Colorimetric Determination of Inorganic Pyrophosphate by a Manual or Automated Method

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A microprocedure for the colorimetric determination of inorganic pyrophosphate (PPi) in the presence or absence of orthophosphate (Pi) has been developed. PPi is estimated quantitatively as the amount of chromophore formed with molybdate reagent, 1-amino-2-naphthol-4-sulfonic acid in bisulfite and thiol reagent (monothioglycerol or 2-mercaptoethanol). The latter is obligatory for color formation. Pi is estimated without thiol reagent. The two chromophores differ in absorption spectra, the greatest difference being at 580 nm. For both, color develops fully by 10 min and is stable up to 1 hr. Just less than 0.4 μM PPi can be determined. The extinction coefficients are 2.70 × 10⁴ and 8.76 × 10³ for PPi and Pi, respectively, both with thiol reagent present, and 2.77 × 10³ for Pi with no thiol reagent.

A ten-fold excess of Pi does not interfere with the determination of PPi, and in fact can be estimated in the same mixture. A 15-fold excess, however, diminishes the accuracy of PPi estimations. Trichloroacetic acid and sodium fluoride inhibit color formation, but this inhibition is overcome by the addition of sodium acetate buffer, pH 4.0. Nucleoside triphosphates and adenosine 3':5'-cyclic monophosphate are stable in the reaction mixture.

The method was tested in assays of Escherichia coli DNA-dependent RNA polymerase (nucleoside triphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). Progress curves measured by either the rate of PPi formation or the rate of synthesis of labeled RNA were very similar. Product PPi formed by as little as 0.6 unit of RNA polymerase in a 225-μl incubation medium could be measured.

An automated version of the method was devised which allows accurate determination of PPi down to 1 μM (without range expander attachment) at a sampling rate of 20–40 tubes/hr.

Earlier, the usual procedure for the colorimetric analysis of PPi involved hydrolyzing it in acid (1) prior to determination of the resulting Pi by the molybdenum blue method of Fiske and Subbarow (2). Later, Flynn et al. (3) introduced a procedure to measure the molybdate–PPi complex that is slowly reduced by cysteine (4). Pi interfered and, because of the lengthy period of color development, so did labile organic

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phosphates. Later, Grindey and Nichol (5) combined the essential features of this procedure with solvent extraction (6) of $P_i$ complexes.

In alternate procedures, $PP_i$ was determined as the amount of reduced $NAD^+$ formed in reactions coupled by several enzymes (7,8); such methods suffer from the disadvantage that extensive purification of coupling enzymes is entailed.

The present method was developed primarily for the direct determination of $PP_i$ as a product of enzymic reactions. Bacterial RNA polymerase was used as a test enzyme. One of the standard radiochemical procedures (9) for the assay of this enzyme was compared to the present colorimetric method.

A preliminary account of our original procedure was presented (10). The method has since been modified as described herein.

**EXPERIMENTAL PROCEDURE**

*Materials*

Deionized, glass-redistilled water and analytical reagent grade chemicals were used throughout. The standard $PP_i$ solution contained 1.0 mM $Na_4P_2O_7\cdot10H_2O$ (British Drug Houses (B.D.H.)), was prepared fresh daily, diluted 1/10 before use and stored in ice. The standard $P_i$ solution contained 1.0 mM anhydrous $Na_2HPO_4$ (B.D.H.), was stored at 2°C and diluted 1/10 before use. Fiske–Subbarow molybdate reagent No. 1 contained 2.5% ammonium molybdate in 5 N $H_2SO_4$ and was purchased from B.D.H.

Gram-pacs of Eikonogen, each containing 0.25 g of 1-amino-2-naphthol-4-sulfonic acid, 0.25 g of sodium sulfite and 14.65 g of sodium bisulfite were purchased from Fisher. One Gram-pac was dissolved in 100 ml of hot distilled water; the solution was cooled to 25°C, filtered and made up to 100 ml. The filtrate was stored in an amber bottle at room temperature and was prepared weekly.

Monothioglycerol was purchased from Sigma, thioglycolic acid from Pierce Chemical, 2-mercaptoethanol from Eastman and dithiothreitol from Nutritional Biochemicals. Solutions were prepared fresh daily and stored in ice.

Tetralithium salts of UTP, GTP and CTP were purchased from Schwarz/Mann, the sodium salt of ATP, highly polymerized calf thymus DNA and bovine serum albumin (Fraction V) from Sigma and tritium-labeled nucleoside triphosphates from New England Nuclear. *Escherichia coli* strain K-12 RNA polymerase containing sigma factor was purchased from Sigma.
Methods

Manual determination of PP\textsubscript{i} and P\textsubscript{i}. To samples containing 0–20 nmoles of PP\textsubscript{i}, 50 \mu l of molybdate reagent and 50 \mu l of thiol reagent (either 0.5 M 2-mercaptoethanol or 0.65 M monothioglycerol) were added, followed immediately by 20 \mu l of Eikonogen in a total volume of 0.5 ml. Absorbance was read after 10–60 min at 580 nm in a Beckman DU spectrophotometer. To determine P\textsubscript{i}, additional samples were treated as above but with thiol reagent omitted. For convenience, all samples including controls were read against reagent blanks lacking thiol reagent.

The extinction coefficients at a wavelength of 580 nm were 2.70 \times 10^4 for PP\textsubscript{i} and 8.76 \times 10^3 for P\textsubscript{i}, both with thiol reagent present, and 2.77 \times 10^3 for P\textsubscript{i} with thiol reagent absent. At 675 nm the extinction coefficients were higher for P\textsubscript{i} (1.39 \times 10^4 and 4.27 \times 10^3, plus and minus thiol reagent, respectively) but lower for PP\textsubscript{i} (2.48 \times 10^4, plus thiol reagent). The latter wavelength could be used to determine low concentrations of P\textsubscript{i} more accurately.

Automated analysis. The Autoanalyzer system (Technicon) consisted of sampler I, proportioning pump I, 2-speed, a 14.5-min-delay coil and colorimeter equipped with recorder. Trichloroacetic acid-acetate samples for assay of RNA polymerase activity were kept in ice until insertion into the turntable of the sampler. There was no deterioration of the samples thereafter for at least 2 hr. With a 40 sample/hr, \(\frac{1}{2}\) cam (sample/wash ratio), two wash cups inserted between each pair of duplicate samples were sufficient to prevent cross-contamination. The wash cups could be omitted with cams of lower sample/wash ratios. The molybdate reagent contained 0.05% sodium dodecyl sulfate (w/v) and the wash water of both the reservoir and wash cups, 0.005%. The tubing and pumping rates used are described in Table 1.

<table>
<thead>
<tr>
<th>Tube i.d. (inches)</th>
<th>Color</th>
<th>Flow rate (ml min(^{-1}))</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.045</td>
<td>Red</td>
<td>0.80</td>
<td>Sample</td>
</tr>
<tr>
<td>0.045</td>
<td>Red</td>
<td>0.80</td>
<td>Air</td>
</tr>
<tr>
<td>0.045</td>
<td>Red</td>
<td>0.80</td>
<td>Return from flow cell</td>
</tr>
<tr>
<td>0.015</td>
<td>Orange-green</td>
<td>0.10</td>
<td>Ammonium molybdate</td>
</tr>
<tr>
<td>0.015</td>
<td>Orange-green</td>
<td>0.10</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>0.0075</td>
<td>Orange-red</td>
<td>0.03</td>
<td>Eikonogen</td>
</tr>
<tr>
<td>0.065</td>
<td>Blue</td>
<td>1.60</td>
<td>Reservoir wash</td>
</tr>
</tbody>
</table>

" Clear standard tubing (Technicon) was used throughout.
RNA polymerase assay. The incubation medium described by Burgess (11) was scaled up to 0.75 ml. The reaction was initiated by the addition of 112.5 nmoles each of tritiated GTP, ATP, UTP and CTP (0.15 μCi of each). After incubation at 37°C the tubes were placed in ice; 100-μl aliquots were removed and processed for radioactivity as described by Blatti et al. (9). Samples were counted in a Beckman LS-250 scintillation counter.

Trichloroacetic acid was added to the remainder of the reaction mixture to give a final concentration of 5% (w/v). After 10 min at 0°C the samples were spun for 10 min at 7,000 rpm in rotor SM-24, equipped with rubber adaptors for glass tubes, in a Sorvall RC-2B refrigerated centrifuge. To 300-μl aliquots of the supernatant fluid, 0.08 ml of 2 M sodium acetate buffer (pH 4.0) was added. Duplicate samples were used for P_i and PP_i estimations.

RESULTS

Colorimetric Procedure

Sulfhydryl compounds. In the procedure of Flynn et al. (3) color development with PP_i and cysteine as reducing agent was not complete even after 90 min. Of the several compounds tested to replace cysteine, 2-mercaptoethanol was found to be the most effective accelerator of the reaction. Monothioglycerol gave comparable results at 1.3 times the concentration of 2-mercaptoethanol. At the optimal concentration of 25–28 μmoles of 2-mercaptoethanol per tube (Fig. 1A) absorbance was

![Fig. 1](image)

Fig. 1. Effect of 2-mercaptoethanol. (A), Time course of color development with 10.8 nmoles of PP_i per tube. (B), Time course of color development with 18.1 nmoles of P_i per tube. For both (A) and (B) the μmoles of 2-mercaptoethanol added per tube are given above each curve; the final volume in each tube was 0.5 ml.
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maximal after 10 min and did not decrease for at least 60 min. There was no detectable color with PP\textsubscript{i} in the absence of thiol reagent (either 2-mercaptopetanol or monothioglycerol).

The absorbance with P\textsubscript{i} and no thiol reagent was low but significant and increased when thiol reagent was added (Fig. 1B). However, the absorbance was still much less than that of comparable concentrations of PP\textsubscript{i}.

When 0.175 M dithiothreitol was used in place of either of the above thiol reagents, precipitates formed, and with 3\% (v/v) thioglycolic acid there was very little acceleration of color development with either PP\textsubscript{i} or P\textsubscript{i}.

*Other reducing agents.* In some experiments the Fiske-Subbarow molybdate and Eikonogen reagents (2) were replaced by quinaldine red (12) to give an extremely sensitive assay for P\textsubscript{i} but, unfortunately, little or no color formation with PP\textsubscript{i} even with thiol reagent present. Color developed with both PP\textsubscript{i} and P\textsubscript{i} with the stannous chloride-hydrazine reducing agent of Hurst (13) or with stannous chloride alone (14) and sulphydryl compounds had no effect. The addition of methyl-p-aminophenol (Elon, Metol (15)) and molybdate reagent plus thiol reagent gave low absorbance readings with PP\textsubscript{i}. No color developed with either P\textsubscript{i} or PP\textsubscript{i} in the bisulfite system of Grindey and Nichol (5) when monothioglycerol was omitted but developed with both when it was added. There was little or no color formation with PP\textsubscript{i} with ascorbic acid as reductant (16).

*Inorganic pyrophosphatase.* Yeast pyrophosphatase (Worthington, Nutritional Biochemicals) was used to hydrolyze PP\textsubscript{i} to P\textsubscript{i} (17). The enzyme preparations available commercially were found to contain ATP-ase activity and, without further purification (18), their usefulness in RNA polymerase assays was precluded. However, it was found in preliminary experiments that the activity of inorganic pyrophosphatase itself could be measured by the present colorimetric method.

*Absorption spectra.* The absorption spectrum for the PP\textsubscript{i} chromophore differed markedly from that of P\textsubscript{i}; the greatest difference was at 580 nm as was also reported by others (5). Even at the highest concentrations of P\textsubscript{i} used no absorption peak at 580 nm was detected; the absorption maximum for the P\textsubscript{i} chromophore, in the presence or absence of thiol reagent, was at 675 nm.

*Standard calibration curves.* Linearity was observed up to 20 nmoles of PP\textsubscript{i} or P\textsubscript{i} per tube for the manual method (Fig. 2). PP\textsubscript{i} could be estimated in the presence of a tenfold excess of P\textsubscript{i} but with a 15-fold excess of P\textsubscript{i} the accuracy of estimation of PP\textsubscript{i} was diminished. The method is amenable to adjustment to increased sensitivity by the use of a range expander (Table 2) which allows 0.4 \mu M PP\textsubscript{i} to be determined.
FIG. 2. Calibration curves by the manual method. Absorbance versus nmoles of PP\textsubscript{i} with 2-mercaptoethanol present (●) or absent (■); absorbance versus nmoles of P\textsubscript{i} with 2-mercaptoethanol present (○) or absent (□). The standard reaction mixture in a final volume of 0.5 ml with 25 μmoles of 2-mercaptoethanol or without mercaptoethanol was used.

The calibration curves were reproducible for the automated system as well. Figure 3 shows a typical tracing of the absorbance of graded concentrations of PP\textsubscript{i}. A linear relationship between maximal absorbance at the peaks of the curves and PP\textsubscript{i} concentration obtained; the calibration curve was almost identical with that shown in Fig. 2 for the manual method. Unfortunately, our range expander attachment could not be used with the Technicon system.

It is worth noting that in the automated method baseline readings between pairs of duplicate samples of high PP\textsubscript{i} concentration sometimes were displaced upwards by about 0.025 optical density unit (Fig. 3), but

TABLE 2
USE OF RANGE EXPANDER ACCESSORY IN THE MANUAL COLORIMETRIC PROCEDURE

<table>
<thead>
<tr>
<th>PP\textsubscript{i} sample (nmoles)</th>
<th>Scale expansion</th>
<th>Absorbance (optical density units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.312</td>
<td>1 ×</td>
<td>0.016</td>
</tr>
<tr>
<td>2 ×</td>
<td>0.033</td>
<td></td>
</tr>
<tr>
<td>5 ×</td>
<td>0.080</td>
<td></td>
</tr>
<tr>
<td>10 ×</td>
<td>0.159</td>
<td></td>
</tr>
<tr>
<td>0.156</td>
<td>10 ×</td>
<td>0.043</td>
</tr>
<tr>
<td>0.312</td>
<td>10 ×</td>
<td>0.157</td>
</tr>
<tr>
<td>0.625</td>
<td>10 ×</td>
<td>0.341</td>
</tr>
<tr>
<td>1.25</td>
<td>10 ×</td>
<td>0.680</td>
</tr>
<tr>
<td>2.50</td>
<td>10 ×</td>
<td>1.305</td>
</tr>
</tbody>
</table>
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Fig. 3. Automated PPᵢ assay. Baseline readings of 0.125–0.130 optical density unit give the absorbance of reagent blanks. The PPᵢ concentrations were, from left to right: 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 and 20.0 nmoles of PPᵢ per sample. Samples were run in duplicate inter-spaced by 2 wash cups. A ½ cam (sample/wash ratio) was used at a pumping rate of 40 samples per hour. The ½ cam is a 2/1 cam with polarity reversed.

Fig. 4. (A), Mixed PPᵢ and Pi systems. Absorbance of 8 nmoles of Pi, no 2-mercaptoethanol (…) and plus 2-mercaptoethanol (---); PPᵢ alone (○) and PPᵢ + 8 nmoles of Pi (□), both with 2-mercaptoethanol present. The volume of the standard mixture was 0.5 ml; 25 μmoles of 2-mercaptoethanol were present as specified above. (B), Assay of RNA polymerase activity. Absorbance of samples in trichloroacetic acid–sodium acetate buffer from 225 μl of RNA polymerase assay mixture, 25 μmoles of 2-mercaptoethanol present (□), incubated without DNA or enzyme or with pancreatic DNase present (■); absorbance minus 2-mercaptoethanol (●), incubated with no DNA or enzyme (○). The radioactivity of product RNA is expressed for 225 μl of assay mixture although the optimal aliquot for counting was 100 μl. Four units of E. coli RNA polymerase were present per 0.75 ml of incubation medium. Counts per minute have been corrected for background radioactivity of nonincubated control samples.
the maximal absorbance of subsequent samples did not deviate from the calibration curve. Only if the absorbance of a sample was less than baseline was the absorbance tracing lost.

*Mixed PP\textsubscript{i} and P\textsubscript{i} samples.* The absorbance of 8 nmoles of P\textsubscript{i} in the standard molybdate system minus 2-mercaptoethanol is shown in Fig. 4A. This figure also shows that the absorbance increased threefold when 2-mercaptoethanol was added. When PP\textsubscript{i} was added to samples containing 8 nmoles of P\textsubscript{i} the absorbance was additive (2-mercaptoethanol present). It was also linear with PP\textsubscript{i} concentration (Fig. 4A).

PP\textsubscript{i} could be determined in such mixed systems by determining the absorbance of P\textsubscript{i} minus thiol reagent, converting this to the absorbance in thiol reagent by using a factor of 3, or by referring to a standard curve and then subtracting this value from the P\textsubscript{i} + PP\textsubscript{i} absorbance in thiol reagent. Alternately, in assays of enzyme activity where only product PP\textsubscript{i} is to be measured, test samples were corrected by the baseline absorbance of controls. The latter was the method of choice in present studies with RNA polymerase.

*Interfering compounds.* The P\textsubscript{i} and PP\textsubscript{i} contents of the nonincubated reaction mixture for RNA polymerase assays were determined. Of the components used, bovine serum albumin was found to contain large amounts of P\textsubscript{i} and accordingly was dialyzed in 100 volumes of 0.05 M Tris–HC\textsubscript{1} buffer (pH 7.9) for 3 hr at 2°C before use. Similarly, some preparations of nucleoside triphosphates either contained significant P\textsubscript{i} initially or showed a progressive increase in P\textsubscript{i} upon storage at −20°C with frequent thawing; lithium salts were much more stable and were used wherever possible. Still, about 5–8 nmoles of P\textsubscript{i}, depending upon the storage age of the nucleoside triphosphates, were found per 225 μl of reaction mixture prior to incubation.

Trichloroacetic acid and sodium fluoride inhibited color formation but the inhibition of both was overcome by sodium acetate buffer (Table 3). In some experiments, use of chloroform (19) instead of trichloroacetic acid to terminate the enzyme reaction was tested, but less reproducible results were obtained.

Standard curves for PP\textsubscript{i} suspended in the RNA polymerase assay medium were similar to the P\textsubscript{i} + PP\textsubscript{i} curve shown in Fig. 4A. Nucleoside triphosphates were found not to break down to form either P\textsubscript{i} or PP\textsubscript{i} during the period of color development with the concentration of molybdate reagent employed. Some hydrolysis to P\textsubscript{i} was noted when twice the amount of molybdate reagent was used. Long standing of samples in this reagent prior to the addition of Eikonogen and thiol reagent was avoided. Nucleoside triphosphates and PP\textsubscript{i} were stable in trichloroacetic acid–acetate buffer at 0°C for several hours. Adenosine 3′:5′-cyclic monophosphate was also stable but 5-phosphoribosyl-1-pyrophosphate was partially hydrolyzed.
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TABLE 3
INHIBITION OF COLOR FORMATION

<table>
<thead>
<tr>
<th>Sample (nmoles of PPi)</th>
<th>Additions</th>
<th>Absorbance</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>0</td>
<td>0.355</td>
<td>-</td>
</tr>
<tr>
<td>7.2</td>
<td>5% Trichloroacetic acid</td>
<td>0.303</td>
<td>14.7</td>
</tr>
<tr>
<td>7.2</td>
<td>5% Trichloroacetic acid + 0.42 M acetate pH 4.0</td>
<td>0.359</td>
<td>0</td>
</tr>
<tr>
<td>6.6</td>
<td>5% Trichloroacetic acid + 10 mM NaF</td>
<td>0.225</td>
<td>26.5</td>
</tr>
<tr>
<td>6.6</td>
<td>5% Trichloroacetic acid + 10 mM NaF + 0.42 M acetate, pH 4.0</td>
<td>0.319</td>
<td>0</td>
</tr>
</tbody>
</table>

Comparison with radiochemical assay for RNA polymerase. Fig. 4B shows the agreement in the progress curves for RNA polymerase when activity was measured either by the absorbance of PPi formed or by the radioactivity of product RNA. There was little or no increase in absorbance over baseline levels when the enzyme was incubated in the absence of DNA or when enzyme was omitted from the incubation mixture. The activity of as little as 0.6 unit of RNA polymerase was measurable by the colorimetric method. The addition of 10 mM NaF to the assay mixture to inhibit inorganic pyrophosphatase activity did not interfere with the colorimetric assay. Moreover, when 10 nmoles of PPi were added to the reaction mixture little or no degradation to Pi was detected. The radiochemical assay method, although much more sensitive, lacks the convenience and economy of time and materials of the colorimetric method.

DISCUSSION

Pi and molybdate react in strong sulfuric acid to form 12-molybdophosphoric acid which is then reduced to molybdenum blue by a number of reducing agents (20). PPi also forms a colored molybdate complex that is reduced at a preferential rate from that of the Pi complex under certain conditions that have been optimized in present work. The two complexes are differentiated by the use of two reducing agents, Eikonogen and thiol reagent, and by measuring absorbance at the wavelength of greatest difference in the absorption spectra of the two chromophores.

It is notable that both PPi and Pi can be determined in mixed systems.

3 One enzyme unit catalyzes the incorporation of 1 nmole of AMP into RNA per 10 min (11).
by the new method. The absorbance is additive with thiol reagent supplementing the Fiske–Subbarow reagents which are used in rather low concentrations in present studies. The amount of P₁ can then be determined with thiol reagent absent, for now there is no color formation from PP₁. Thus, difficulties encountered currently in the determination of both compounds, for example in studies of inorganic pyrophosphatase activity (21,22), can be overcome.

The method is rapid and economical and about three times more sensitive for PP₁ determination than that of Grindey and Nichol (5). It is less sensitive than the isotope exchange method for PP₁ estimation developed by Flodgard (24), but this method as well as those in which PP₄ measurement is coupled with the reduction of NAD⁺ (7,8,23) entail the preparation of purified coupling enzymes.

Because it is amenable to automation the method is suitable for the analysis of large series of samples. Time-consuming steps of other procedures consisting of differential solvent extraction of molybdate complexes (5,6), precipitation of PP₁ as manganous salts (1,25) or adsorption of impurities by charcoal (26) and isolation of PP₁ by ion-exchange chromatography (27) are eliminated as long as the P₁:PP₁ ratio does not exceed 10.

In regard to the assay of RNA polymerase and similar enzymes, the present method would have particular usefulness in the monitoring of activity of samples obtained during enzyme purification.

ACKNOWLEDGMENT

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REFERENCES