increased renal tubular resorption of phosphate (23). The association of joint disease closely resembling osteoarthritis with active acromegaly suggests that the elevation of PPi in these conditions may share a common pathway. Acromegaly has been reported in association with pseudogout (24), but no systemic search for calcium pyrophosphate crystal deposition has been made in acromegalic patients.

Note added in proof. The type I PPiase used here is no longer available from the Sigma Chemical Company. The

[3] Unpublished observations of the authors.

type III lyophilized enzyme, specific activity 500 U/mg protein, has been substituted.

The concentration of the type III enzyme needed to effectively hydrolyze the PPi was reduced to 0.1 U/200 μl sample by adding 50 μl of 50 mM EDTA and 50 μl of 65 mM $MgCl_2$ to the isobutanol concentrate (approximately 500 μl). The inhibition of color development by 0.1 U of pyrophosphatase was negligible (about 0.005 OD units) and HCl was substituted for $HClO_4$ in the colorimetric reaction, which reduced the sample dilution and increased the sensitivity of the method. With decreased sample dilution, it was necessary to control the molybdate concentration more closely and this was done simply by first measuring the Pi in each sample and then varying the ammonium molybdate used to precipitate it; 4% (wt/vol) was used for 0–0.7 mM Pi, 7.5% for 0.7–1.2 mM Pi, and 10% for 1.2–1.5 mM Pi.

The mean OD observed in 30 consecutive plasma samples was 0.202 with a range of 0.067–0.307, and the background OD was reduced to 0.049 with a range of 0.029–0.082.

ACKNOWLEDGMENTS

The authors are indebted to Miss Olivia Pamatmat for expert technical assistance, and to Drs. R. G. G. Russell, S. Bisaz, and H. Fleisch for their helpful suggestions.

This work was supported by U. S. Public Health Service Grants AM-13069 and AM-05621 and by grants from the Illinois Chapter of The Arthritis Foundation.

REFERENCES

1. McCarty, D. J. J., 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.
2. Kohn, N. N., R. E. Hughes, D. J. McCarty, Jr., and J. S. Faires. 1962. The significance of calcium phosphate crystals in the synovial fluid of arthritic patients: the "pseudogout syndrome." II. Identification of crystals. *Ann. Intern. Med.* **56**: 738.
3. McCarty, D. J. 1966. Calcium pyrophosphate crystal deposition disease pseudogout, articular chondrocalcinosis. *In* Arthritis and Allied Conditions. J. L. Hollander, editor. Lea & Febiger, Philadelphia. 7th edition. 947.
4. Russell, R. G. G., S. Bisaz, H. Fleisch, H. L. F. Currey, H. M. Rubinstein, A. A. Dietz, I. Boussina, A. Micheli, and G. Fallet. 1970. Inorganic pyrophosphate in plasma, urine, and synovial fluid of patients with pyrophosphate arthropathy (chondrocalcinosis or pseudogout). *Lancet.* **2**: 899.
5. McCarty, D. J., S. D. Solomon, M. L. Warnock, and E. Paloyan. 1970. Inorganic pyrophosphate concentrations in the synovial fluid of arthritic patients. *J. Lab. Clin. Med.* **78**: 216.
6. Altman, R. D., O. Muniz, J. C. Pita, and D. S. Howell. 1972. Ultramicro analysis of inorganic pyrophosphate (PPi) in synovial fluid and blood. *Arthritis Rheum.* **15**: 101. (Abstr.)
7. Russell, R. G. G., S. Bisaz, A. Donath, D. B. Morgan, and H. Fleisch. 1971. Inorganic pyrophosphate in plasma in normal persons and in patients with hypophosphatasia, osteogenesis imperfecta, and other disorders of bone. *J. Clin. Invest.* **50**: 961.
8. Johnson, J. C., M. Shanoff, S. T. Bass, J. A. Boezi, and R. G. Hansen. 1968. An enzymatic method for determination of inorganic pyrophosphate and its use as an assay for RNA polymerase. *Anal. Biochem.* **26**: 137.
9. Flodgaard, H. 1970. Isotope derivative method for determination of microquantities of inorganic pyrophosphate in biological material. *Eur. J. Biochem.* **15**: 273.
10. Silcox, D. C., S. Jacobelli, and D. J. McCarty. 1972. The identification of inorganic pyrophosphate (PPi) in human platelets. *J. Clin. Invest.* **51**: 90 a. (Abstr.)
11. Sugino, Y., and Y. Miyoshi. 1964. The specific precipitation of orthophosphate and some biochemical applications. *J. Biol. Chem.* **239**: 2360.
12. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**: 1756.
13. Grindey, G. B., and C. A. Nichol. 1970. Microprocedure for determination of pyrophosphate and orthophosphate. *Anal. Biochem.* **33**: 114.
14. Flynn, R. M., M. E. Jones, and F. Lipmann. 1954. A colorimetric determination of inorganic pyrophosphate. *J. Biol. Chem.* **211**: 791.
15. Schlesinger, M. J., and M. J. Coon. 1960. Hydrolysis of nucleotide di- and triphosphates by crystalline preparations of yeast inorganic pyrophosphatase. *Biochim. Biophys. Acta.* **41**: 30.
16. Kunitz, M. 1962. Hydrolysis of adenosine triphosphate by crystalline yeast pyrophosphatase. *J. Gen. Physiol.* **45**(Pt. 2, Suppl.): 31.
17. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**: 375.
18. Ropes, M. W., G. A. Bennett, S. Cobb, R. Jacox, and R. A. Jessar. 1958. 1958 revision of diagnostic criteria for rheumatoid arthritis. *Bull. Rheum. Dis.* **9**: 175.
19. McCarty, D. J., and P. F. Pepe. 1972. Erythrocyte neutral inorganic pyrophosphatase in pseudogout. *J. Lab. Clin. Med.* **79**: 277.
20. Holman, H. H. 1962. Estimation of error by duplicate measurement. Biological Research Method, Hafner Publishing Co., New York. 75.
21. Fleisch, H., and S. Bisaz. 1962. Isolation from urine of pyrophosphate, a calcification inhibitor. *Am. J. Physiol.* **203**: 671.
22. Russell, R. G. G., S. Bisaz, and H. Fleisch. 1969. Pyrophosphate and diphosphonates in calcium metabolism and their possible role in renal failure. *Arch. Intern. Med.* **124**: 571.
23. Corvilain, J., and M. Abramow. 1962. Some effects of human growth hormone on renal hemodynamics and on tubular phosphate transport in man. *J. Clin. Invest.* **41**: 1230.
24. Lamotte, M., J. M. Segrestaa, and G. Krassinine. 1966. Arthrite microcristalline calcique (pseudo-goutte) chez un acromégale. *Sem. Hôp. Paris.* **42**: 2420.