Enzymic determination of inorganic phosphates, organic phosphates and phosphate-liberating enzymes by use of nucleoside phosphorylase–xanthine oxidase (dehydrogenase)-coupled reactions

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(Received 25 February 1985/18 April 1985; accepted 8 May 1985)

Coupled enzyme assays are described for measuring inorganic phosphates, organic phosphates and phosphate-liberating enzymes in biological material. The assays all determine P, by its reaction with inosine, catalysed by nucleoside phosphorylase; this yields ribose 1-phosphate and hypoxanthine. The hypoxanthine is oxidized to uric acid by xanthine oxidase, and may be measured either by the absorbance of the uric acid, or by the formazan formed when a tetrazolium salt is used as the oxidant. The coupled enzyme assays are characterized by (1) high sensitivity, (2) quantitative utilization of phosphates and stoichiometric formation of the measurable products, (3) measurement at pH 6.0–8.5, (4) determination of phosphates within a single analytical step, and (5) continuous measurement of phosphohydrolase activity in a corresponding rate assay. Examples include determinations of substrates such as P, PP, and AMP, and of enzymes such as 5’-nucleotidase, inorganic pyrophosphatase and glucose-6-phosphatase. Directions for further examples are given.

In biological material P, is usually determined chemically by the formation of phosphomolybdate under acid conditions and its subsequent reduction to Molybdenum Blue (e.g. Fiske & SubbaRow, 1925; Berenblum & Chain, 1938; Taussky & Shorr, 1953; Dryer et al., 1957; Marsh, 1959; Baginski et al., 1967). The molybdate methods are also used to assay the activity of P,-liberating enzymes such as 5’-nucleotidase (Heppel & Hilmoe, 1955), inorganic pyrophosphatase (Schlesinger & Coon, 1960) and glucose-6-phosphatase (Nordie & Arion, 1966). These methods permit only discontinuous measurement of enzyme activity, and they are time-consuming. Moreover, because of the assay conditions, unspecific non-enzymic hydrolysis of labile phosphoesters may occur (Black & Jones, 1983; Lane & Wattmough, 1984).

In the present paper nucleoside phosphorylase and xanthine oxidase are used to couple P, uptake with the formation of photometrically measurable products. These reactions not only allow P, to be determined, but also are well suited to assay the activity of phosphohydrolases continuously and to measure their phosphoester substrates within a single analytical step.

Analytical principle

The analytical principle is based on the phosphorolytic cleavage of nucleosides such as inosine to hypoxanthine and ribose 1-phosphate by nucleoside phosphorylase and the subsequent xanthine oxidase-catalysed conversion of hypoxanthine into uric acid. Two alternative procedures of the analytical principle are utilized. They differ in the electron acceptor of the indicator reaction and the product to be measured. In the first procedure, the NP–XO assay, molecular O, serves as the oxidant, and the product monitored in the u.v. region is uric acid (Hwang & Cha, 1973). In the second procedure, the NP–XDH assay, xanthine oxidase functions as a dehydrogenase (xanthine dehydrogenase), and tetrazolium salts such as INT are reduced instead of molecular O, and the formazan thus formed is measured between 470 and 550 nm (Fried, 1966).

Abbreviations used: INT, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride; NP, nucleoside phosphorylase; XDH, xanthine dehydrogenase; XO, xanthine oxidase.
NP-XO assay:
\[ P_1 + \text{inosine} \xrightarrow{\text{NP}} \text{hypoxanthine} + \text{ribose 1'-phosphate} \]

Hypoxanthine + 2 H₂O + 2 O₂ \xrightarrow{\text{xO}} uric acid + 2 H₂O₂ (O₂⁻)

NP-XDH assay:
\[ P_1 + \text{inosine} \xrightarrow{\text{NP}} \text{hypoxanthine} + \text{ribose 1'-phosphate} \]

Hypoxanthine + 2 tetrazolium salt \xrightarrow{\text{XDH}} uric acid + 2 formazan

The assays may be used to measure P₁ output continuously, and hence follow the action of a phosphohydrolase; similarly the substrate (R-P) of a phosphohydrolase may be determined by the total amount of P₁ released.

\[ \text{R-P} + \text{H}_2\text{O} \xrightarrow{\text{Phosphohydrolase}} \text{P}_1 + \text{substrate} \]

\[ \text{P}_1 \xrightarrow{\text{direct enzymic measurement}} \]

**Materials and methods**

**Materials**

AMP, glucose 6-phosphate, inorganic pyrophosphatase (EC 3.6.1.1), inosine, mannose 6-phosphate, nucleoside phosphorylase (EC 2.4.2.1) and xanthine oxidase (EC 1.2.3.2) were from Boehringer (Mannheim, Germany); hypoxanthine, INT, 5'-nucleotidase (EC 3.1.3.5) and xanthine were from Sigma (München, Germany); KH₂PO₄, Mops, Na₃P₂O₅, and uric acid were from Merck (Darmstadt, Germany); bovine serum albumin was from Behring (Marburg, Germany). Renex 690 was a gift from Atlas-Chemie (Essen, Germany).

The enzymes were freed from P₁ and (NH₄)₂SO₄ by dialysis at 4°C against 104mM-KCl/50mM-Tris/HCl buffer, pH 7.4, for about 4h, or, alternatively, by washing twice with 2vol. of 3.2M-(NH₄)₂SO₄ in 50mM-Tris/HCl buffer, pH 7.4, and resuspending in 1vol. of 104mM-KCl/50mM-Tris/HCl buffer, pH 7.4. KH₂PO₄ was dried for 2h at 110°C. All the other chemicals were of analytical grade and used without further purification.

Microsomal fractions were isolated from male Wistar rats (de Groot & Haas, 1980). Detergent treatment of microsomal fractions was performed as previously described (de Groot & Noll, 1985).

**Methods**

Phosphate determinations and enzyme activity measurements were carried out at 25 or 37°C in a volume of 1.0ml of reaction mixture, with 5µl, 10µl or 20µl sample. One unit of enzyme is defined as that amount which causes transformation of 1µmol of substrate/min. If not otherwise stated, the following two reaction mixtures were used: NP-XO assay, 104mM-KCl/50mM-Tris/HCl buffer, pH 7.4, 1mM-inosine, 1mM-O₂ (equilibrated by bubbling 100% O₂ through the solution for 10min before the addition of enzymes), nucleoside phosphorylase (500 units/l) and xanthine oxidase (50 units/l); NP-XDH assay, 104mM-KCl/50mM-Tris/HCl buffer, pH 7.4, 1mM-inosine, 1mM-INT, nucleoside phosphorylase (500 units/l) and xanthine dehydrogenase (50 units/l).

Uric acid formed in the NP-XO assay was measured at 302nm, and formazan formed in the NP-XDH assay was measured at 546nm, by using the respective absorption coefficients \(ε_{302} = 8.1 \times 10^{3} \text{M}^{-1} \text{cm}^{-1}\) and \(ε_{546} = 9.2 \times 10^{3} \text{M}^{-1} \text{cm}^{-1}\). The absorption coefficient of uric acid was determined with weighed amounts of the substance. The absorption coefficient of formazan (here originating from INT) was determined as described by Nachlas et al. (1960), or, alternatively, by the xanthine dehydrogenase-catalysed reaction of INT with weighed amounts of xanthine. Xanthine, as well as hypoxanthine and uric acid, was dissolved in 0.01M-KOH.

For the measurement of PP₁, the assay mixtures were further supplemented with inorganic pyrophosphatase (400 units/l) and 1.5mM-MgCl₂. For the measurement of AMP they were supplemented with 5'-nucleotidase (100 units/l) and 10mM-MgCl₂. In the end-point analysis of phosphates (P₁, AMP) the incubation time was 10min and readings were made against reagent blanks.

The catalytic activities of phosphohydrolases were determined with either assay supplemented with the appropriate substrate: 1.5mM-PP₁ for the determination of inorganic pyrophosphatase, 1mM-AMP for the determination of 5'-nucleotidase, and either 6mM-glucose 6-phosphate or 4mM-mannose 6-phosphate for the determination of glucose-6-phosphatase. In the 5'-nucleotidase assay 10mM-MgCl₂ was added. The inorganic pyrophosphatase assay mixture included 1.5mM-MgCl₂, and in the glucose-6-phosphatase assay the 104mM-KCl/50mM-Tris/HCl buffer, pH 7.4, was replaced by 104mM-KCl/50mM-Mops/KOH buffer, pH 6.7.

Nucleoside phosphorylase activity was determined with a modified NP-XDH assay mixture comprising 1mM-inosine, 1mM-INT, xanthine dehydrogenase (50 units/l) and either 104mM-KCl/50mM-Tris/HCl buffer, pH 7.4, or 104mM-KCl/50mM-Mops/KOH buffer, pH 6.7. Xanthine oxidase activity was determined in the presence of 1mM-xanthine, and xanthine dehydrogenase activ-
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Fig. 1 shows the time courses of uric acid formation by the NP-XO assay and of formazan formation by the NP-XDH assay after the addition of P_i. In both cases the reaction sequence started at maximal rate almost immediately, and the formation of the end products, uric acid or formazan, ceased after about 3 min. From the maximum absorbances obtained it is concluded that, for every 1 mol of P_i esterified, 1 mol of uric acid or 2 mol of formazan was formed by the NP-XO assay and the NP-XDH assay respectively. The quantitative recovery of weighed amounts of P_i by both procedures is also demonstrated in Fig. 2. Both assays were linear up to P_i concentrations in the sample of at least 15 mM. The respective P_i concentrations in the reaction mixtures were 149 μM (NP-XO assay, ΔA_{302} = 1.20) and 75 μM (NP-XDH assay, ΔA_{546} = 1.37).

Uric acid formed was measured at 302 nm with a

![Graph](image1)

**Fig. 1. Time course of uric acid formation by the NP-XO assay and of formazan formation by the NP-XDH assay after addition of 50 μM-P_i**

The NP-XO assay mixture contained 104 mM-KCl/50 mM-Tris/HCl buffer, pH 7.4, 1 mM-inosine, 1 mM-O_2, nucleoside phosphorylase (500 units/l) and xanthine oxidase (50 units/l). The NP-XDH assay mixture contained 104 mM-KCl/50 mM-Tris/HCl buffer, pH 7.4, 1 mM-inosine, 1 mM-Na_2, nucleoside phosphorylase (500 units/l) and xanthine dehydrogenase (50 units/l). Uric acid formation was measured at 302 nm, and formazan formation at 546 nm.

![Graph](image2)

**Fig. 2. Relation between weighed P_i added and P_i determined by the NP-XO assay or the NP-XDH assay**

P_i standard solutions were prepared with dried KH_2PO_4. A 10 μl portion (NP-XO assay) or 5 μl (NP-XDH assay) of P_i standard solution was added to 1.0 ml of the respective reaction mixture composed as described in the legend to Fig. 1. After 10 min at 37°C absorbances were measured against appropriate reagent blanks at 302 and 546 nm respectively. P_i determined was calculated from ΔA_{302} (NP-XO assay) by using an ε_{302} for uric acid of 8.1 × 10^4 M^-1 cm^-1 and from ΔA_{546} (NP-XDH assay) by using an ε_{546} for formazan of 9.2 × 10^4 M^-1 cm^-1. Analysis of regression: y = 1.01x - 0.10, r = 0.99 (NP-XO assay); y = 0.98x - 0.02, r = 0.99 (NP-XDH assay).
filter photometer (Eppendorf 1101 M) equipped with a mercury-vapour lamp as light-source and a photomultiplier sensitive in the u.v. region. With appropriate spectrophotometers uric acid may be read at 297 nm or at its maximum absorbance at 293 nm. The respective absorption coefficients are ε_{297} = 11.7 \times 10^{3} \text{M}^{-1}\text{cm}^{-1} and ε_{293} = 12.6 \times 10^{3} \text{M}^{-1}\text{cm}^{-1} (Scheibe et al., 1974). Likewise, the sensitivity of the NP–XO assay can be further increased by measuring formazan closer to its peak absorbance around 500 nm. Instead of INT, alternative tetrazolium salts such as Nitro Blue Tetrazolium may also be employed. Since absorption coefficients of formazans significantly depend on the reaction conditions, it is recommended that they be determined individually.

**Kinetic evaluations**

Each of the auxiliary substrates inosine, molecular O₂ and INT was present in the respective assay at a concentration of 1 mM. Their Michaelis constants (K_m) are 0.04 mM, 0.1 mM and about 0.3 mM respectively under the present conditions (determinations not shown). Comparison of these data with the amounts of P_i consumed (e.g. 149 μM in the NP–XO assay and 75 μM in the NP–XDH assay; see above) reveals that the auxiliary substrates of the coupling reactions do not become critical during the reaction period of the P_i measurements (Figs. 1 and 2), even taking into consideration a 1:2 stoichiometry between P_i and molecular O₂ and INT respectively.

For the intermediates hypoxanthine and xanthine we determined K_m values of 0.002 and 0.003 mM (determinations not shown). For P_i we obtained a K_m of 0.2 mM. The low K_m for hypoxanthine and xanthine explain the absence of a lag phase (Fig. 1). From the K_m for hypoxanthine and xanthine together with the K_m for P_i and the activities of nucleoside phosphorylase (500 units/l) and xanthine oxidase (dehydrogenase) (50 units/l) used, it also follows that P_i added has to be metabolized to more than 99% after about 3 min (Bergmeyer, 1974) (compare Fig. 1).

The K_m values can be used to predict the behaviour of the nucleoside phosphorylase/xanthine oxidase (dehydrogenase)-coupled reactions when they are utilized for measurements of activities of P_i-liberating enzymes in a corresponding rate assay. Assuming a limiting velocity (V_max) of the phosphohydrolase of 10 units/l (in the assay) and with the activities of nucleoside phosphorylase and xanthine oxidase (dehydrogenase) (500 units/l and 50 units/l respectively), it can be calculated, in accordance with Takagahara et al. (1983), that the indicator enzyme, here xanthine oxidase (dehydrogenase), should have reached 90% of V_max of the

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**Fig. 3. Progress curve of the 5′-nucleotidase reaction**

5'-Nucleotidase activity was measured with the NP–XDH assay supplemented with 1 mM-AMP and 10 mM-MgCl₂. Portions (5, 10 and 20 μl, as indicated in the Figure) of a 5'-nucleotidase stock solution were added to 1.0 ml of the reaction mixture at the arrow. Further experimental details are as indicated in the legends to Figs. 1 and 2.

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target enzyme after 1 min and 99% of V_max after about 2.4 min.

**Determination of 5′-nucleotidase, inorganic pyrophosphatase and glucose-6-phosphatase**

The enzymes 5'-nucleotidase and inorganic pyrophosphatase were chosen to validate the ability of the present method to measure the catalytic activity of P_i-liberating enzymes continuously. As expected, about 1 min after the addition of either 5'-nucleotidase or inorganic pyrophosphatase to a reaction mixture (NP–XO assay or NP–XDH assay) supplemented with their respective substrates AMP (1 mM) or PP_i (1.5 mM), constant P_i liberation was recorded; this is exemplified for 5'-nucleotidase and the NP–XDH assay in Fig. 3. A linear relationship was obtained between the amount of phosphohydrolase and the activity detected.

As a further example we measured the catalytic activity of the membrane-bound microsomal glucose-6-phosphatase utilizing either glucose 6-phosphate or mannose 6-phosphate as substrate (Fig. 4). We reproduced the known characteristics of this complex enzyme system (Arion et al., 1975): (a) in native microsomal fractions high activity was observed with glucose 6-phosphate as substrate and low activity with mannose 6-phosphate as substrate; (b) after disruption of the microsomal membrane by detergent there was only a slight increase in the glucose-6-phosphatase activity, but
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Enzyme activities were measured at 25°C in native (——) and detergent-treated (---) microsomal fractions with the NP-XDH assay mixture supplemented with either 6 mM-glucose 6-phosphate or 4 mM-mannose 6-phosphate. The NP-XDH assay mixture contained 104 mM-KCl/50 mM-Mops/KOH buffer, pH 6.7, 1 mM-inosine, 1 mM-INT, nucleoside phosphorylase (500 units/l) and xanthine dehydrogenase (50 units/l). Samples (10 µl; 4 mg of microsomal protein/ml) were added to 1.0 ml of reaction mixture. Further experimental details are as indicated in the legends to Figs. 1 and 2.

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a marked increase in the mannose-6-phosphatase activity. The glucose-6-phosphatase measurements were performed at pH 6.7. Both the NP-XO assay and the NP-XDH assay converted P_i readily within the pH range of at least 6.0–8.5 (results not shown).

Determination of inorganic pyrophosphate and AMP

The phosphoester substrates of phosphatases were determined with an experimental set-up similar to that used for measuring the rate of P_i release. In Fig. 5 the measurement of P_i is exemplified with the use of inorganic pyrophosphatase (400 units/l) as additional auxiliary en-
zyme in the NP-XDH assay. Since PP_{i} was contaminated by P_{i} to a disturbing degree, the measurements of PP_{i} were started by the addition of inorganic pyrophosphatase after the contaminating P_{i} had been completely converted into ribose 1-phosphate. In routine phosphoester assays P_{i} contaminations can be taken into account by use of appropriate sample blanks (without phosphohydrolase). The addition of PP_{i} resulted in a 1:4 stoichiometric formation of formazan. As a second example it is demonstrated (Fig. 6) that weighed amounts of AMP were quantitatively recovered by a reaction system consisting of the NP-XO assay and 5'-nucleotidase (100 units/l). Since AMP was free from contaminating P_{i} in this example, readings could be performed against a reagent blank.

Discussion

The results demonstrate that both versions of our analytical principle, the NP-XO assay and the NP-XDH assay, are well suited for the determination of inorganic and organic phosphates, and are readily applicable for measurement of the catalytic activity of P_{i}-liberating enzymes continuously in a corresponding rate assay.

With the selected activities of nucleoside phosphorylase (500 units/l) and xanthine oxidase (dehydrogenase) (50 units/l), conversion of P_{i} was almost complete after about 3 min. These relatively high catalytic activities, however, are not a basic requirement. By accepting a somewhat longer reaction time, the activities of both enzymes can be significantly diminished. With nucleoside phosphorylase at 200 units/l and xanthine oxidase (dehydrogenase) at 20 units/l, completion of the reaction is to be expected after about 5 min. In the present paper P_{i} was determined by the end-point method. Because of the K_{m} for P_{i} of 0.2 mM it is also possible to estimate the P_{i} concentration of a sample kinetically. This potential may be especially useful for the measurement of P_{i} by the NP-XO assay or the NP-XDH assay with an automated analysis system.

For the measurement of the catalytic activity of P_{i}-liberating enzymes, three examples were chosen: inorganic pyrophosphatase, 5'-nucleotidase and the complex system of glucose-6-phosphatase. Further potential examples are nucleoside diphosphatase, ATPase and sedoheptulose-1,7-bisphosphatase. In fact, almost any P_{i}-liberating process within the pH range 6.0-8.5 should be assayable with the present analytical principle. The recommended catalytic activities of nucleoside phosphorylase and xanthine oxidase (dehydrogenase) permit a maximum velocity of the target enzyme of 10 units/l in the reaction mixture. Suitable activities of nucleoside phosphorylase and xanthine oxidase (dehydrogenase) to represent a higher catalytic activity of the enzyme to be tested can be derived from equations set up by Takagahara et al. (1983).

Although we have used both versions of our assay for the assays described, the NP-XDH assay is more convenient because (1) it dispenses with the need to saturate solutions with O_{2}, (2) it gives a visible product, and (3) it is more sensitive because of the high absorption coefficient of the formazan and the fact that two molecules of it are produced per molecule of P_{i}, reacting. This high sensitivity means that it can usually be run without a sample blank. There are conditions, however, when only the NP-XO assay can be used, e.g. in experiments with microsomal fractions in the presence of significant amounts of NAD(P)H.

We thank Professor H. Sies for helpful criticism and advice during the preparation of the manuscript.

References