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EFFECT OF INORGANIC PYROPHOSPHATE ON RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN HIGHER PLANTS

STANISLAW KOWALCZYK and PIOTR MASLOWSKI

Department of Biochemistry, Institute of Biology, Mikołaj Kopernik University, Toruń, Poland

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Key Word Index—*Zea mays*; Gramineae; corn; inorganic pyrophosphate; respiratory control; translocation.

Abstract—In coupled mitochondria of maize, inorganic pyrophosphate has no effect on electron transport whereas it competitively inhibits state 3 (with addition of ADP) respiration. The degree of inhibition depends on the ADP concentration in the reaction medium. At 150 and 300 μM ADP, the inhibition constant (K_i) has a value of 1.1×10^{-4} M. Pyrophosphate either does not penetrate through the membranes or penetrates through them in only very small amounts. It does not inhibit the exchange $^{32}Pi \rightleftharpoons Pi$; however, it undergoes an exchange with ADP (2 nmol PPi/mg protein for 10 min at 30°).

INTRODUCTION

It is well known that the chromatophores of *R. rubrum* [1, 2] as well as the chloroplasts [3] and mitochondria of higher plants [4] and the mitochondria of animals [5] are able to carry out an electron transport-dependent synthesis of PPi which is intimately interrelated to the synthesis of ATP. There is evidence for the participation in the phosphorylation process of two independent enzyme systems, i.e. ATPase and inorganic alkaline pyrophosphatase (PPase) [2, 6], although the presence of some interdependence between both these systems seems to be quite probable. The present study was designed to elucidate the possible role of PPi in respiration and phosphorylation in the mitochondria of higher plants.

RESULTS

Effect of PPi in oxygen uptake and respiratory control. Changes in oxygen uptake in state **4** (without addition of ADP to the reaction medium) and in state 3 (with addition of ADP) mitochondria of maize were studied. The respiratory control (RC, quotient of respiration in states **3** and **4**) was 4.75 and the ratio ADP:O was 1.93. The latter value was very close to the theoretical one [7]. The oxygen uptake (64.2–77.3 nmol O_2/mg protein per min) in state **4** mitochondria was unaffected by 0.05–2 mM PPi (Fig. 1). On the other hand, oxygen uptake under conditions of oxidative phosphorylation (state 3) was inhibited even at 200 μM PPi. This inhibition increased with increase in PPi concentration. At 1 mM and higher, oxygen uptake dropped below the values characteristic for state **4**. These data clearly indicate that at the concentrations used, PPi exerted no effect on substrate oxidation, whereas it substantially inhibited respiration in state **3**. This inhibition was unlikely to be due to chelation of Mg^{2+} in the reaction medium, because of the relatively high concentration of Mg^{2+} (5 mM) as compared with the effective concentration of PPi.

The plot of RC/RC_i as a function of the PPi concentration at two ADP concentrations indicated that this inhibition was competitive (Fig. 2). The inhibition constant (K_i) obtained by Dixon's method was 1.1×10^{-4} M (Fig. 3).

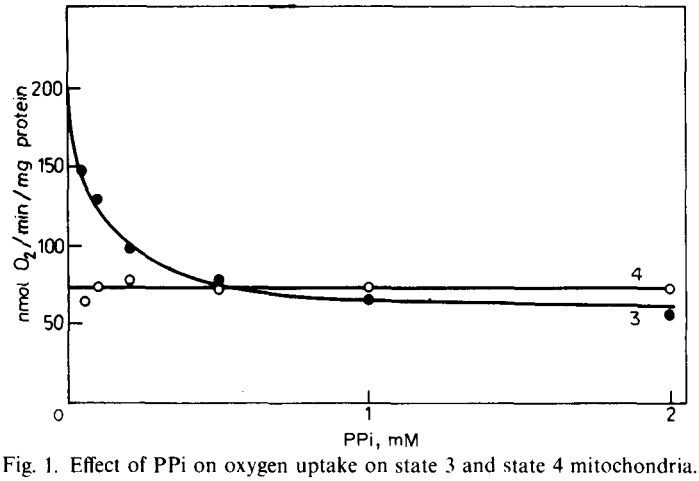

Fig. 1. Effect of PPi on oxygen uptake on state 3 and state 4 mitochondria.

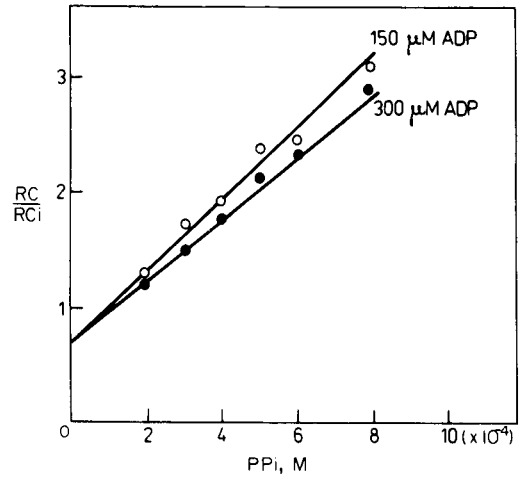

Fig. 2. Dixon's plot for determination of the type of inhibition of phosphorylation by PPi.

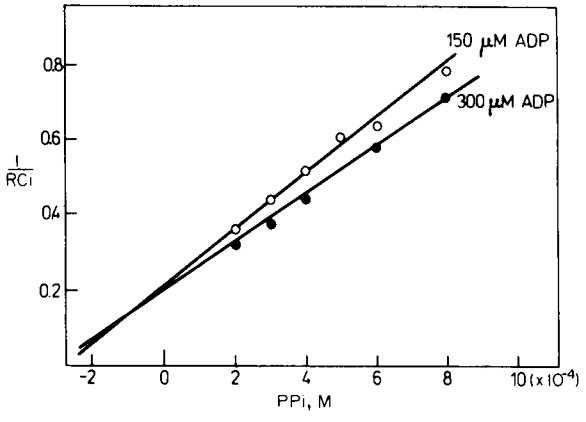

Fig. 3. Dixon's plot of the constant of inhibition (K_i) of phosphorylation by PPi.

Confirmation that PPi affected phosphorylation but not electron transport was provided by the demonstration that PPi inhibited oxygen uptake (state 3) was stimulated by the addition of the uncoupling agent dinitrophenol (Fig. 4).

PPi transport through the internal membrane of mitochondria

The experiments illustrated in Fig. 5 were performed on the basis of the previous finding [4] that inorganic pyrophosphatase is localized either on the internal side of the internal membrane or within the matrix. Thus, if exogenous PPi was unable to penetrate through the internal membrane then it would not be hydrolysed to Pi. A suspension of mitochondria was divided into six equal parts and centrifuged at 10000 *g*. The pellets were suspended in equal volumes of 0.4, 0.3, 0.2, 0.1 and 0.05 M sucrose and in distilled water and the specific activities of PPase and cytochrome oxidase in each suspension determined. The level of the cytochrome oxidase activity was taken as a measure of the morphological integrity of the mitochondria, since added cytochrome-*c* (due to the size of the molecule) can only act as an exogenous donor of electrons to broken mitochondria. The results showed that the activities of both enzymes were low in isotonic medium (0.25–0.4 M sucrose), and rapidly increased as the hypotonicity of the medium was increased (Fig. 5). These findings indicated that PPi either does not penetrate through the membrane or penetrates through it to a very small degree. The low PPase activity in isotonic medium may have been derived from cytoplasmic PPase adsorbed on the membranes or may have originated from partly damaged structures.

In addition to the above experiment, the transport of PPi into the mitochondria was measured by the swelling method in 0.1 M ammonium phosphate or pyrophosphate, according to Le Quoc *et al.* [8]. The results showed that PPi, in contrast to Pi, caused no mitochondrial

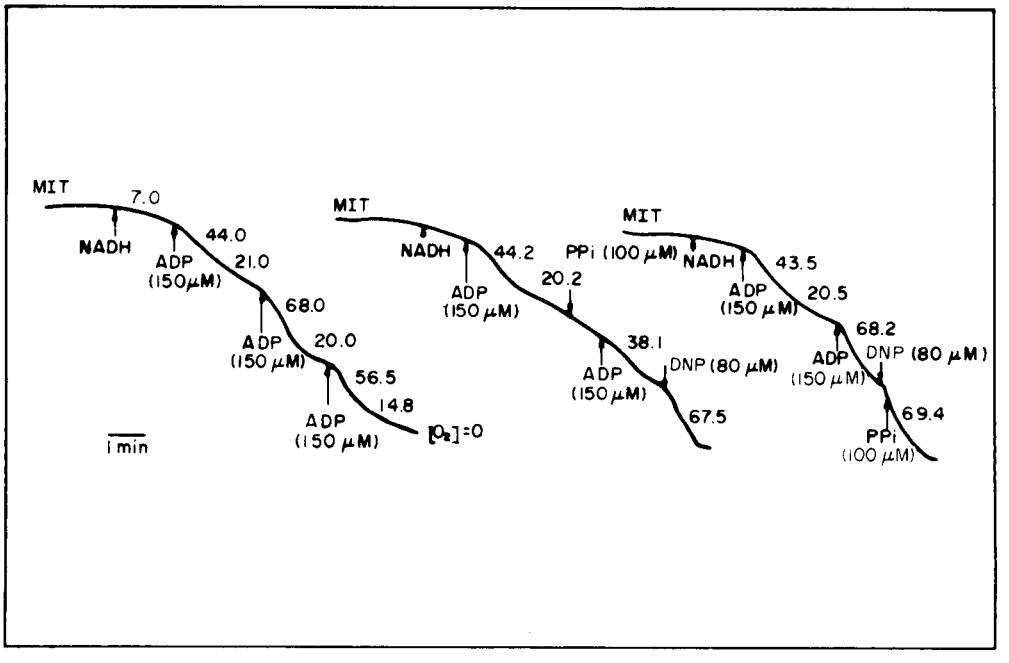

Fig. 4. Effect of PPi on NADH oxidation by maize mitochondria.

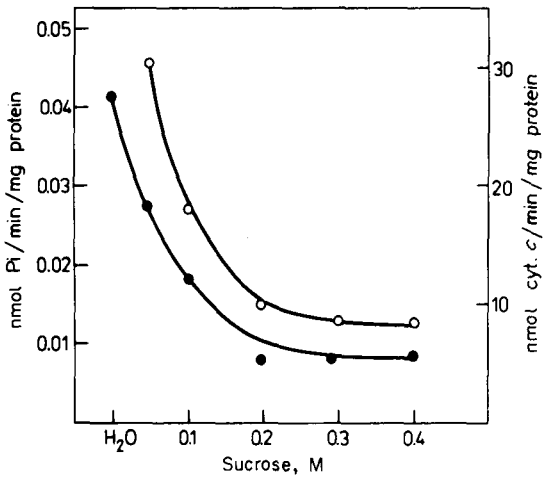

Fig. 5. The effect of osmolarity on PPase and cytochrome oxidase activities of mitochondria. ●——●, PPase activity (μmol Pi/min per mg protein); ○——○, cytochrome oxidase activity (nmol cytochrome-*c*/min per mg protein).

swelling (Fig. 6), and thus provided further evidence that PPi did not penetrate into the mitochondria. To confirm the above observations, the mitochondria were incubated at 0–4° in the presence of 0.5 mM PPi–$^{32}P^{32}Pi$ (849 600 cpm). The radioactivity of the samples after filtration was minimal, not exceeding 0.46% of the total activity of the sample applied on to the filter. Moreover, the degree of labelling of the mitochondria did not increase upon prolongation of the incubation time up to 15 min. It was probable that pyrophosphate was to a small extent adsorbed on the filter and chelated by divalent ions present in the mitochondrial membranes.

Effect of PPi on phosphate exchange

When mitochondria which had been loaded with unlabelled Pi and then washed were incubated (0–150 sec) in the presence of ^{32}Pi, the ^{32}Pi content in the mitochondria rapidly increased (Fig. 7). This observation

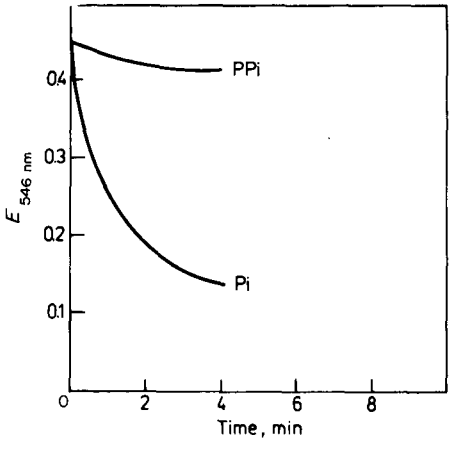

Fig. 6. Comparative effects of Pi and PPi on osmotic swelling of mitochondria. Mitochondria (about 5 mg/ml) were incubated in 62 mM sucrose, 8 mM $MgCl_2$, 50 mM KCl in 20 mM TES–KOH buffer, pH 7.0 at 30° for 10 min. At the end of this time 0.1 ml and the incubation mixture was mixed with 0.9 ml of 0.5 mM EGTA–0.1 M ammonium phosphate or PPi and the ΔE_{546nm} measured with time.

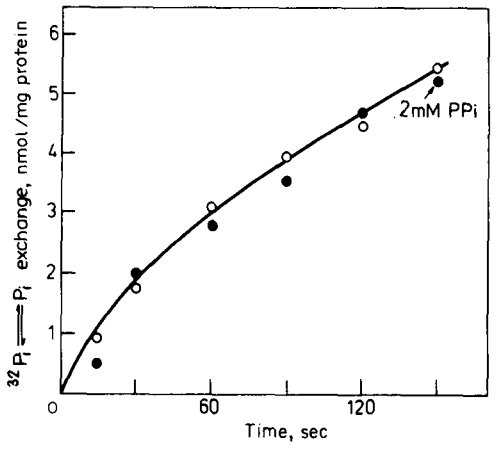

Fig. 7. Time course of exchange in mitochondria. Mitochondria were preincubated for 15 min at room temp. in 0.25 M sucrose, 1 mM EDTA, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 0.1% BSA in 20 mM imidazole–HCl buffer, pH 6.5 After washing and centrifugation at 0°, the mitochondria were suspended in the above medium free from KH_2PO_4. Measurements of the Pi exchange were initiated by addition of KH_2PO_4–$KH_2{}^{32}PO_4$ (50 μM final concentration) to 0.2 ml of the mitochondrial suspension (0.3 mg protein). At given times the reaction was inhibited by addition of mersalyl at 5 mM final concentration. After filtration and washing (2–3 sec), the filter was assayed for radioactivity.

pointed to a rapid exchange between the exogenous and endogenous pool of orthophosphate, probably by means of the orthophosphate translocator [9, 10]. This translocator had no affinity for PPi, since 0.5–2 mM PPi had no effect on the rate of exchange.

$PPi \rightleftharpoons ATP$, $PPi \rightleftharpoons ADP$, $PPi \rightleftharpoons AMP$

Because of a lack of labelled nucleotides, the experiments on the effect of PPi on the exchange of nucleotides were substituted by tests designed to verify the possibility of the occurrence of the exchanges $^{32}P^{32}Pi \rightleftharpoons ATP$, $^{32}P^{32}Pi \rightleftharpoons ADP$ and $^{32}P^{32}Pi \rightleftharpoons AMP$. After preliminary preincubation of the mitochondria with the respective nucleotide (2 mM) at 0–4°, they were washed and then incubated with 500 μM PPi–$^{32}P^{32}Pi$. The total activity of the sample taken for filtration was 160 000 cpm. A significant uptake of $^{32}P^{32}Pi$ was found only for the ADP-containing mitochondria. The exchange was inhibited some 50% (Fig. 8) by atractyloside, an inhibitor of the nucleotide translocator. This suggested that the transport of PPi into the mitochondria can proceed by participation of the nucleotide translocator found in plants [11, 12] since this translocator is less sensitive to atractyloside than the animal translocator. These findings permit certain conclusions to be drawn concerning the mechanism of the inhibition of phosphorylation by PPi, however, for a better insight into the exchange rate and affinity to PPi, studies using ^{14}C-labelled nucleotides are required. These are now in progress and will be reported in a further communication.

Phosphotransferase reaction $ADP + PPi \rightleftharpoons ATP + Pi$

The occurrence of this reaction would represent another possibility of the inhibition of oxidative phosphorylation by PPi, since ADP would be phosphorylated by PPi without the participation of the

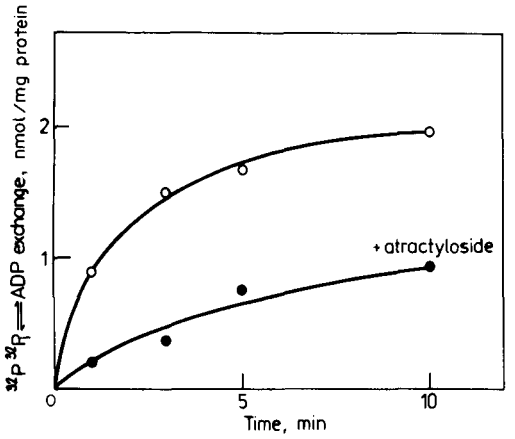

Fig. 8. Time course of $^{32}P^{32}Pi \rightleftharpoons ADP$ exchange in mitochondria. Mitochondria were incubated for 4 min at 0° in a medium containing 0.25 M sucrose, 1 mM EDTA, 0.1% BSA, 30 μM ADP in 20 mM imidazole–HCl buffer, pH 6.5. The mitochondrial suspension was then diluted with the above medium (without ADP) and centrifuged for 10 min at 20000 g. The mitochondrial pellet was incubated at 25° in a medium containing 0.25 M sucrose, 0.1% BSA, 1 mM EDTA, 50 μM PPi/$^{32}P^{32}Pi$ in 10 mM imidazole–HCl buffer, pH 6.5. At given times 0.2 ml samples were taken, filtered and washed with 5 ml of 0.25 M sucrose. The filter was then assayed for radioactivity. In some cases, 50 nmol atractyloside/mg protein were introduced into the reaction medium.

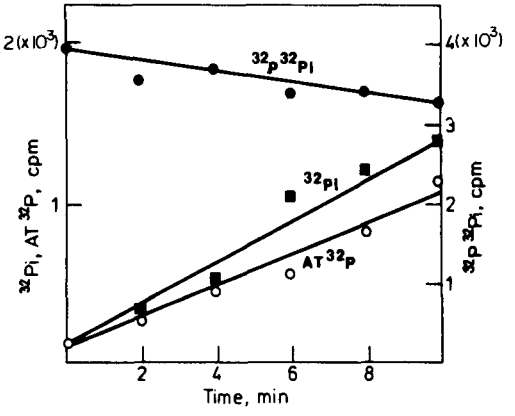

Fig. 9. Phosphotransferase reaction ADP + PPi $\rightleftharpoons$ ATP + Pi. Mitochondria (0.3 mg protein) were incubated at 30° in a medium containing 0.3 M sucrose, 5 mM $MgCl_2$, 0.1% BSA, 250 μM ADP, 1 mM PPi–$^{32}P^{32}Pi$, antimycin A (1 mg/ml) in 25 mM TES–KOH, pH 7.6. The reaction was stopped with 2 M $HClO_4$ and the solution was neutralized with 2 M KOH. After removal of protein by centrifugation, 10 μl samples were taken and subjected to high-voltage paper electrophoresis. The bands of ATP, ADP, PPi and Pi (located under UV) were cut out and assayed for radioactivity.

electron-transport chain. After incubation of the appropriate reaction mixture at 30° in the presence of 0.13 μM antimycin A to inhibit the respiratory chain, the reaction was stopped by transferring 0.2 ml samples to 0.25 ml of 10% TCA. Electrophoretic separation and measurements of the radioactivity of the different fractions permitted the determination of the amount of $^{32}P^{32}Pi$ undergoing hydrolysis and enabled an estimate to be made of the levels of AT^{32}P and ^{32}Pi formed. The results obtained suggested some interesting possibilities for PPi utilization in cells (Fig. 9). However, on account of the low reaction rates, they do not provide a reliable explanation of the mechanism of the inhibition of phosphorylation by PPi.

DISCUSSION

As stated in the Introduction, PPi biosynthesis in plant chloroplasts and mitochondria is well established. However, there is evidence of a role for this compound in the phosphorylation processes. The rate of phosphorylation is determined by the ratio [ATP]:[ADP][Pi] and PPi would influence this rate indirectly via changes in the Pi concentration. Recently, however, attention has been drawn to the possibility of a more direct participation of PPi in the above process; namely, it has been shown that the coupling factor CF_0 contains closely bound nucleotides which can be exchanged for PPi [13, 14]. The present results clearly indicate that PPi competitively inhibits oxidative phosphorylation, and that the degree of inhibition depends on the ADP concentration in the reaction medium. At 150 and 300 μM ADP, the inhibition constant (K_i) is 1.5×10^{-4} M. It could thus be assumed that PPi competes with ADP for a binding site on the translocator or that, in the case of penetration into the mitochondria, it can block the nucleotide-binding centre on factor F_1 [13, 14].

The PPase activity associated with the internal membranes of intact mitochondria and of those damaged to different extents indicate that PPi either does not penetrate through the membrane or penetrates to a very small extent only. The low activity of PPase in isotonic medium may originate from cytoplasmic PPase adsorbed on the membrane or from partly damaged mitochondria. In isotope uptake experiments, the small amounts of radioactivity remaining on the filter, probably result from the adsorption of PPi on the membranes. Such adsorption is possible, on account of the Ca^{2+} and Mg^{2+} ions found in the membranes [15].

Heldt and Rapley [9] and Fliege *et al.* [10] studied the transport of various metabolites through chloroplast membranes, and found that PPi competitively inhibits the transport of orthophosphate to the stroma compartment. Moreover, it has been found that PPi penetrates into the chloroplasts at a rate of 1.3 μmol/mg chlorophyl per hr, and that 0.2 mM orthophosphate inhibits this penetration by 70%. The above authors suggest that PPi competes with orthophosphate for the Pi translocator. In the present experiments, in contrast to the chloroplasts, PPi exerted no effect on Pi transport through the internal membrane of the mitochondria. Thus 0.5–2 mM PPi did not exhibit the exchange $^{32}Pi \rightleftharpoons Pi$. It can thus be assumed that the translocator exhibits no affinity to PPi and does not represent a possible pathway of PPi penetration through the membrane.

On the other hand, PPi undergoes exchange with ADP at a rate of 2 nmol PPi/mg protein per 10 min at 30°. Inhibition of the exchange by atractyloside may indicate that this exchange proceeds with participation of the nucleotide translocator. These findings suggest that PPi can inhibit phosphorylation by way of competition with ADP for the binding site on the nucleotide translocator.

We found no effect of 0.05–2 mM PPi on electron transport, whereas Susheela *et al.* [16] and Sivaramakrishnan *et al.* [17] have reported that 0.3 and 1 mM PPi activates succinic dehydrogenase in animal mitochondria.

It is noteworthy that the phosphotransferase reaction takes place in maize mitochondria (Fig. 8). On account of its relatively low rate, this reaction does not explain the immediate inhibition of phosphorylation coupled with electron transport by PPi. However, it may be of great importance since it represents another possibility of PPi utilization in the cell.

EXPERIMENTAL

Plant material. Etiolated 5-day seedlings of maize (*Zea mays* Wir 42) were used.

Isolation of mitochondria. Maize seedlings were cut into small pieces and ground in a cooled mortar, lined with a thick nylon net, in a soln (2 ml/g tissue) of 0.4 M sucrose, 5 mM EGTA and 0.1% BSA (defatted according to ref. [18]) in 50 mM KPi buffer, pH 8.0. After squeezing the suspension through the nylon net and adjustment of the filtrate to pH 7.6, the mitochondria were isolated and purified by differential centrifugation [19].

Respiration measurements. Respiration was measured with a Clark oxygen electrode. The reaction medium (1.5 ml) consisted of 0.25 M sucrose, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 0.1% BSA, 1 mM NADH, 0.1–0.13 mg/ml mitochondrial protein in 10 mM TES–KOH buffer, pH 7.2. Measurements were performed at 30°. The initial O_2 concn was 240 μM. State 3 respiration was initiated by addition of ADP after 3 min preincubation.

Protein determination. Protein was assayed according to ref. [20].

Determination of PPase activity. This was determined from the amount of Pi formed by enzymic hydrolysis of PPi [21].

Determination of cytochrome oxidase activity. This was assayed spectrophotometrically from the spectral differences between the reduced and oxidized form of cytochrome, according to ref. [22], without addition of digitonin to the reaction medium.

PPi determination. PPi was determined by the method of ref. [23].

High-voltage paper electrophoresis of Pi, PPi, ADP and ATP was performed in 50 mM citrate buffer, pH 4.2 using a voltage of 60 V/cm of filter paper length, according to ref. [6].

Synthesis of $^{32}P^{32}Pi$. An aq. soln of $KH_2{}^{32}PO_4$ (1 μCi/mg) was evapd at 50–60° and then heated in a muffle furnace for 20 hr at 210°. After cooling and dissolution in a small vol. of H_2O, the soln was quantitatively applied on to a Dowex 1 × 8 (Cl) column (1 × 10 cm). Pi was washed from the column with 0.05 M KCl–0.01 M HCl. PPi was then eluted with 0.2 M KCl–0.05 M HCl. Eluates with labelled PPi were neutralized with Tris to pH 7.5 and concd under vacuum at 6°. The yield of $^{32}P^{32}Pi$ was about 70%. For verification of the efficiency of separation, unlabelled pyrophosphate and labelled $KH_2{}^{32}PO_4$ were chromatographed on Dowex 1 × 8, the fractions containing unlabelled PPi were free from ^{32}Pi.

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