Continuous enzyme-coupled assay of phosphate- or pyrophosphate-releasing enzymes

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A coupled enzyme assay able to monitor the kinetics of reactions catalyzed by phosphate- or pyrophosphate-releasing enzymes is presented here. The assay is based on the concerted action of inorganic pyrophosphatase (PPase), purine nucleoside phosphorylase (PNPase), and xanthine oxidase (XOD). In the presence of phosphate, PNPase catalyzes the phosphorolysis of inosine, generating hypoxanthine, which is oxidized to uric acid by XOD. The uric acid accordingly formed can be spectrophotometrically monitored at 293 nm, taking advantage of a molar extinction coefficient which is independent of pH between 6 and 9. The coupled assay was tested using DNA polymerases as a model system. The activity of Klenow enzyme was quantitatively determined, and it was found in agreement with the corresponding activity determined by traditional methods. Moreover, the continuous coupled assay was used to determine $K_m$ and $V_{max}$ of Klenow enzyme, yielding values in good agreement with previous observations. Finally, the coupled assay was also used to determine the activity of partially purified DNA polymerases, revealing its potential use to monitor purification of phosphate- or pyrophosphate-releasing enzymes.

The detection of inorganic phosphates represents an analytical challenge in the environmental, agricultural, and industrial areas. Accordingly, the relevance of phosphates as analytical targets has prompted the development of chemical methods for their determination. In addition, chemosensors for phosphates were recently designed, synthesized, and tested (1,2). Using traditional or chemosensor-assisted chemical methods, phosphates can be detected using sensitive, simple, and cost-effective procedures, some of which can also be miniaturized (3) and fully automated (4). Nevertheless, these procedures are discontinuous, rendering rather cumbersome the kinetic evaluation of reactions involving phosphates.

Over the years, different enzymatic methods have been proposed for the detection of inorganic phosphates or pyrophosphates. By coupling the reactions catalyzed by purine nucleoside phosphorylase (PNPase; EC 2.4.2.1) and xanthine oxidase (XOD; EC 1.17.3.2, formerly 1.2.3.2), de Groot et al. were able to quantitatively assay inorganic and organic phosphate (5). Including inorganic pyrophosphatase (PPase; EC 3.6.1.1) in their assay, de Groot et al. were also able to quantitate inorganic pyrophosphate (5).

This method relies on the following steps (Supplementary Figure S1): (i) pyrophosphate is converted to phosphate by PPase; (ii) PNPase catalyzes the phosphorolysis of inosine to hypoxanthine and ribose-1-phosphate; and (iii) XOD oxidizes hypoxanthine to uric acid, the absorbance of which can be spectrophotometrically monitored (e.g., at 293 nm). This assay performed well to analyze total phosphate or pyrophosphate concentrations, i.e., by determining the absorbance increase at reaction completion. Unfortunately, de Groot et al. did not investigate in detail the potential use of their method to study the kinetics of reactions catalyzed by enzymes releasing phosphate or pyrophosphate (5).

Moreover, they used PNPase (500 mU/mL) in excess over XOD (50 mU/mL), while the opposite should be for this enzyme-coupled assay (6,7). Similarly, the optimal amount of auxiliary enzymes was not determined for the coupled assay of pyrophosphate relying on sulfate adenylyltransferase (8).

A simple kinetic assay for phosphate-releasing enzymes was proposed by Webb (9). In this case, PNPase was used to convert phosphate and 2-amino-6-mercaptopurine ribonucleoside (e.g., methylthioguanosine, MESG) into ribose-1-phosphate and 2-amino-6-mercaptopurine. At pH 7.6 and 360 nm, the difference in absorbance between MESG and the corresponding base generated by PNPase yields a $\Delta \epsilon$ equal to 11,000 M$^{-1}$ cm$^{-1}$ (9). Accordingly, the assay proposed by Webb eliminates the need to couple PNPase and XOD reactions. However, this method features the disadvantage that, at pH values below 7.6, the $\Delta \epsilon$ between MESG and the reaction product strongly decreases (9).

More recently, Tagiri-Endo reported on the use of hypoxanthine-guanine phosphoribosyl transferase (HGPRT; EC 2.4.2.8) to detect the pyrophosphate released during the action of DNA polymerases (10). However, this assay was specifically designed to estimate the amount of DNA amplified by PCR, and no attempts were made to evaluate the kinetics of reactions catalyzed by DNA polymerases.

A continuous enzyme-coupled assay of phosphate- or pyrophosphate-releasing enzymes (PREs) is presented here. In
particular, the PPase-PNPase-XOD (PPX) system was investigated in detail to: (i) identify the concentration of each auxiliary enzyme necessary to correctly detect the kinetics of reactions catalyzed by PREs; (ii) define the pH interval where the activity of PREs can be determined; (iii) compare the PPX assay with a well known reference method (11). Finally, DNA polymerases were chosen as a model to test the PPX system.

Materials and methods

Materials

*Escherichia coli* PPase (recombinant), bacterial PNPase, bacterial xXOD, type XV activated calf thymus DNA (12), and analytical grade reagents were from Sigma-Aldrich. The Klenow fragment of *E. coli* DNA polymerase I was from New England Biolabs (Ipswich, MA, USA). Crude *E. coli* DNA polymerase III holoenzyme was prepared as previously described (13).

Determination of phosphate with Malachite green

The procedure described by Baykov et al. (3) was used as a reference method to detect orthophosphate in aqueous samples. Briefly, a 5x assay mixture was obtained by mixing 10 mL of Malachite green solution (1.2 g in 1 L of 3 M sulphuric acid) with 2.5 mL of a freshly-prepared ammonium molybdate solution (7.5%, w/v in H2O) and 0.2 mL of 10% (v/v) Tween 20. To detect orthophosphate, 40 µL of the 5x assay mixture were added to 160 µL of each sample; upon incubation at 37°C for 30 min, the absorbance at 600 nm was determined using a Bio-Rad (Hercules, CA, USA) 550 microplate reader. To quantitate orthophosphate, an appropriate calibration curve (ranging from 0 to 12.5 µM) was used. The activity of DNA polymerases was determined in the presence of 100 mM Tris-HCl pH 7.8, 65 µg/mL of activated calf thymus DNA (12), and analytical grade reagents were from Sigma-Aldrich. The Klenow fragment of *E. coli* DNA polymerase I was from New England Biolabs (Ipswich, MA, USA). Crude *E. coli* DNA polymerase III holoenzyme was prepared as previously described (13).

Enzyme assays

PPase, PNPase, and XOD units (U) are defined as the amount of enzyme able to produce per min, at 25°C, 1 µmol of product at pH 9.0, 7.4, and 7.5, respectively. The activity of DNA polymerases was assayed in the presence of activated calf thymus DNA (65 µg/mL) or in the presence of 2.5 µM poly-dA:oligo-dT (60-mer and 20-mer, respectively). The assay mixture contained 100 mM Tris-HCl pH 7.8, 5 mM MgCl2, 0.25 mM inosine, 0.1 mM dNTPs (or 0.1 mM dTTP), and 5, 50, and 500 mU/mL of PPase, PNPase, and XOD, respectively. The reactions were started by addition of

dTTP, and monitored at 293 nm using a PerkinElmer (Waltham, MA, USA) lambda spectrophotometer. At this wavelength, the ε of uric acid was assumed equal to 12.6 x 10^3 M^-1 cm^-1 (14).

DNA polymerization reactions were also assayed in the presence of 0.5 mM 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), and monitored at 460 nm. At this wavelength, the ε of INT-formazan was assumed equal to 12.5 x 10^3 M^-1 cm^-1 (15).

Microplate assays

The *E. coli* DNA polymerase III catalytic core (comprising the α, ε, and θ subunits) was overexpressed in *E. coli* TOP10 as previously described (16). Upon overexpression, soluble proteins were extracted (using 50 mM Tris-Cl pH 8, 50 mM NaCl, 1 mM EDTA) and loaded onto a Q-Sepharose FF column. A linear NaCl gradient (from 50 to 600 mM) was then applied to the column, and 70 fractions were collected.

![Figure 1](image1.png)

**Figure 1. Initial velocities of reactions catalyzed by XOD (A), PNPase (B), and PPase (C), as a function of substrate (A, B) or enzyme (C) concentration.**

(A) Initial velocities of hypoxanthine oxidation by XOD as a function of substrate concentration. The XOD activity was monitored at 460 nm. The reaction mixtures contained 100 mM Tris-HCl pH 7.8, 10 mM MgCl2, 250 µM dNTPs, 60 µm of PPase. The reactions were stopped by the addition of the Malachite green assay mixture. Controls were carried out in the absence of MgCl2, and in the presence of 10 mM EDTA.

(B) Initial velocities of PNPase activity at pH 7.8, 7.4, and 7.5, respectively. The PNPase activity was monitored at 293 nm using a Bio-Rad (Hercules, CA, USA) 550 microplate reader. To quantitate PNPase, an appropriate calibration curve (ranging from 0 to 12.5 µM) was used. The activity of DNA polymerases was determined in the presence of 100 mM Tris-HCl pH 7.8, 65 µg/mL of activated calf thymus DNA (12), and analytical grade reagents were from Sigma-Aldrich. The Klenow fragment of *E. coli* DNA polymerase I was from New England Biolabs (Ipswich, MA, USA). Crude *E. coli* DNA polymerase III holoenzyme was prepared as previously described (13).

(C) Initial velocities of PPase activity at pH 7.8, 7.4, and 7.5, respectively. The PPase activity was monitored at 293 nm using a Bio-Rad (Hercules, CA, USA) 550 microplate reader. To quantitate PPase, an appropriate calibration curve (ranging from 0 to 12.5 µM) was used. The activity of DNA polymerases was determined in the presence of 100 mM Tris-HCl pH 7.8, 65 µg/mL of activated calf thymus DNA (12), and analytical grade reagents were from Sigma-Aldrich. The Klenow fragment of *E. coli* DNA polymerase I was from New England Biolabs (Ipswich, MA, USA). Crude *E. coli* DNA polymerase III holoenzyme was prepared as previously described (13).

![Figure 2](image2.png)

**Figure 2. Kinetics of DNA replication catalyzed by Klenow enzyme.**

(A) Time course of the absorbance at 293 nm of a reaction mixture containing 100 mM Tris-HCl (pH 7.8), Klenow polymerase, 2.5 µM oligo-dT, 5 mM MgCl2, 0.25 mM inosine, 5, 50 and 500 mU/mL of PPase, PNPase, and XOD, respectively. (B) Initial velocities of the absorbance at 293 nm of the assay mixture of Figure 2A, supplemented with 2.5 µM poly-dA. (C) Initial velocities of Klenow-catalyzed DNA replication in the presence of 1.25 or 2.5 µM poly-dA-oligo-dT and 100 µm dTTP. Other conditions as in Figure 2B.
DNA polymerase I of phosphates were determined in dNTPs activated DNA (Supplementary Figure 3), and the concentration of phosphates was found to be very low (≤ 1%, data not shown). To avoid contamination by phosphates, it is therefore important to prepare frozen aliquots of dNTP stock solutions. Upon repeated freezing and thawing of these solutions, a significant increase in the concentration of phosphates was observed (data not shown).

Figure 3. Absorption spectra of 25, 50, 75, and 100 µM uric acid at pH 6 (A) and 9 (B). Buffering was provided, at both pH values, by the universal Mes-Tris buffer.

The exonuclease activity of the ε subunit was assayed with thymidine 5′-monophosphate p-nitrophenyl ester, as described by Hamdan et al. (17). The polymerase activity of the α subunit was determined using our coupled assay in the presence of Tris-HCl, and we used dNTPs from New England Biolabs. The concentration of phosphates in these dNTPs was indeed observed (data not shown). To avoid contamination by phosphates, it is however important to prepare frozen aliquots of dNTP stock solutions. Upon repeated freezing and thawing of these solutions, a significant increase in the concentration of phosphates was observed (data not shown).

To determine the appropriate amount of each enzyme to be used in the coupled assay, we first determined the relevant catalytic constants of XOD and PNPase at pH 7.8 (100 mM Tris-HCl). First, we assayed XOD activity, as a function of oxanthine concentration, in the presence of 25 mM dNTPs. Under these conditions, we estimated $K_m$ and $V_{max}$ equal to $52.7 \pm 5.7 \mu M$ and $33.5 \pm 1.5 \mu M/s$, respectively (Figure 1A). Next, we assayed PNPase activity, as a function of phosphate concentration, in the presence of 5 mM MgCl$_2$, 0.25 mM inosine, 500 µM/mL of XOD and 10 µM/mL of PNPase. Under these conditions, $K_m$ and $V_{max}$ were estimated equal to $170 \pm 8 \mu M$ and $285 \pm 3 \mu M/s$, respectively (Figure 1B). According to Lee et al. (18), the PNPase supplied by Sigma-Aldrich is isolated from *Cellulomonas* sp., and features a $K_m$ for phosphate equal to $167 \pm 22 \mu M$ at pH 7.6. Therefore, our use of XOD to assay PNPase activity appears quantitatively reliable, and suggests the optimal concentration of these two enzymes when performing the PPX assay.

According to McClure (6), when two auxiliary enzymes are used in a coupled assay, the lag time necessary to reach steady-state conditions approaches a minimum when the first-order rate constant ($V_{max} / K_m$) of the secondary auxiliary enzyme is about 4-fold the corresponding constant of the primary auxiliary enzyme. This means, taking into account the constants reported here ($V_{max} / K_m$ equal to $6.4 \times 10^{-4}$ and $4.2 \times 10^{-3}$ for XOD and PNPase at 25 mM/mL, respectively), that a 25-fold excess of XOD over PNPase should be used to minimize the lag time. Alternatively, to save auxiliary enzymes, McClure proposed the use of amounts giving equal first-order rate constants (6). In our case, this translates into using XOD 6–7 times in excess over PNPase. Similar considerations arise when the theory of Storer and Cornish-Bowden (7) is applied. Indeed, an excess of about 10-fold of XOD over PNPase should be used to attain maximal velocities for both enzyme-catalyzed reactions in approximately equal times. Therefore, we decided to test PNPase activity in the presence of 50 and 500 µM/mL of PNPase and XOD, respectively. As Figure 1C shows, a linear dependence of XOD activity in the presence of 50 and 500 µM/mL of PNPase and XOD, respectively. As Figure 1C shows, a linear dependence of XOD activity as a function of phosphate concentration was observed (data not shown). To avoid contamination by phosphates, it is however important to prepare frozen aliquots of dNTP stock solutions. Upon repeated freezing and thawing of these solutions, a significant increase in the concentration of phosphates was observed (data not shown).

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Results and discussion

As a first step, we decided to evaluate the presence of phosphates in the reagents that we used to determine DNA polymerase activity. To this aim, each reagent was analyzed by the conventional Malachite green method (3), and the concentration of phosphates was quantitated using a calibration curve, ranging from 0 to 12.5 µM ($Abs = 0.019 + 0.046 \times [P]$, Supplementary Figure S2A). No significant contamination was detected in Tris-HCl, MgCl$_2$, and calf thymus activated DNA (Supplementary Figure S2B). On the contrary, significant levels of phosphates were determined in dNTPs (purchased from two different suppliers), and in both DNA polymerases tested, i.e., *E. coli* DNA polymerase III (DNA Pol-III) and Klenow fragment of DNA polymerase I (Supplementary Figure S2C). Therefore, to avoid contamination by phosphates, we extensively dialyzed the two polymerases against Tris-HCl, and we used dNTPs from New England Biolabs. The concentration of phosphates in these dNTPs was indeed found to be very low (≤ 1%, data not shown).

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The appropriate amount of each enzyme to be used in the coupled assay, we first determined the relevant catalytic constants of XOD and PNPase at pH 7.8 (100 mM Tris-HCl). First, we assayed XOD activity, as a function of oxanthine concentration, in the presence of 25 mM dNTPs. Under these conditions, we estimated $K_m$ and $V_{max}$ equal to $52.7 \pm 5.7 \mu M$ and $33.5 \pm 1.5 \mu M/s$, respectively (Figure 1A). Next, we assayed PNPase activity, as a function of phosphate concentration, in the presence of 5 mM MgCl$_2$, 0.25 mM inosine, 500 µM/mL of XOD and 10 µM/mL of PNPase. Under these conditions, $K_m$ and $V_{max}$ were estimated equal to $170 \pm 8 \mu M$ and $285 \pm 3 \mu M/s$, respectively (Figure 1B). According to Lee et al. (18), the PNPase supplied by Sigma-Aldrich is isolated from *Cellulomonas* sp., and features a $K_m$ for phosphate equal to $167 \pm 22 \mu M$ at pH 7.6. Therefore, our use of XOD to assay PNPase activity appears quantitatively reliable, and suggests the optimal concentration of these two enzymes when performing the PPX assay.

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As a model system to test our enzyme coupled assay, we selected the Klenow fragment of *E. coli* DNA polymerase I.
According to the reaction velocities observed with different concentrations of coupling enzymes (see above) we decided to use 5, 50, and 500 mU of PPase, PNPase, and XOD, respectively. Furthermore, in order to perform a set of appropriate controls, poly-dA:oligo-dT was chosen as substrate. As a first test, we incubated Klenow enzyme with 2.5 µM oligo-dT, in the absence of both poly-dA and dTTP. No appreciable activity was detected under these conditions (Figure 2A). Moreover, when 2.5 µM poly-dA was added to the reaction mixture (in the presence of 2.5 µM oligo-dT), no significant activity was detected in the absence of dTTP (Figure 2B). On the contrary, when 2.5 µM poly-dA:oligo-dT and 100 µM dTTP were present, a linear increase in absorbance at 293 nm was readily observed (Figure 2C).

Under the same conditions, the polymerase activity detected in the presence of 1.25 µM poly-dA:oligo-dT was slightly lower when compared with the activity observed with 2.5 µM DNA (Figure 2C), indicating that the kinetics is zero-order. To further inspect the kinetics of DNA elongation catalyzed by Klenow, we assayed polymerase activity as a function of poly-dA:oligo-dT concentration (at 100 µM dTTP). Under these conditions, \( k_{\text{cat}} \) and \( V_{\text{max}} \) were calculated equal to 12.4 nM and 6.1 nM/s, respectively (Supplementary Figure S3). Our estimation of \( k_{\text{cat}} \) is about 2-fold higher than previously published values (5–6 nM), referring to synthetic oligonucleotides (19–21). It is however worthy to note that we used a substrate different from previously used DNAs (19–21). Unfortunately, we were unable to determine the concentration of Klenow enzyme used in our assays. The protein concentration of the dialyzed stock enzyme solution was indeed below the sensitivity limit of the micro-Bradford method (1 µg/mL). Taking into account the volume of stock enzyme solution used per assay, we estimate that the final concentration of Klenow polymerase was below 2.4 nM in each assay. This, in turn, means that the magnitude of \( k_{\text{cat}} \) is ≥ 2.5 s⁻¹.

To compare our enzyme coupled assay with a reference method, we used the conventional Malachite green procedure to detect phosphates. In particular, the activity of the Klenow enzyme and of a partially-purified \textit{E. coli} DNA Pol-III (13) were tested using both the coupled enzyme assay and the Malachite green method. Moreover, we wanted to evaluate if the two methods, when applied to Klenow enzyme, yielded activity values compatible with those indicated by the manufacturer (New England Biolabs). To this aim, the polymerase activity was assayed under the conditions indicated by New England Biolabs, i.e., at 37°C, using activated calf thymus DNA as substrate, and incubating the reaction mixture for 30 min. When 0.1 units of Klenow were used, we detected the release of 0.54 ± 0.07 nmol of pyrophosphate in 30 min with the Malachite green assay. When the enzyme coupled assay was performed under the same conditions, 0.1 units of Klenow enzyme produced 0.50 ± 0.01 nmol of pyrophosphate in 30 min. This value indicates that the coupled assay reliably estimates DNA polymerase activity. In addition, the values of activity obtained by the conventional Malachite green method and by the coupled assay are in reasonable agreement with the activity indicated by the manufacturer, i.e., 10 nM of pyrophosphate released by 1 unit of Klenow in 30 min at 37°C. It should be noted that our enzyme coupled assay can be performed using electron acceptors other than oxygen, e.g., 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT). In this case the assay would rely on the detection of INT-formazan, which can be conveniently monitored at 458 (22) or 460 nm (15). Therefore, we determined the activity of Klenow enzyme using our coupled assay in the presence of activated calf thymus DNA as substrate and 0.5 mM INT as electron acceptor. Under these conditions, 0.1 units of enzyme yielded 0.29 ± 0.01 nmol of pyrophosphate in 30 min (Supplementary Figure S4). This value is significantly lower than those obtained with the Malachite Green method and with our coupled assay performed in the presence of oxygen as the terminal electron acceptor (see above). We propose that this discrepancy is linked to the \( \epsilon \) of INT-formazan (we used 12.5 × 10⁵ M⁻¹ cm⁻¹), which has been difficult to determine (15).

It is important to note that INT-formazan can be detected at wavelengths compatible with assays dealing with partially purified enzyme preparations. To test this point, we overexpressed \textit{E. coli} DNA polymerase III catalytic core, containing the \( \alpha \) (polymerase), \( \epsilon \) (3′-5′ exonuclease), and \( \theta \) subunits. In particular, soluble proteins were extracted from \textit{E. coli} TOP10 subjected to overexpression of the core, and the extract accordingly obtained was loaded onto a Q-Sepharose FF column and eluted with a linear NaCl gradient. An aliquot of each collected fraction was then subjected to our coupled assay performed in microplates in the presence of 0.5 mM INT (see Materials and methods). As a reference assay, we determined in parallel the exonuclease activity of \( \epsilon \), under the conditions reported by Hamdan et al. (17). The polymerase and the exonuclease activity peaks were found to overlap satisfactorily (Supplementary Figure S5), indicating that our INT-based microplate assay of polymerase activity is reliable, and can be conveniently used to monitor purification of phosphate- or pyrophosphate-releasing enzymes.

The Malachite green method was also used to test the activity, in the presence of activated calf thymus DNA, of a partially purified preparation of \textit{E. coli} DNA pol-III (13). After 30 min of incubation at 37°C, we estimated the release of 1 ± 0.15 nmol of pyrophosphate. When the enzyme-coupled assay was performed under the same conditions (in the presence of oxygen as electron acceptor), we observed polymerase activity equal to 0.7 ± 0.04 nmol of pyrophosphate generated in 30 min. Taking into account the comparisons presented here, we propose the PPX coupled assay as a reliable approach to continuously determine the activity of DNA polymerases.

To evaluate the potential use of our enzyme coupled assay over a wide pH interval, we determined the molar extinction coefficient of uric acid at pH 6 and 9. To this aim, we used the previously described Mes-Tris universal buffer (23), which is known to span a wide pH interval at constant ionic strength (23). As Figures 3A-3B show, at both pH values uric acid features maximal absorbance at 290 nm. This observation was confirmed, for both pH values, by inspection of the first-derivative spectra (data not shown). Our estimate of \( \epsilon_p \) significantly differs from previously reported values, indicating 293 nm as the wavelength of maximal molar absorbance by uric acid (14). However, it should be mentioned that Smith observed a significant shift (from 292 to 295 nm) of \( \epsilon_{\text{uric }} \), when comparing the spectra of uric acid in phosphate and carbonate buffers (24). Moreover, a concentration dependence shift of the specific molar absorbance of uric acid was reported (25). We therefore propose 290 nm as the \( \lambda_{\text{uric }} \) for uric acid at pH 6 and 9, in Mes-Tris buffer. In addition, according to the data reported in Figures 3A-3B, we propose 12 × 10⁵ and 12.4 × 10⁵ M⁻¹ cm⁻¹ as the \( \epsilon \) of uric acid at 290 nm, at pH 6 and 9, respectively.

The activities of \textit{Cellulomonas} sp. PNPase (18) and bacterial XOD feature pH optimum at 7.5–8.0. On the contrary, the pH optimum of \textit{E. coli} PPase is equal to 8.5–9.0 (26). Therefore, to further test the pH-dependence of our enzyme-coupled assay, we determined PPase activity as a function of pH, using the Mes-Tris universal buffer. As Figure 4 shows, PPase activity features a maximum at pH 8.5–9.0, in agreement with previous observations reported by Josse (26). In addition, at pH 6 the activity drops to 15% of the maximum (Figure 4). It is interesting to note that Josse, using maleate buffer, reported at pH 6 a relative activity equal to less than 10% of the maximum (26). The difference between these and our observations could be because of our use of a universal buffer, which
avoids potentially inhibitory or stimulatory effects exerted by the different buffers used in activity assays performed as a function of pH. Therefore, we propose that our enzyme coupled assay could be used at pH 6–7 upon increasing the concentration of PPase, e.g. to 10–50 mU/mL instead of 2–10 mU/mL, as optimal for assays at pH 7–8.

According to the observations reported here, the PPX enzyme coupled assay was shown to represent a robust and sensitive method to perform activity assays of phosphate- or pyrophosphate-releasing enzymes. Using this coupled assay, enzyme activities can indeed be determined continuously, quantitatively, and over a wide pH range. Moreover, the assay can be performed in microplates to determine the activity of partially purified enzyme preparations.

Competing interests
The authors declare no competing interests.

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