

## A Sensitive and Rapid Method for Determination of Pyrophosphatase Activity

YURI A. SHAKHOV\* and PÁL NYRÉN

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

A new, rapid and sensitive method of measuring the rate and amount of  $PP_i$  hydrolysis is described. The method is based on registration of small pH changes, caused by decrease of hydrogen-ion concentration during the pyrophosphatase reaction. With this detected value of hydrogen-ion concentration change and the buffering capacity of each reaction mixture determined by titration, the rate and amount of  $PP_i$  hydrolysis can be calculated. The results obtained by pH method are in good agreement with values determined by direct phosphate analysis. The described method can be applied to the continuous determination of pyrophosphatase activity in chromatophores, mitochondria, crude extracts or purified enzymes.

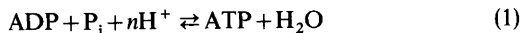
### ABBREVIATIONS

$P_i$ , inorganic orthophosphate;  $PP_i$ , inorganic pyrophosphate; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, morpholinopropane sulfonic acid; PIPES, piperazine-*N,N'*-bis[2-ethanesulfonic acid]; TCA, trichloroacetic acid; Bchl, bacteriochlorophyll.

It is well known that many tissues and cells contain inorganic pyrophosphatases (E.C. 3.6.1.1). Two forms of inorganic pyrophosphatase, which are customarily called soluble and membrane-bound have been intensively investigated during the last decade. The recent interest in membrane-bound pyrophosphatases is due to the fact that this enzyme

has  $PP_i$ -synthesizing activity. It has been shown that synthesis of  $PP_i$  coupled to the electron-transport chain is carried out in the chromatophores of *Rhodospirillum rubrum*<sup>1</sup> and mitochondria of animal tissues and yeast.<sup>2,3</sup> Solubilization and characterization of the membrane-bound pyrophosphatases, as well as the purification and characterization of the soluble ones, are being carried out in few laboratories.<sup>4–7</sup> However, investigations of pyrophosphatases are inhibited by the absence of the simple and efficient method for measuring the pyrophosphatase activity. All today's methods of pyrophosphatase assay are based on determination of  $P_i$  as a product of the enzyme reaction. Some of the  $P_i$ -determination methods include an extraction procedure of the phosphomolybdate complex, which takes time and is rather complicated.<sup>8,9</sup> The other known methods have limitations with regard to the purity of the pyrophosphatase preparation that is used, or require delicate tools or enzymes.<sup>10–14</sup>

Saris and Nishamura *et al.*<sup>15,16</sup> were the first who applied the pH recording method to the study of phosphorylation and ATPase reaction in bacterial chromatophores and animal mitochondria. According to reaction (1), a certain amount of  $H^+$  is



released or bound in the ATPase reaction or in phosphorylation, respectively. The *n*-value in eqn. (1) is dependent on pH and ionic strength.

We have developed a rapid method for determination of the rate and amount of  $PP_i$  hydrolysis by chromatophore pyrophosphatase, with the use of a sensitive recording pH-meter. This method has been applied to the continuous

\*Present address: A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117 234, U.S.S.R.

measuring of the pyrophosphatase activity in chromatophores, mitochondria, crude extracts or partly purified enzymes.

## EXPERIMENTAL

*General.* The principle of this method is to measure small pH changes by the decrease of hydrogen-ion concentration when the reaction (2) takes place in the physiological pH range; the value of  $n = \Delta H^+ / \Delta PP_i$  is calculated theoretically and the buffering capacity (added  $H^+ / \Delta pH$ ) is determined experimentally by titration.



From the Debye-Hückel theory,<sup>17</sup> it is expected that the values of  $pK_a$  decrease as the ionic strength increases in dilute solution. It has been shown that there is a considerable decrease of  $pK_a$  for ATP- and ADP-magnesium complexes, compared to  $pK_a$  for ATP and ADP, respectively. The values of  $pK_a$  which have been found in the literature, are shown in Table 1. As is seen in Table 1, differences between  $pK_{aATP}$  and  $pK_{aATP \cdot Mg^{2+}}$ , as well as between  $pK_{aADP}$  and  $pK_{aADP \cdot Mg^{2+}}$ , reach about 2 pH units. Proceeding from similar structure and charge distribution in molecules of ATP, ADP and  $PP_i$ <sup>18,19</sup> we propose the decrease of  $pK_a$  for  $PP_i \cdot Mg$  complex up to values about 7.5 and 5.1 for the first and second proton dissociation. We do not consider the difference between the  $pK_{aPi}$  and  $pK_{aPi \cdot Mg}$  because, according to Ref. 21, phosphate should remain in uncomplexed form. Then in a certain pH range (up to pH 8.0), the reactions (3) should be predominant for hydrolysis of the  $PP_i \cdot Mg$  complex which is the true substrate for the enzyme.<sup>22</sup>

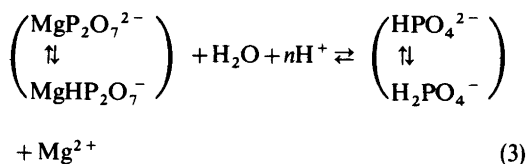


Table 1. Values for  $pK_a$  for ATP, ADP,  $PP_i$  and  $P_i$ .

| Acid              | $pK_a$ | Ref.   | Acid                | $pK_a$ | Ref.          |
|-------------------|--------|--------|---------------------|--------|---------------|
| ATP <sup>-3</sup> | 7.68   | 18, 19 | ATPMg <sup>-1</sup> | 5.44   | 18, 19        |
| ADP <sup>-2</sup> | 7.2    | 18, 19 | ADPMg               | 5.38   | 18, 19        |
| $P_i^{-1}$        | 7.21   | 20     | $P_i^{-2}$          | 12.38  | 20            |
| $PP_i^{-3}$       | 8.95   | 20     | $PP_iMg^{-1}$       | 7.5    | Assumed by us |
| $PP_i^{-2}$       | 6.1    | 22     | $PP_iMg$            | 5.1    | Assumed by us |

At pH > 8.0 hydrolysis of  $PP_i$  does not need protons e.g. the reaction appears as neutral; reaction (4).



The buffering capacity of the reaction mixture was determined experimentally: a known amount of hydrochloric acid (usually 25  $\mu$ l of 10 mN HCl = 250 ng ions  $H^+$ ) was added to the reaction mixture after each measurement and the pH change induced by this addition was recorded.

The amount of protons (n) in eqn. (3) can be calculated as (number of hydrogen ions disappeared)/(number of  $PP_i$  ions hydrolyzed) by assuming the relationship (5) for weak acid HA.

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \quad (5)$$

*Chromatophore isolation.* For pyrophosphatase activity measurements chromatophores isolated from the non-sulfur purple bacterium *R. rubrum* strain SI were used. The bacteria was grown anaerobically in light at 30 °C in the medium described by Bose *et al.*<sup>23</sup> After 40 h of growth (the end of the logarithmic phase) cells were harvested, washed and chromatophores were prepared by mechanical disruption in a Ribi cell fractionator in 0.2 M glycylglycine, pH 7.4 at 138 MPa. Cell debris was removed by centrifugation at 10 000g for 60 min and the supernatant was further centrifuged at 100 000g for 90 min. The pellet was washed twice by 0.3 M NaCl in 0.2 M glycylglycine pH 7.4 and by 0.2 M glycylglycine pH 7.4, respectively, with following centrifugations at 100 000g for 60 min. The preparation was stored at 0 °C in 0.2 M glycylglycine pH 7.4.

*Pyrophosphatase activity measurement.* The pyrophosphatase assay was carried out in a medium with weak buffering action, containing 1 mM PIPES buffer, 1 mM  $Na_4P_2O_7$  and 1 mM  $MgSO_4$  at 30 °C. Total volume 3 ml, chromatophores 2–40  $\mu$ l (0.8 mg Bchl/ml, 35 mg protein/ml) were added and pH adjusted to 6.80.

An open cylindrical thermostatic cuvette, a glass electrode GK 2322C and pH meter model 26 both from Radiometer and recorder model 2210 from LKB were used. The sensitivity of measurements could be changed, but a full-scale reading on the recorder shart corresponding to 0.1–0.15 pH units was the most frequent. Each measurement was usually done in a pH range of  $6.80 \pm 0.05$  and there was no detectable change of the linear, rate of the reaction caused by pH change. If, during the reaction, pH changes surpassed 0.1 pH units some changes in the velocity of the  $PP_i$  hydrolysis could be detected.

For comparison, the pyrophosphatase activity was determined by direct analysis of phosphate by using the method described in Ref. 10. In this case the assay medium contained 50 mM PIPES buffer pH 6.80, 1 mM  $Na_4P_2O_7$ , 1 mM  $MgSO_4$  and 2–4  $\mu$ l chromatophores. Total volume 2 ml. The reaction was carried out for 10 min at 30 °C and arrested by addition of 1 ml of 10% cold TCA.

RESULTS AND DISCUSSION

A typical example of pH change during  $PP_i$  hydrolysis is shown in Fig. 1. The vertical basic line shows the absence of pH changes in the assay medium, caused by ingredient interactions of atmospheric carbonation. The fast shift of the pH is due to addition of chromatophores, suspended in 0.2 M glycylglycine buffer pH 7.4. This method has been applied to verify the effects of uncoupler and inhibitor on pyrophosphatase activity in chromatophores. As is seen in Fig. 1 the pyrophosphatase is stimulated by 1  $\mu$ M FCCP from Boehringer Mannheim GmbH) and inhibited by 0.1 mM DCCD (from Fluka AG).

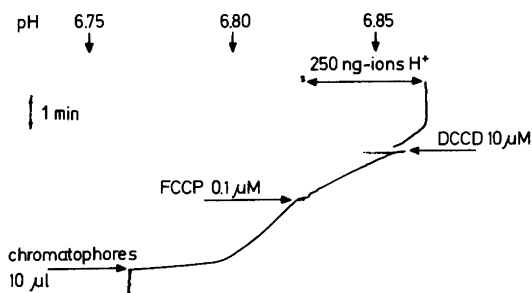


Fig. 1. Recordings of the pH change during the pyrophosphatase reaction in chromatophores of *R. rubrum* and the effects of FCCP and DCCD. Chromatophores corresponding to 0.35 mg of protein were added to 3 ml of assay medium.

It follows from eqn. (3) and values of pH presented in Table 1, that the low pH should be optimal for measuring the  $\Delta$  pH which appears during the pyrophosphatase reaction. As is shown in Fig. 2, pH optimum for this method was about pH 6.0–6.3, when various buffers were used. In contrast to the  $P_i$ -determination method of pyrophosphatase activity (see Fig. 3), the buffers, obviously, have a strong effect on pyrophosphatase assay using pH method. The highest sensitivity occurred with MOPS, but in this case there was some change of rate of the reaction caused by pH-change. Therefore we used PIPES, in the presence of which there practically is no change in the reaction velocity in the pH range 6.0–7.0 as seen in Fig. 2.

To understand the cause of the dramatic decrease of pyrophosphatase activity at a pH lower than 6.0 we studied the pH optimum of its activity by the  $P_i$ -

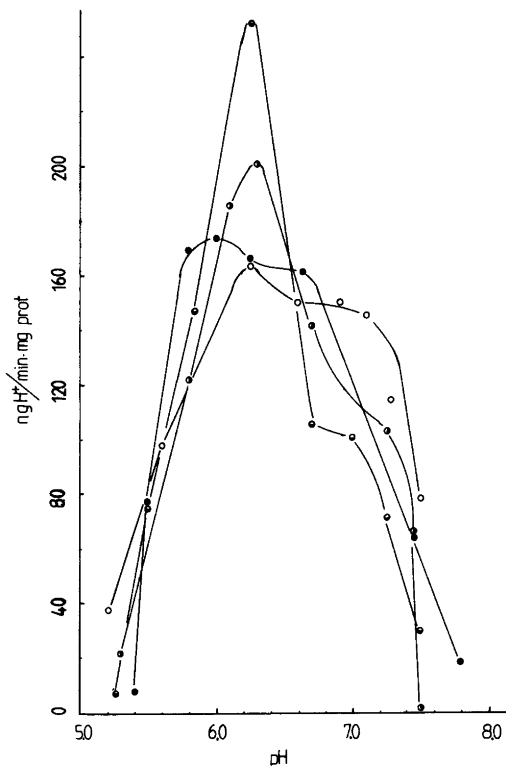


Fig. 2. pH optima for pyrophosphatase activity determined by pH method. 1 mM buffers were used: ●, tris-HCl; ○ PIPES-NaOH; ●, MES-NaOH; ○, MOPS-NaOH. Chromatophores corresponding to 1.4 mg of protein were added to 3 ml of assay medium. FCCP 0.1  $\mu$ M.

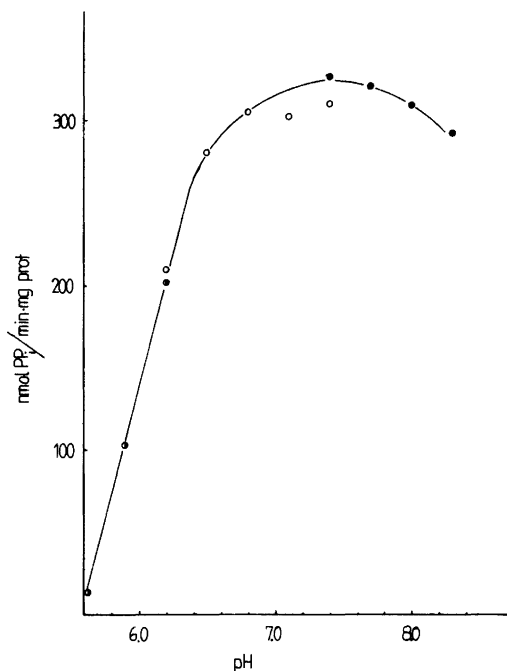


Fig. 3. Activity of chromatophore pyrophosphatase as a function of pH. Determination by direct phosphate analysis. 50 mM buffers were used: ●, tris-HCl; ○, PIPES-NaOH; ●, MES-NaOH. Chromatophores corresponding to 0.07 mg of protein were added to 3 ml of assay medium. FCCP 0.1  $\mu$ M.

determination method. As seen in Fig. 3, the pyrophosphatase activity has a pH optimum at pH 6.8–8.2 and decreased rapidly at low pH. Thus the observed decrease in the activity as measured by the

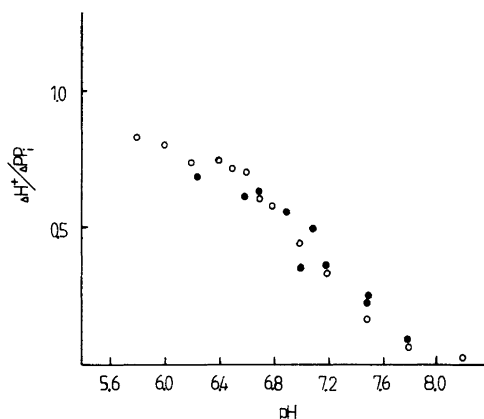


Fig. 4. Values of  $n = \Delta H^+ / \Delta PP_i$  used for calculations of rate of  $PP_i$  hydrolysis. Points ○ are derived from theoretical calculations using eqns. (3)–(5) and values of pK, presented in Table 1. Points ● correspond to the experimentally determined values of  $n$  at various pH from the comparison of  $PP_i$  hydrolysis rate obtained by two independent methods (pH recording and direct  $P_i$  determination).

pH-method actually occurs at low pH. At a pH range of 7–8 the pH change should be decreased according to eqn. (4).

The rates of  $PP_i$  hydrolysis, as determined by the two independent methods ( $P_i$ -determination and pH measurement), with the same chromatophore samples at pH  $6.80 \pm 0.01$  are compared in Table 2. In the first and the second columns, the rates of photophosphorylation determined by the  $P_i$  analysis and pH measurement are indicated. The determination of  $n = \Delta H^+ / \Delta PP_i$  is possible in

Table 2. Comparison of pyrophosphatase activity rates determined by two methods and experimental determination of  $n = \Delta H^+ / \Delta PP_i$ . All experiments done at pH  $6.80 \pm 0.01$ .

| Experiment No. | Rate of $PP_i$ hydrolysis ( $\Delta PP_i$ /min mg prot) |                | $n = \frac{\Delta H^+}{\Delta PP_i}$<br>determined experimentally |
|----------------|---------------------------------------------------------|----------------|-------------------------------------------------------------------|
|                | $P_i$ determination                                     | pH measurement |                                                                   |
| 1              | 76.05                                                   | 47.8           | 0.62                                                              |
| 2              | 69.14                                                   | 40.5           | 0.58                                                              |
| 3              | 70.25                                                   | 37.2           | 0.52                                                              |
| 4              | 133.5                                                   | 81.0           | 0.60                                                              |
| 5              | 133.9                                                   | 78.5           | 0.58                                                              |
| 6              | 113.0                                                   | 80.8           | 0.71                                                              |
| 7              | 304.0                                                   | 172.1          | 0.57                                                              |
| 8              | 106.2                                                   | 75.8           | 0.71                                                              |

parallel experiments from the chemically analyzed amount of  $P_i$  released, from one side, and the measurements of pH change and buffering capacity, from the other. The  $n$ 's determined by this method with no assumptions of  $pK_a$  or equations are presented in the last column of Table 2. The mean value of  $n$ , obtained in 17 experiments, was 0.59 (standard deviation 0.09) at pH  $6.8 \pm 0.01$ . The  $n$ -values were calculated also at different pH values by assuming eqns. (3) and (5) and using the  $pK_a$  values, presented in Table 1. The result of the calculation of  $n = \Delta H^+ / \Delta PP_i$  is presented in Fig. 4, as well as the results of the experimental determination of  $n$ -values at the same pH. It is seen that  $n$ -values calculated theoretically and obtained experimentally are in good agreement with each other. According to theoretical calculations, changes of ionic strength (when 2 or 40  $\mu$ l chromatophores were added) shall not exceed 7% of  $n$ -coefficient changes to a lower value.

Moyle *et al.*<sup>23</sup> studied proton-translocating pyrophosphatase of *R. rubrum* by measuring changes in the pH value. In the presence of 1  $\mu$ M FCCP the hydrolysis of  $PP_i$  by the chromatophore pyrophosphatase produced an alkalization of the media. This effect was not discussed, but we think it may be explained by the same  $H^+$ -binding as in our experiments. Using 3.3 mM glycylglycine buffer pH 7.0–7.1 in the presence of FCCP, Moyle *et al.* have obtained the coefficient  $n$  about 0.3 (g ion  $H^+$  disappeared)/(mol  $PP_i$  hydrolyzed). In our case  $n$ -values for this pH level are 0.39, 0.36 and 0.34 for 1 mM PIPES, MES and MOPS buffers, respectively.

It should be remembered that consumption of  $H^+$  may be accompanying some reactions and as a result the  $\Delta H^+ / \Delta PP_i$  ratio may be changed. Actually, one example is in 23, where gradient of  $H^+$  was formed in the vesicle preparation during  $PP_i$  hydrolysis. In our experiments with chromatophores we abolished the  $H^+$ -gradient formation by addition of uncoupler.

It may be mentioned that the described pH method was successfully used by us for measurement of pyrophosphatase activity in rat liver mitochondria which were isolated as described by Ernster and Löw<sup>24</sup> and suspended in 0.25 sucrose.

Extensive application of this method should contribute to a fuller understanding of the role of pyrophosphatases in cell metabolism, as its rapidity and sensitivity make it suitable both for quantitative, kinetic and screening experiments.

*Acknowledgements.* This work was supported by grants from the Swedish Natural Research Council to Prof. M. Baltscheffsky. One of us (Y.S.) would like to thank the Swedish Institute for 10 months scholarship. We wish to thank Professors H. and M. Baltscheffsky for helpful discussions and criticism. We also thank Dr. P. Muchiri for isolation of mitochondria.

## REFERENCES

- Baltscheffsky, M., Baltscheffsky, H. and von Stedingk, L. V. *Brookhaven Symp. Biol.* 19 (1966) 246.
- Mansurova, S. E., Shakhov, Yu. A., Beljakova, T. N. and Kulaev, I. S. *FEBS Lett.* 55 (1975) 94.
- Mansurova, S. E., Ermakova, S. A., Zviagilskaya, R. A. and Kulaev, I. S. *Microbiology USSR* 44 (1975) 874.
- Rao, P. V. and Keister, D. L. *Biochem. Biophys. Res. Commun.* 84 (1978) 465.
- Efremovich, N. V., Volk, S. E., Baykov, A. A. and Shakhov, Yu. A. *Biokhimiya (Engl. Trans.)* 45 (1980) 785.
- Shakhov, Yu. A., Mansurova, S. E. and Kulaev, I. S. *Biochem. International* 3 (1981) 139.
- Kondrashin, A. A., Remennikov, V. G., Samuilov, V. D. and Skulachev, V. P. *Eur. J. Biochem.* 113 (1980) 219.
- Berenblum, I. and Chain, E. *Biochem. J.* 32 (1938) 295.
- Melgunov, V. I. and Kulaev, I. S. *Neurospora News Lett.* 20 (1973) 35.
- Rathbun, W. B. and Betlach, V. M. *Anal. Biochem.* 28 (1969) 436.
- Schulz, D. W., Passonneau, J. V. and Lowry, O. H. *Anal. Biochem.* 13 (1967) 300.
- Kuttner, T. and Cohen, H. R. *J. Biol. Chem.* 75 (1927) 517.
- Delsal, J. L. and Manhoury, H. *Arch. Inst. Razi* 12 (1980) 43.
- Baykov, A. A. and Avaeva, S. M. *Eur. J. Biochem.* 32 (1973) 136.
- Saris, N. E. *Suom. Kemistiseuran Tied.* 68 (1959) 98.
- Nishimura, M., Ito, T. and Chance, B. *Biochim. Biophys. Acta* 59 (1962) 177.
- Debye, P. and Hückel, E. *Phys. Z.* 24 (1923) 185.
- Phillips, R. C., Phillips, S. J., George, P. and Rutman, R. J. *J. Am. Chem. Soc.* 88 (1966) 2631.
- Phillips, R. C., Phillips, S. J., Eisenberg, P., George, P. and Rutman, R. J. *J. Biol. Chem.* 240 (1976) 4393.
- Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M. *Data for Biochemical Research*, Clarendon Press, Oxford 1969.

21. Smith, R. M. and Alberty, R. A. *J. Am. Chem. Soc.* 78 (1956) 2376.
22. Klemme, J.-H. and Gest, H. *Proc. Natl. Acad. Sci. U.S.A.* 68 (1971) 721.
23. Bose, S. K., Gest, H. and Ormerod, J. G. *J. Biol. Chem.* 236 (1961) 13.
24. Moyle, J., Mitchell, R. and Mitchell, P. *FEBS Lett.* 23 (1972) 233.
25. Ernster, L. and Löw, H. *Exp. Cell. Res. Suppl.* 3 (1955) 133.

Received March 16, 1982.