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 P-labeled pyrophosphate, has been developed for the measurement of inorganic pyrophosphate (PPi) 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 PPi was ±13%. There were no differences in plasma PPi 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 PPi 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 PPi may be important in calcium metabolism because PPi 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 PPi was therefore measured in several disorders of bone. Normal values were found in osteogenesis imperfecta, osteoporosis, "acute" osteoporosis, and primary hyperparathyroidism. Plasma PPi was invariably raised in hypophosphatasia. The excess of PPi 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 (PPi) 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 PPi in intact animals. Recent studies have suggested that PPi may be important in regulating calcium metabolism. Thus small amounts of PPi inhibit the precipitation of calcium phosphate from solution (2, 3) and bind strongly to crystals of hydroxyapatite (4). Apatite crystals to which PPi has adsorbed grow and dissolve more slowly than nontreated crystals (4, 5). This suggests that the PPi 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 PPi might lead to changes in the concentrations of PPi 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 PPi in human diseases in which the turnover of bone is abnormal.

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

Methods
Introduction
The main difficulties in developing a satisfactory technique for measuring PPi in plasma were that the concentrations of PPi were very low, that other compounds appeared to interfere with the chemical determination, and that PPi...
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\(_1\) with calcium phosphate, treatment with a cation exchange resin to remove calcium and nucleotides, and finally, separation of PP\(_1\) from other phosphate compounds by chromatography on an anion exchange resin. The specific radioactivity of the PP\(_1\), eluted from the columns was determined and the concentration of PP\(_1\) 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\(_1\) in human plasma

30–50 ml of venous blood was collected into glass vessels surrounded by ice. These vessels contained heparin and sufficient known small amounts of \(^{32}P\)-labeled pyrophosphate (from the Radiochemical Centre, Amersham, England, initial specific activities 5–205 mCi/m mole) to allow a total initial activity of around 100,000 cpm. The \(^{32}P\)-labeled pyrophosphate was added in order to correct for subsequent losses of PP\(_1\). The collection of whole blood directly into the \(^{32}P\)-labeled pyrophosphate 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\(_1\) in the blood. Thus preliminary studies had shown that, in plasma from patients with elevated alkaline phosphatase, the hydrolysis of PP\(_1\), 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 \(^{32}P\)-labeled pyrophosphate 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 × 50 μl). 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 leukocytes. 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 × 50 μl) 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\(_1\). Ultrafiltration was carried out in a special apparatus constructed

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

Figure 1. Elution of orthophosphate (P₁) and pyrophosphate (P₂) from ion-exchange columns (Dowex 1 × 10, 100–200 mesh, chloride form) after application of plasma extract prepared as described in the text. °P[P₂] 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 (°P) in the pyrophosphate peak in (a), and neither phosphate nor °P appears in this position after treatment with pyrophosphatase (b).

traces of P₁ due to adsorption of P₁ to the columns. After elution of P₁, P₂ 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 P₂ in the fractions was hydrolyzed to P₁ by heating for 30 min in a boiling water bath. A volume of a reagent mixture (0.5% w/v ammonium molybate 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 P₁ was determined by measuring the extinction at 820 mμ (molar extinction coefficient, E₇₅₀ = 2.7 × 10⁶) using semi-microcuvettes in a Beckman DU spectrophotometer. Whenever radioactivity due to °P was measured, 50-µl samples were applied in duplicate or triplicate to washed aluminium planchets sprayed with plastic film. When dry, the planchets were counted in an automatic methane gas flow counter (Frieske and Hoepfner, Erlangen-Bruck, West Germany).

To calculate the concentration of P₂ 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 °P[P₂] 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 P₂. 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 P₂, 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 P₂ was eluted with 0.09 N HCl in place of 0.25 N HCl, each fraction that contained °P[P₂] had a specific activity not more than 10% higher than the °P[P₂] 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 P₂, 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 P₂. By allowing the hydrolysis of added °P[P₂] 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 P₂ will be minimal.
TABLE I
Maximum Specific Radioactivities of $^{32}P[PP_i]$ Eluted from Ion-Exchange Columns under a Variety of Conditions

<table>
<thead>
<tr>
<th>Elution conditions</th>
<th>Maximum specific radioactivity of PP$_i$ (cpm/μg P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three separate chromatographic runs of a single plasma extract. Ortho- phosphate was removed by elution with 200 ml of 0.133 M KCl containing 25 mM Na$_2$B$_4$O$_7$, followed by 25 ml H$_2$O (see text for details). Elution of pyrophosphate with 0.25 N HCl.</td>
<td>27,500</td>
</tr>
<tr>
<td>0.25 N HCl</td>
<td>27,800</td>
</tr>
<tr>
<td>0.25 N HCl</td>
<td>29,850</td>
</tr>
<tr>
<td>0.09 N HCl</td>
<td>28,900</td>
</tr>
<tr>
<td>0.09 N HCl</td>
<td>28,500</td>
</tr>
<tr>
<td>0.09 N HCl</td>
<td>27,800</td>
</tr>
<tr>
<td>0.09 N HCl</td>
<td>30,400</td>
</tr>
</tbody>
</table>

A single plasma extract was used throughout.

Yeast inorganic pyrophosphatase came from two sources: 3 X crystallized enzyme was kindly supplied by Dr. M. Kunitz, The Rockefeller University, New York, and 2 X 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/ml) was added to 1 ml of a reagent mixture containing 3.3% (w/v) ammonium molybdate and 27 N H$_2$SO$_4$. 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) mmole P/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 lyophilized at −5°C. The residue was dissolved in a minimum volume of water and applied to paper chromatograms (papier 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$_2$O, 16 g of trichloroacetic acid, and 1.2 ml of 20% NH$_4$OH; the second consisted of a 70:30 (v/v) mixture of methanol and 2 N NH$_4$OH). 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, chromatically reacting phosphorus spots were localized by maintaining 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)

<table>
<thead>
<tr>
<th>Concentration of ADP or ATP added to whole blood</th>
<th>ATP added</th>
<th>ADP added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma 1</td>
<td>Plasma 2</td>
</tr>
<tr>
<td>mole/μl</td>
<td>μg P/ml</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.254</td>
<td>0.175</td>
</tr>
<tr>
<td>$^{10}$-4</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>$^{10}$-3</td>
<td>0.244</td>
<td>0.176</td>
</tr>
<tr>
<td>$^{10}$-2</td>
<td>0.266</td>
<td>0.179</td>
</tr>
</tbody>
</table>

* Separation of PP$_i$ from nucleotide incomplete (see Methods).
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 concen-
tration 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 $10^4$, $10^3$, and $10^2$
mole/liter (Table II). Significant detectable interference
only occurred at the highest concentration of each nucleo-
tide ($10^4$ 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 pyrophos-
phate. At $10^4$ and $10^2$ n mole/liter concentrations, this
effect does not occur.

Recovery of PP$_1$ and reproducibility

Although the amount of $^{32}$P[PP$_1$] 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$_1$ in plasma could still be calculated since the specific
activity of the PP$_1$ could be measured accurately in the
column eluates. When known amounts of nonradioactive PP$_1$
were added to blood, the observed fall in the specific ac-
tivity of the $^{32}$P[PP$_1$] 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$_1$ was valid.

The standard deviation for a single estimation, calculated
from 20 duplicate determinations of PP$_1$ in plasma, was
0.18 $\mu$moles/liter. Using this value of standard deviation,
the result obtained for a single determination of plasma PP$_1$
would, with 99% probability, lie within $\pm 0.47$ $\mu$moles/liter
of the true value. This is equivalent to approximately $\pm 13%$
at the mean plasma concentration of 3.56 $\mu$moles/liter.

Patients

Patients were studied as out patients at three main cen-
tres: Berne, Oxford, and University College Hospital, Lon-
don. The diagnosis was established in each case by recog-
nized criteria. The cases of hypophosphatasia included six
on whom urinary PP$_1$ 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 cellu-
lose phosphate for treatment.

None of the five cases of hyperparathyroidism had evi-
dence 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$_1$. 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$_1$ in normal human plasma.
The results are shown in Fig. 2. The mean ($\pm$SEM)
concentration of PP$_1$ in the plasma of 36 normal men
was 3.48 ±0.15 $\mu$moles/liter (SD of mean = 0.89). For
37 normal women the values (mean ±SEM) were 3.50
±0.15 $\mu$moles/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 $\mu$moles/liter (SD of
mean = 0.80). The normal range (99% limits) for this
population is therefore 1.19-5.65 $\mu$moles/liter (0.074–
0.350 $\mu$P/ml).

Because other workers may not always be able to add
$^{32}$P[PP$_1$] to blood at the time of collection, we measured
PP$_1$ in 21 samples to which $^{32}$P[PP$_1$] 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$_1$ were lower (2.68 ±0.19 $\mu$moles/liter) than when

![Figure 2](https://example.com/figure2.png)

**Figure 2.** The relation between age and the concentration
of PP$_1$ in plasma in normal men ($\bullet$) and women ($\bigcirc$).

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**Table III**

Recovery of Inorganic Pyrophosphate (PP$_1$) Added to Blood

<table>
<thead>
<tr>
<th>PP$_1$ added</th>
<th>PP$_1$ found</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$P/ml plasma</td>
<td>$\mu$P/ml plasma</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>0.146</td>
<td>—</td>
</tr>
<tr>
<td>0.0975</td>
<td>0.244</td>
<td>101</td>
</tr>
<tr>
<td>0.245</td>
<td>0.378</td>
<td>96</td>
</tr>
<tr>
<td>0.730</td>
<td>0.877</td>
<td>100</td>
</tr>
</tbody>
</table>

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EDTA and $^{32}$P[PP$_1$] were added at the time of collection. This confirms that hydrolysis of PP$_1$ occurs during preparation of the plasma. Indeed similar studies on bloods containing large amount of alkaline phosphatase showed that plasma PP$_1$ 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$_1$ 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. 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$_1$ requires large volumes of blood and is very laborious.

**Plasma PP$_1$ in bone diseases.** Fig. 4 shows plasma PP$_1$ 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$_1$ was invariably higher than normal in hypophosphatasia but was normal in osteogenesis imperfecta and osteopetrosis. Higher than normal values for plasma PP$_1$ 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$_1$ was also above the upper limit of normal (plasma PP$_1$ = 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$_1$ was within the normal range in the other conditions studied.

**DISCUSSION**

The method finally adopted for the determination of PP$_1$ 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$_1$ in body fluids. It cannot be stated with certainty that no other compound is measured along with PP$_1$, 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$_1$] 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$_1$ in human blood is that of Solomons and Syner (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$_1$] 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$_1$ concentration of 0-
11.0 \(\mu\)moles/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 ±0.79 \(\mu\)moles/liter, and in two pooled blood samples from Wistar rats the concentrations were 3.18 and 3.23 \(\mu\)moles/liter).

The mean ± SE of mean concentration of PP\(_i\) found in human plasma was 3.50 ±0.11 \(\mu\)moles/liter. The normal range (99% confidence limits) lies between 1.19 and 5.65 \(\mu\)moles/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 \(^{32}\)PP[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 $^{32}P$[PPi] in blood
   $+\text{EDTA} = r_{B+EDTA}$ cpm/ml.
(5) Concentration of $^{32}P$[PPi] in plasma
   $+\text{EDTA} = r_{P+EDTA}$ cpm/ml, and
(6) mean specific activity (cpm/$\mu$g P) of PPi, eluted in fractions 3-7 from ion-exchange chromatography
   $= S_{AP Pi}$ cpm/$\mu$g.

Then
(7) Total radioactivity in whole blood plus EDTA
   ($= R_{B+EDTA}$) at the beginning of analysis = $r_{B+EDTA}$
   $(V_B + V_{EDTA})$ ml of plasma plus EDTA where
   $R_P = r_{P+EDTA}(V_P + V_{EDTA})$ 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.
(9) Then, the total PPi ($\mu$g) in $V_P$ ml of plasma, defined as
   $T_{PP i} = \frac{R_P}{S_{AP Pi}}$ amount ($\mu$g P) of $^{32}P$[PPi] added initially.

Thus concentration of PPi in plasma
   $= \frac{T_{PP i}}{V_P} \mu$g P/ml.

**ACKNOWLEDGMENTS**

We are grateful to Professor C. E. Dent, the late Professor F. Fourman, Doctors R. Smith, J. Badenoch, and M. Bishop, and Mr. J. Morgan of the Nuffield Orthopedic Centre for allowing us to study the patients. We also thank Paola Bronz, Vreni Wick, Ursula Zwicky, Marion Kemery, and Christopher Preston, who provided skilled assistance at various stages of this work.

This investigation was supported by the Swiss National Fund (No. 3567), the U. S. Public Health Service (Grant AM 7266), by the Emil Barrell Foundation of the Hoffmann-La Roche Company, by the Medical Research Council of Great Britain, and by the Welcome Trust.

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