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**Research Article**

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# Inorganic Pyrophosphate in Plasma in Normal Persons and in Patients with Hypophosphatasia, Osteogenesis Imperfecta, and Other Disorders of Bone

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Abstract An isotope dilution method, using $^{32}P$-labeled pyrophosphate, has been developed for the measurement of inorganic pyrophosphate ($PP_i$) in human plasma. The specificity of the method was better than 90% as assessed by elution patterns during ion-exchange chromatography, by paper chromatography, and by incubation with inorganic pyrophosphatase. The 99% confidence limits for a single estimation of plasma $PP_i$ was ±13%. There were no differences in plasma $PP_i$ between men and women, but the values in young people (0–15 yr) were slightly higher than in older people. The mean concentration (±SE) of $PP_i$ in the plasma of 73 men and women was 3.50 ±0.11 μmoles/liter (0.217 ±0.007 μg P/ml) and the normal range (99% limits) was 1.19–5.65 μmoles/liter (0.074–0.350 μg P/ml).

It has been suggested that $PP_i$ may be important in calcium metabolism because $PP_i$ can prevent the precipitation of calcium phosphates in vitro and in vivo, and can slow the rates at which hydroxyapatite crystals grow and dissolve. Plasma $PP_i$ was therefore measured in several disorders of bone. Normal values were found in osteogenesis imperfecta, osteopetrosis, "acute" osteoporosis, and primary hyperparathyroidism. Plasma $PP_i$ was invariably raised in hypophosphatasia. The excess of $PP_i$ in plasma might be the cause of the defective mineralization in hypophosphatasia and the function of alkaline phosphatase in bone may be to act as a pyrophosphatase at sites of calcium deposition.

## INTRODUCTION

Inorganic pyrophosphate ($PP_i$) is known to be produced as a by-product of many biosynthetic reactions in vivo (1). Although its role in individual enzyme reactions is well established, very little is known about the metabolism of $PP_i$ in intact animals. Recent studies have suggested that $PP_i$ may be important in regulating calcium metabolism. Thus small amounts of $PP_i$ inhibit the precipitation of calcium phosphate from solution (2, 3) and bind strongly to crystals of hydroxyapatite (4). Apatite crystals to which $PP_i$ has adsorbed grow and dissolve more slowly than nontreated crystals (4, 5). This suggests that the $PP_i$ known to be present in bone may be able to control the rates at which bone crystals grow and dissolve, and may be important in calcium homeostasis (6, 7). It is possible that disturbances in the metabolism of $PP_i$ might lead to changes in the concentrations of $PP_i$ in bone and might alter the rates of mineral accretion and dissolution in bone. It is therefore important to be able to study the metabolism of $PP_i$ in human diseases in which the turnover of bone is abnormal.

Studies on the metabolism of $PP_i$ in man have so far been restricted by the lack of a suitable method for measuring the low concentrations of $PP_i$ present in plasma. The only published method (8) for plasma $PP_i$ is probably nonspecific, as will be discussed later. In this paper, we described a new specific method and present some measurements of plasma $PP_i$ made in normal persons and in various diseases of bone, particularly three congenital conditions, hypophosphatasia, osteogenesis imperfecta, and osteopetrosis.

## METHODS

### Introduction

The main difficulties in developing a satisfactory technique for measuring $PP_i$ in plasma were that the concentrations of $PP_i$ were very low, that other compounds appeared to interfere with the chemical determination, and that $PP_i$

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was subject to variable and often extensive hydrolysis during the analysis.

The method finally adopted was based on isotope dilution. In outline the essential steps were addition of $^{32}$P-labeled pyrophosphate to the blood at the time of collection, preparation of the plasma, deproteinization of the plasma by ultrafiltration, two coprecipitations of $PP_i$ with calcium phosphate, treatment with a cation exchange resin to remove calcium and nucleotides, and finally, separation of $PP_i$ from other phosphate compounds by chromatography on an anion exchange resin. The specific radioactivity of the $PP_i$ eluted from the columns was determined and the concentration of $PP_i$ in the original plasma could then be calculated.

The technique will be described in detail with the results of various tests applied to test the reproducibility and sensitivity of the method.

## The measurement of $PP_i$ in human plasma

30–50 ml of venous blood was collected into glass vessels[1] surrounded by ice. These vessels contained heparin and sufficient known small amounts of $^{32}$P[$PP_i$] (from the Radiochemical Centre, Amersham, England, initial specific activities 5–205 mCi/mmole) to allow a total initial activity of around 100,000 cpm. The $^{32}$P[$PP_i$] was added in order to correct for subsequent losses of $PP_i$. The collection of whole blood directly into the $^{32}$P[$PP_i$] appeared a valid procedure since the entire radioactivity remained outside the cells and could be completely recovered in the plasma after separation. Immediately after collection of the blood, ethylenediaminetetraacetate (EDTA) was added (1 ml of 200 mM EDTA disodium salt, adjusted to pH 7.4 with NaOH, was added for every 10 ml of blood). This addition of EDTA reduced the rate of hydrolysis of $PP_i$ in the blood. Thus preliminary studies had shown that, in plasma from patients with elevated alkaline phosphatase, the hydrolysis of $PP_i$ by the end of ultrafiltration (see below) could reach 100%. This hydrolysis was reduced by the routine addition of EDTA to the blood. For example in three different blood samples kept at 0°–4°C (without EDTA) the hydrolysis of added $^{32}$P[$PP_i$] ranged from 8 to 28% after 5 hr and from 28 to 67% after 22 hr. The addition of EDTA reduced this hydrolysis to 5–10% during 22 hr.

An aliquot of blood was removed for determination of packed red cell volume and radioactivity ($3 \times 50$ $\mu$l). The remainder of the blood was immediately centrifuged at 0°–4°C and the plasma separated. At this stage it is important that there should be no hemolysis, in order to avoid potential interference by red cell nucleotides. The blood should be centrifuged sufficiently hard to remove platelets and leucocytes. In practice, the centrifugation was either carried out in the presence of Plasaid beads (Stayne Laboratories Ltd., High Wycombe, Bucks, England) or the plasma was centrifuged twice.

If the analysis could not be performed immediately the plasma was frozen (at about −20°C) after separation. After taking aliquots of plasma ($3 \times 50$ $\mu$l) for determination of radioactivity, the plasma was equilibrated with a mixture of 5% $CO_2$ and 95% air and was then ultrafiltered at 2°–6°C. The equilibration with 5% $CO_2$ before ultrafiltration was necessary in order to prevent the precipitation of calcium phosphate and coprecipitation of $PP_i$. Ultrafiltration was carried out in a special apparatus constructed of Perspex (Plexiglas). A yield of 11–26 ml of ultrafiltrate could be obtained from 15–30 ml of plasma ultrafiltered overnight through Visking dialysis membranes of about 6 cm diameter under a pressure of 5–8 atmospheres (compressed air). Ultrafiltration proved superior to other methods of deproteinisation such as precipitation with alcohol or trichloroacetic acid.

The mean (±SEM) concentration of radioactivity in the ultrafiltrate from 43 plasma samples was 82 ±1% of the radioactivity in the original plasma. Some hydrolysis of the $^{32}$P[$PP_i$] occurred during separation of the plasma and during ultrafiltration, even though all steps were carried out in the cold. This illustrates the importance of using an isotope dilution technique to correct for losses.

The $PP_i$ was coprecipitated from the ultrafiltrate with calcium phosphate. This step is an important one since it allows the $PP_i$ to be removed from many of the salts and other contaminating substances in the ultrafiltrate. It was based on the observation that calcium phosphates adsorb $PP_i$ very strongly (4, 9). 0.5 N KOH was added until the ultrafiltrate was just yellow with methyl red as indicator, 250 mM $CaCl_2$ was then added drop by drop until a copious precipitate had formed. The precipitate was recovered by centrifugation, dissolved in the cold in a minimum amount of 0.5 N HCl, and the volume made to 25 ml with water. A further 0.25 mmole of calcium was added, and the precipitation repeated by adding 0.5 N KOH. The precipitate was again dissolved in 0.5 N HCl and diluted to 5 ml with water.

An excess (500 mg) of Dowex 50-W × 4 (200–400 mesh, sodium form, previously washed with 1 N NaOH, $H_2O$, 1 N HCl, $H_2O$, 1 M NaCl and $H_2O$) was then shaken with the dissolved precipitates for 2 min to remove calcium and nucleotides. Treatment with Dowex 50-W proved a better method of removing added adenosine di- and triphosphates (ADP and ATP) than acid-washed charcoal. The resin was removed by filtration through a glass sinter (G4) and washed four times with 3 ml of water. The filtrate and washings were collected in the cold in a vessel containing 0.5 ml of 0.2 M tris(hydroxymethyl)aminomethane base (Tris). The volume was made to 20 ml with $H_2O$ and the pH, measured with indicator paper, was checked to ensure that it was greater than 7.

The neutralized 20 ml of solution remaining after treatment with Dowex 50-W will be referred to later as "plasma extract." It was applied to columns containing anion exchange resin (Dowex 1 × 10, 100–200 mesh, chloride form). The resin was prepared by repeated alternate washings with 1 N NaOH, $H_2O$, 4 N HCl, and $H_2O$. Between runs the columns were regenerated with at least 100 ml of 4 N HCl, followed by washing with $H_2O$ until the effluent was chloride free. The columns were 17–20 cm long and had an internal diameter of 8.0–8.5 mm. Each column contained 10 ml of wet resin bed and had a flow rate of 0.5–1 ml/min. These columns are similar to those used for determination of $PP_i$ in urine, as described previously (10, 11). The columns were always of Pyrex glass, since some other types of glass, e.g. soda glass, adsorbed $P_i$ and $PP_i$ and this interfered with their separation. After washing the columns with 10 ml of water, $P_i$ could be eluted either with 100 ml of 0.05 N HCl or with 200 ml of a solution of 0.133 M KCl and 25 mM $Na_2B_4O_7$ (in the latter case followed by 25 ml of $H_2O$ to remove salts if paper chromatography or repeat ion-exchange chromatography was to be carried out). For routine purposes we always use 0.05 N HCl to elute $P_i$ because, although the KCl-borate solution gave better recoveries of $PP_i$, the $PP_i$ fractions sometimes contained

[1] Glassware must not be washed in commercial detergents containing phosphates or polyphosphates.

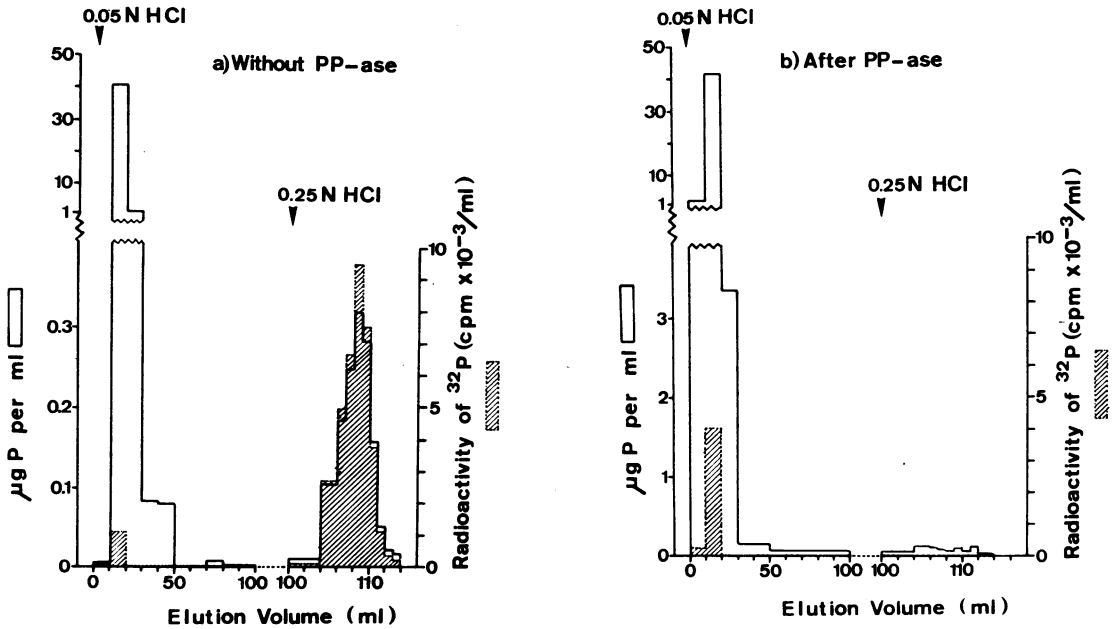


FIGURE 1 Elution of orthophosphate ($P_i$) and pyrophosphate ($PP_i$) from ion-exchange columns (Dowex 1 × 10, 100–200 mesh, chloride form) after application of plasma extract prepared as described in the text. $^{32}P[PP_i]$ was added to the blood at the time of collection and the elution of radioactivity is shown (*a*) without and (*b*) with incubation with inorganic pyrophosphatase (at ultrafiltrate stage, see text). There is a close relation between the elution of phosphate-reacting material and radioactivity ($^{32}P$) in the pyrophosphate peak in (*a*), and neither phosphate nor $^{32}P$ appears in this position after treatment with pyrophosphatase (*b*).

traces of $P_i$ due to adsorption of $P_i$ to the columns. After elution of $P_i$, $PP_i$ was eluted with 0.25 N HCl, fractions being collected as 1 × 4 ml (fraction 1, 100–104 ml), 1 × 2 ml (fraction 2, 105–106 ml), 5 × 1 ml (fractions 3–7, 107–111 ml), and 3 × 2 ml (fractions 8–10, 112–117 ml). Aliquots (2 × 50 μl) were taken from fractions 3–7 for determination of radioactivity. Concentrated HCl was then added to all the fractions to bring the HCl concentration in each to about 0.5 N HCl. The $PP_i$ in the fractions was hydrolyzed to $P_i$ by heating for 30 min in a boiling water bath. A volume of a reagent mixture (0.5% w/v ammonium molybdate and 2% w/v ascorbic acid dissolved in 1 N HCl) equal to that of the fraction was then added and the whole heated for 10 min in a boiling water bath. After cooling the $P_i$ was determined by measuring the extinction at 820 mμ (molar extinction coefficient, $E_{1\,cm} = 2.7 \times 10^4$) using semi-microcuvettes in a Beckman DU spectrophotometer. Whenever radioactivity due to $^{32}P$ was measured, 50-μl samples were applied in duplicate or triplicate to washed aluminum planchets sprayed with plastic film. When dry, the planchets were counted in an automatic methane gas flow counter (Frieseke and Hoepfner, Erlangen-Bruck, West Germany).

To calculate the concentration of $PP_i$ in the original plasma, use was made of the principle of isotope dilution. The calculations are shown in the Appendix.

The value for specific activity of $^{32}P[PP_i]$ from ion-exchange chromatography was taken as the mean activity of fractions 3–7 (107–111 ml) from the 0.25 N HCl eluates. The specific activities of these fractions were usually remarkably uniform, suggesting that only a single compound was eluted in these fractions (see Fig. 1). Occasionally, however, in our earlier studies, when the alkaline borate-KCl mixture was used to elute orthophosphate, there was an obvious progressive decrease in specific activity from fractions 5 through 7, presumably because these later fractions contained phosphate-reacting material that was not $PP_i$. Detailed studies showed that this material was orthophosphate adsorbed to the columns. Interference from this source has now been eliminated by using 0.05 N HCl rather than the alkaline borate-KCl mixture to elute orthophosphate.

In order to determine whether other compounds are eluted with $PP_i$, various other tests of specificity were carried out. These included a second ion-exchange chromatography (Table I), incubation with yeast pyrophosphatase, paper chromatography, and tests with added nucleotides.

*Repeat chromatography.* When pooled fractions 3 and 4 from the 0.25 N HCl elution from several columns were subjected to ion-exchange chromatography for a second time and the $PP_i$ was eluted with 0.09 N HCl in place of 0.25 N HCl, each fraction that contained $^{32}P[PP_i]$ had a specific activity not more than 10% higher than the $^{32}P[PP_i]$ in the pooled fractions (Table I).

*Incubation with pyrophosphatase.* Yeast inorganic pyrophosphatase (12) was used to check the specificity of the method. This enzyme is highly specific for $PP_i$, but under special conditions it will hydrolyze ATP (13). Even under optimum conditions, however, the hydrolysis of ATP is about 350 times slower than the hydrolysis of $PP_i$. By allowing the hydrolysis of added $^{32}P[PP_i]$ to just reach completion or by stopping the reaction before it reaches completion, it is reasonable to assume that the hydrolysis of compounds other than $PP_i$ will be minimal.

TABLE I

*Maximum Specific Radioactivities of $^{32}P[PP_i]$ Eluted from Ion-Exchange Columns under a Variety of Conditions*

| Elution conditions | Maximum specific radioactivity of $PP_i$ |
|---|---|
| | *cpm/μg P* |
| Three separate chromatographic runs of a single plasma extract. Orthophosphate was removed by elution with 200 ml of 0.133 M KCl containing 25 mM $Na_2B_4O_7$, followed by 25 ml $H_2O$ (see text for details). Elution of pyrophosphate with 0.25 N HCl. | 27,500<br>28,400<br>27,100 |
| First chromatographic run as above. Peak activity fractions from the three columns pooled and then reapplied to six other columns, which were eluted with 200 ml of 0.133 M KCl containing 25 mM $Na_2B_4O_7$, followed by 25 ml $H_2O$ (see text for details). $PP_i$ was then eluted with | |
| 0.25 N HCl | 27,800 |
| 0.25 N HCl | 29,850 |
| 0.09 N HCl | 28,900 |
| 0.09 N HCl | 28,500 |
| 0.09 N HCl | 27,800 |
| 0.09 N HCl | 30,400 |

A single plasma extract was used throughout.

Yeast inorganic pyrophosphatase came from two sources: 3× crystallized enzyme was kindly supplied by Dr. M. Kunitz, The Rockefeller University, New York, and 2× crystallized enzyme was obtained from the Sigma Chemical Co., St. Louis, Mo.

Incubation with enzyme was carried out on ultrafiltrates or on the neutralized plasma extract (pH 7.2). In the latter case, to satisfy cofactor requirements and to avoid bacterial growth 0.1 M $MgCl_2$ was added (final concentration 0.1 mmole/liter) together with neomycin sulphate (final concentration 1 mg/ml). The enzyme was added in aliquots of about 100 μg and the solution incubated at 30°C on a shaker, until the hydrolysis of the $^{32}P[PP_i]$ was practically complete (95–100%) as measured by an isobutanol–petroleum ether extraction procedure described below.

With this extraction procedure, adapted from the method described by Hall (14), radioactivity due to $^{32}P[P_i]$ and $^{32}P[PP_i]$ can be determined separately. 1 ml of the sample to be analyzed (which must contain less than 100 μg $P_i$/ml) was added to 1 ml of a reagent mixture containing 3.3% (w/v) ammonium molybdate and 2.7 N $H_2SO_4$. To this was added 2 ml of a mixture of isobutanol and petroleum ether (4:1 by volume; petroleum ether was a 80°–100°C boiling fraction, British Drug Houses Ltd., Poole, England). This mixture was shaken for at least 1 min and the organic and aqueous phases were then separated by centrifugation. The entire procedure was carried out in an ice bath. Two 50-μl aliquots were taken from each layer for determination of radioactivity. With this method, $^{32}P[P_i]$ is quantitatively extracted as phosphomolybdic acid into the organic phase, whereas $^{32}P[PP_i]$ remains in the aqueous phase and it is therefore possible to calculate the extent of hydrolysis of the $^{32}P[PP_i]$ in the original sample.

When hydrolysis of the $^{32}P[PP_i]$ in the plasma extract was practically complete, the solution was applied immediately to the ion-exchange columns, and the residual phosphate and radioactivity measured in fractions eluting with 0.25 N HCl between 100 and 117 ml (Fig. 1).

After incubation of the plasma extract with pyrophosphatase before ion-exchange chromatography, the amount of phosphate-reacting material remaining in fractions 3–7 was equivalent to 0.24 ±0.05 (mean ±SE) mμmoles $PP_i$/ml of plasma. This represented 7 ±1% (mean ±SE) of the concentration of $PP_i$ in similar plasma extracts not treated with pyrophosphatase. An example of the effect of PP-ase is shown in Fig. 1. It is clear that the major part of the $^{32}P$-labeled pyrophosphate and the phosphate-reacting material that eluted with 0.25 N HCl no longer does so after treatment with pyrophosphatase.

*Ascending paper chromatography.* Paper chromatography was carried out as follows. $PP_i$-containing eluates from ion-exchange chromatography were collected under ice and were lyophilized at −5°C. The residue was dissolved in a minimum volume of water and applied to paper chromatograms (paper 2043b, Schleicher-Schüll, Feldmeilen ZH, Switzerland). Internal standards of $^{32}P[P_i]$ and $^{32}P[PP_i]$ were incorporated in some of the aliquots. Two solvent systems were used (the first contained 280 ml of isopropanol, 120 ml of $H_2O$, 16 g of trichloroacetic acid, and 1.2 ml of 20% $NH_4OH$; the second consisted of a 70:30 (v/v) mixture of methanol and 2 N $NH_4OH$). When the second solvent was used 200 μg of disodium EDTA was added to each spot. After ascending chromatography had been carried out the $^{32}P$-containing spots were localized by radioautography. After this, chemically reacting phosphorus spots were localized by moistening the chromatogram with a mixture containing 1 g of ammonium molybdate, 3 ml of concentrated HCl, 3 ml of 70% perchloric acid, and 8 ml of water, all diluted to 100 ml with acetone. The chromatograms were then exposed to an UV lamp for 10 min and the resulting blue spots were stabilized by contact with $NH_3$ vapor.

When fractions 3 and 4 from the 0.25 N HCl elution were pooled from several columns and treated in the way described above, the only detectable phosphorus-containing (blue) spot other than orthophosphate was superimposable upon the spot observed radioautographically and due to the tracer $^{32}P[PP_i]$ added to the blood. No spots were seen

TABLE II

*Effect of Addition of Adenine Nucleotides (ADP and ATP) to Whole Blood on the Recovery of Plasma $PP_i$ (μg P/ml)*

| Concentration of ADP or ATP added to whole blood | Plasma $PP_i$ | | | |
|---|---|---|---|---|
| | ATP added | | ADP added | |
| | Plasma 1 | Plasma 2 | Plasma 3 | Plasma 4 |
| *mole/liter* | *μg P/ml* | | | |
| None | 0.254 | 0.175 | 0.164 | 0.123 |
| $10^{-4}$ | * | * | * | * |
| $10^{-5}$ | 0.244 | 0.176 | 0.189 | 0.132 |
| $10^{-6}$ | 0.266 | 0.179 | 0.159 | 0.124 |

* Separation of $PP_i$ from nucleotide incomplete (see Methods).

TABLE III

*Recovery of Inorganic Pyrophosphate ($PP_i$) Added to Blood*

| $PP_i$ added | $PP_i$ found | Recovery |
|---|---|---|
| *μg P/ml plasma* | *μg P/ml plasma* | % |
| None | 0.146 | — |
| 0.0975 | 0.244 | 101 |
| 0.245 | 0.378 | 96 |
| 0.730 | 0.877 | 100 |

either in ultraviolet light or by the phosphate reaction in the positions expected for ADP or ATP, but it is possible that contamination in the order of 5% might have escaped detection by this technique.

*Effects of added nucleotides.* One of the most serious potential sources of interference with the specificity of the method would be from adenine nucleotides. The concentration of such nucleotides is very low in plasma but is high in erythrocytes, and this is one reason why hemolyzed bloods were not taken for the analyses. Experiments were carried out in which ATP and ADP were added to whole blood to provide concentrations in blood of $10^{-6}$, $10^{-5}$, and $10^{-4}$ mole/liter (Table II). Significant detectable interference only occurred at the highest concentration of each nucleotide ($10^{-4}$ mole/liter). At this concentration, separation of pyrophosphate and nucleotide on the column is only partial, so that the variable dilution of pyrophosphate by nucleotide causes variability in specific activity of the fractions, and it becomes impossible to calculate a value for pyrophosphate. At $10^{-5}$ and $10^{-6}$ M nucleotide concentrations, this effect does not occur.

## Recovery of $PP_i$ and reproducibility

Although the amount of $^{32}P[PP_i]$ recovered in the 0.25 N HCl fractions from the columns was often as low as 20–30% of that present in the original blood, the concentration of $PP_i$ in plasma could still be calculated since the specific activity of the $PP_i$ could be measured accurately in the column eluates. When known amounts of nonradioactive $PP_i$ were added to blood, the observed fall in the specific activity of the $^{32}P[PP_i]$ recovered from the columns was exactly as predicted (Table III). This provided reassuring evidence that the use of the principle of isotope dilution to measure $PP_i$ was valid.

The standard deviation for a single estimation, calculated from 20 duplicate determinations of $PP_i$ in plasma, was 0.18 μmole/liter. Using this value of standard deviation, the result obtained for a single determination of plasma $PP_i$ would, with 99% probability, lie within ±0.47 μmole/liter of the true value. This is equivalent to approximately ±13% at the mean plasma concentration of 3.56 μmoles/liter.

## Patients

Patients were studied as out patients at three main centres: Berne, Oxford, and University College Hospital, London. The diagnosis was established in each case by recognized criteria. The cases of hypophosphatasia included six on whom urinary $PP_i$ measurements have been reported previously (15).

Five of the cases of osteogenesis imperfecta were from a single family, in which a dominant mode of inheritance was present. Five of the remaining cases were the only known affected members in their families. Some of these cases were severely affected and three of the adults had had 40–70 fractures each.

The two cases of osteopetrosis were both mild, one had been recognized when aged 7 yr and the other when aged 43 yr. The younger was on a low calcium diet plus cellulose phosphate for treatment.

None of the five cases of hyperparathyroidism had evidence of bone disease and the three cases of "acute" or "juvenile" osteoporosis (16) were studied after the phase of most rapid demineralization had occurred.

Normal persons were always bled at the same sessions as the patients, as an additional check on the technique for plasma $PP_i$. Blood was usually taken in the morning, in the fasting state whenever possible. Urine pyrophosphate was measured by the technique of Fleisch and Bisaz (10).

## RESULTS

*The concentration of $PP_i$ in normal human plasma.* The results are shown in Fig. 2. The mean (±SEM) concentration of $PP_i$ in the plasma of 36 normal men was 3.48 ±0.15 μmoles/liter (SD of mean = 0.89). For 37 normal women the values (mean ±SE) were 3.50 ±0.15 μmoles/liter (SD of mean = 0.92). There was no significant differences between men and women ($P > 0.7$, Student's $t$ test). The values in young persons (0–15 yr) were slightly higher than in older persons but the numbers are too small to be certain about the influence of age. The pooled results for the 73 men and women gave a population mean ±SE of 3.50 ±0.11 μmoles/liter (SD of mean = 0.80). The normal range (99% limits) for this population is therefore 1.19–5.65 μmoles/liter (0.074–0.350 μg P/ml).

Because other workers may not always be able to add $^{32}P[PP_i]$ to blood at the time of collection, we measured $PP_i$ in 21 samples to which $^{32}P[PP_i]$ was added to the plasma only after centrifugation (at 2°–6°C). No EDTA was added at any stage. The mean ±SE values for plasma $PP_i$ were lower (2.68 ±0.19 μmoles/liter) than when

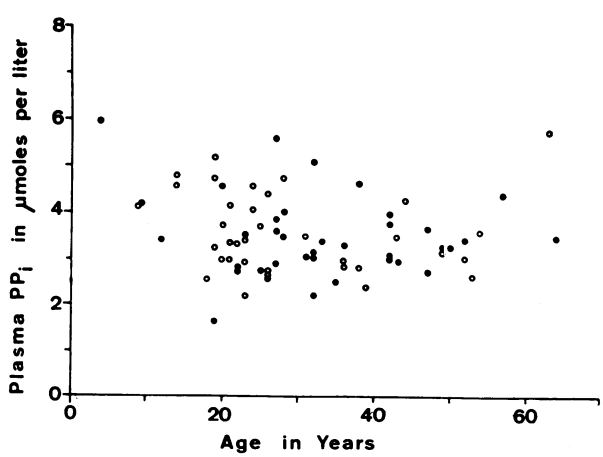


FIGURE 2 The relation between age and the concentration of $PP_i$ in plasma in normal men (●) and women (○).

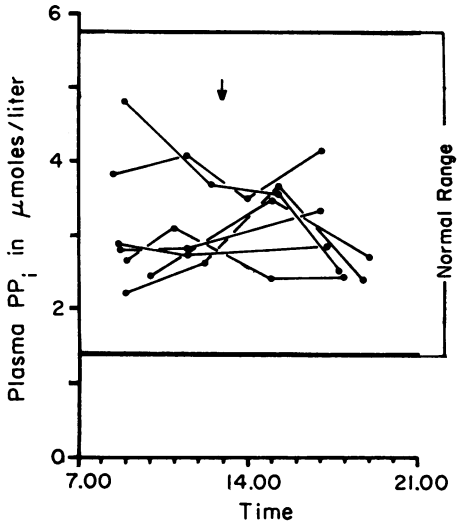


FIGURE 3 Variation in plasma $PP_i$ in seven normal persons during a normal day. Breakfast was not eaten, and the time of the mid-day meal is shown by the arrow.

EDTA and $^{32}P[PP_i]$ were added at the time of collection. This confirms that hydrolysis of $PP_i$ occurs during preparation of the plasma. Indeed similar studies on bloods containing large amount of alkaline phosphatase showed that plasma $PP_i$ concentrations approaching zero can be found, unless precautions are taken to cool the blood and to add EDTA at the beginning of the analysis.

When plasma $PP_i$ was taken from seven normal persons several times during a single day, the variation found was greater than could be attributed to variation in the technique alone (Fig. 3). These variations seemed unrelated to the time of day or time of meals so that other, as yet unidentified, factors may be playing a part.

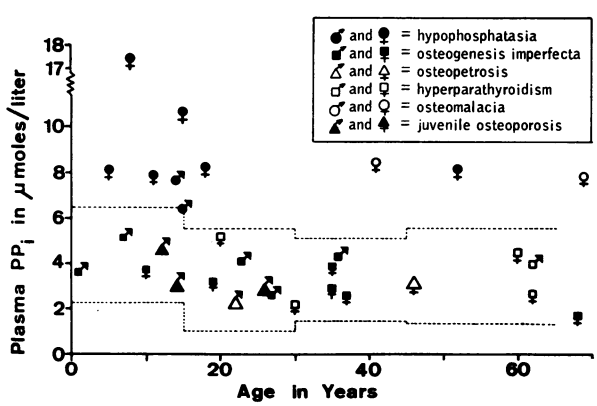


FIGURE 4 The concentration of $PP_i$ in the plasma of patients with hypophosphatasia, osteogenesis imperfecta, osteopetrosis, hyperparathyroidism, osteomalacia, and juvenile osteoporosis. The normal ranges (99% limits) calculated for each 15 yr age group is shown between the dotted lines.

Such factors will assume importance if repeated studies in single individuals are undertaken. Detailed studies of this sort are difficult to do at present because the technique for plasma $PP_i$ requires large volumes of blood and is very laborious.

*Plasma $PP_i$ in bone diseases.* Fig. 4 shows plasma $PP_i$ values in eight patients with hypophosphatasia (17), 11 patients with osteogenesis imperfecta, 2 patients each with osteopetrosis or osteomalacia, 3 with "acute" osteoporosis of the juvenile type (16), and 5 with primary hyperparathyroidism (without evidence of bone disease). Plasma $PP_i$ was invariably higher than normal in hypophosphatasia but was normal in osteogenesis imperfecta and osteopetrosis. Higher than normal values for plasma $PP_i$ were found in two out of six relatives of patients with hypophosphatasia. All these six relatives were thought to be carriers (heterozygotes) of the disease (four mothers, one father, and one sister).

Plasma $PP_i$ was also above the upper limit of normal (plasma $PP_i = 8.35$ and 7.6 $\mu$moles/liter) in the two cases of osteomalacia due to intestinal malabsorption (one postgastrectomy and one due to gluten sensitivity). Plasma $PP_i$ was within the normal range in the other conditions studied.

## DISCUSSION

The method finally adopted for the determination of $PP_i$ in plasma gave satisfactory results, considering the small quantities involved. This method, although laborious, makes it possible to study, for the first time, the factors that control the concentration of $PP_i$ in body fluids. It cannot be stated with certainty that no other compound is measured along with $PP_i$, but the method appears better than 90% specific as judged by the elution patterns from the ion-exchange columns, by paper chromatography, and by incubation with inorganic pyrophosphatase. Interference from ATP and ADP at the concentrations likely to be encountered in blood was not detectable.

Apart from specificity, the other feature of major importance in the method is that $^{32}P[PP_i]$ is added at the moment the blood is collected. This allows corrections to be made for all losses, especially the considerable hydrolysis (up to 100% when the plasma alkaline phosphatase is raised) that occurs in absence of EDTA during the preparation of the plasma and ultrafiltrate. The only published report on measuring $PP_i$ in human blood is that of Solomons and Styner (8). These authors used serum for analysis and made no corrections for hydrolysis. The specificity of their technique was not adequately assessed, and the specific activity of $^{32}P[PP_i]$ in their various column fraction (Fig. 2, reference 8) varied, suggesting that more than one compound was present. Indeed they found a mean serum $PP_i$ concentration of

11.0 μmoles/liter, which is twice our upper limit of normal. This is not simply a geographical difference since we have found similar concentrations of $PP_i$ in plasma from normal persons in the USA, UK, and Switzerland. The concentrations of $PP_i$ in the plasma of other species so far examined are also in the same range as in man (in seven adult dogs, mean ±SE plasma $PP_i = 2.26 \pm 0.79$ μmoles/liter, and in two pooled blood samples from Wistar rats the concentrations were 3.18 and 3.23 μmoles/liter).

The mean ±SE of mean concentration of $PP_i$ found in human plasma was 3.50 ±0.11 μmoles/liter. The normal range (99% confidence limits) lies between 1.19 and 5.65 μmoles/liter. These concentrations, although small, are in the range in which $PP_i$ inhibits calcium phosphate precipitation in vitro (2, 3), and slows the growth and dissolution of apatite crystals (4). This supports the proposal that $PP_i$ might be one of the substances presumed to be required to prevent mineralization of soft tissues and that alterations in $PP_i$ concentration might regulate tissue calcification and the rates of entry and exit of calcium in bone.

Before discussing the changes that occur in bone disease, it is worth emphasising that some caution is necessary in interpreting changes in plasma $PP_i$. It is possible that changes might occur in the metabolism of $PP_i$ at various sites in the body including bone, without alteration in the concentration of $PP_i$ in plasma. Conversely, any changes that occur might be restricted to plasma, so that changes in plasma $PP_i$ would not necessarily mean that there are disturbances in the metabolism of $PP_i$ in bone. Changes in turnover of $PP_i$ could occur without changes in the concentration of $PP_i$ in plasma. In dogs the turnover of plasma $PP_i$, measured using $^{32}P[PP_i]$, is very rapid and the entire plasma pool is replaced every 1–3 min (18). Unfortunately, it is more difficult to obtain information of this sort in man. Thus it is not known whether $PP_i$ in plasma is in equilibrium with the $PP_i$ in bone and whether the concentration of $PP_i$ in plasma is affected by local changes in the metabolism of $PP_i$ in bone. In future studies these points will have to be clarified.

Hypophosphatasia was the only disease we studied in detail in which plasma $PP_i$ was invariably above normal. This observation is consistent with previous studies in which it was shown that urinary $PP_i$ is always higher than normal in this condition (15, 19). Because there is a deficiency of alkaline phosphatase in hypophosphatasia associated with high amounts of $PP_i$, it is reasonable to assume that $PP_i$ is one of the natural substrates for alkaline phosphatase in vivo. Indeed there is now excellent evidence that many mammalian alkaline phosphatases are able to hydrolyse $PP_i$ (20–23). The accumulation of $PP_i$ in hypophosphatasia may be the cause of the defective mineralization of bone in this disease, since several experimental studies have shown that $PP_i$ can inhibit calcification in various living systems (24–26). One function of alkaline phosphatase in bone may be to remove $PP_i$ so that deposition of calcium salts can take place. Although the defective mineralization in hypophosphatasia resembles that in rickets, it is a notable feature of hypophosphatasia that the mineralization defect persists, even though plasma concentrations of calcium and phosphate are often higher than in normal persons (17).

With regard to the therapy of hypophosphatasia, there has been a favorable report of the use of phosphate supplementation in hypophosphatasia (19). However, we have found that feeding phosphate does not significantly change plasma $PP_i$. If feeding phosphate is an effective form of treatment, some explanation other than an effect on plasma $PP_i$ must be sought.

Our results on osteogenesis imperfecta do not agree with those of Solomons and Styner (8), who claimed that patients with osteogenesis imperfecta had elevated serum $PP_i$ concentrations. Our patients had normal plasma $PP_i$. Because the technique of Solomons and Styner is probably not specific for $PP_i$, their results may be in error. Solomons and Styner also claimed that patients with osteogenesis imperfecta excrete relatively more $PP_i$ in their urine than normal, when their urine orthophosphate is taken into account. Urine $PP_i$ is easier to measure than plasma $PP_i$ and there is no reason why their urine technique should not have been valid. However, it seems that they compared affected children (age 2 days–14 yr) with normal adults as controls, and neglected the fact that the ratio of $PP_i/P_i$ in urine is 2–3 times higher in children than in adults (10, 11). We measured urinary $PP_i$ in five of our cases of osteogenesis imperfecta. None of them excreted more than 0.8 mg $PP_i$ per 100 mg of orthophosphate, which is the upper limit of normal in young persons (15). At present, therefore, there is very little evidence to support the idea that an abnormality in $PP_i$ metabolism may be the cause of osteogenesis imperfecta.

Plasma $PP_i$ was normal in the other bone diseases we studied, with the exception of both the patients with osteomalacia. The significance of these changes will become evident only after further studies, but the findings in osteomalacia could suggest a role for vitamin D in removing $PP_i$ so that calcification can proceed. Elevated plasma $PP_i$ concentrations have also been reported in some patients with renal failure (7), a condition in which abnormalities in the metabolism of vitamin D are also present (27).

## APPENDIX

The calculation of the concentration of $PP_i$ in plasma, although straightforward is shown in detail so that the essential measurements and assumptions may be recognized.

(1) Total volume of blood from patient = $V_B$ ml.

(2) Volume of 0.2 M EDTA added = $V_{EDTA}$ ml.

(3) Packed cell volume (PCV, %, measured on whole blood *after* addition of EDTA) = PCV.

(4) Concentration of $^{32}P[PP_i]$ in blood +EDTA = $r_{B+EDTA}$ cpm/ml.

(5) Concentration of $^{32}P[PP_i]$ in plasma + EDTA = $r_{P+EDTA}$ cpm/ml, and

(6) mean specific activity (cpm/$\mu$g P) of $PP_i$, eluted in fractions 3–7 from ion-exchange chromatography = $SA_{PPi}$ cpm/$\mu$g.

Then

(7) Total radioactivity in whole blood plus EDTA ($= R_{B+EDTA}$) at the beginning of analysis = $r_{B+EDTA} \cdot (V_B + V_{EDTA})$ cpm.

(8) Total yield of plasma + EDTA should be

$$\frac{(V_B + V_{EDTA})\ (100 - PCV)}{100} \text{ ml} = V_P + V_{EDTA},$$

where $V_P$ is the true volume of plasma present in the $V_B$ ml of blood taken from the patient.

(9) If all the $^{32}P[PP_i]$ added initially to the blood remains outside cells and confined to plasma, then the total radioactivity ($R_P$) in ($V_P + V_{EDTA}$) ml of plasma plus EDTA where

$$R_P = r_{P+EDTA} \cdot (V_P + V_{EDTA}) \text{ cpm},$$

should be found to equal the total radioactivity at the beginning of the analysis, thus $R_P = R_{B+EDTA}$.
Experimentally this was found to be so.

(10) Then, the total $PP_i$ ($\mu$g) in $V_P$ ml of plasma, defined as $T_{PPi} = \frac{R_P}{SA_{PPi}}$ − amount ($\mu$g P) of $^{32}P[PP_i]$ added added initially.

(11) Thus concentration of $PP_i$ in plasma = $\frac{T_{PPi}}{V_P}$ $\mu$g P/ml.

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