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The Phosphate–Pyrophosphate Exchange and Hydrolytic Reactions of the Membrane-Bound Pyrophosphatase of *Rhodospirillum rubrum*: Effects of pH and Divalent Cations

H. Celis[1] and I. Romero[1]

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Abstract

The relation that exist between the Pi–PPi exchange reaction and pyrophosphate hydrolysis by the membrane-bound pyrophosphatase of chromatophores of *Rhodospirillum rubrum* was studied. The two reactions have a markedly different requirement for pH. The optimal pH for hydrolysis was 6.5 while the Pi–PPi exchange reaction was at 7.5; the pH affects mainly the K_m of Mg^{2+} or Pi for the enzyme; Mn^{2+} and Co^{2+} support the Pi–PPi exchange reaction partially (50%), but the reaction is slower than with Mg^{2+}; other divalent cations like Zn^{2+} or Ca^{2+} do not support the exchange reaction. In the hydrolytic reaction, Zn^{2+}, at low concentration, substitutes for Mg^{2+} as substrate, and Co^{2+} also substitutes in limited amount (50%). Other cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, etc.) do not act as substrates in complex with PPi. The Zn^{2+} at high concentrations inhibited the hydrolytic reaction, probably due to uncomplexed free Zn^{2+}. In the presence of high concentration of substrate for the hydrolysis (Mg–PPi) the divalent cations are inhibitory in the following order: $Zn^{2+} > Mn^{2+} > Ca^{2+} \geqq Co^{2+} > Fe^{2+} > Cu^{2+} > Mg^{2+}$. The data in this work suggest that H^+ and divalent cations in their free form induced changes in the kinetic properties of the enzyme.

Key Words: Chromatophores; membrane-bound pyrophosphatase; phosphate–pyrophosphate exchange; pyrophosphate hydrolysis; pH; divalent cations.

Introduction

The membrane-bound pyrophosphatase of *Rhodospirillum rubrum* chromatophores is a molecular complex clearly distinguishable from the H^+-ATPase (Keister and Minton, 1971; Fisher and Guillory, 1969) which catalyzes the

[1] Departamento de Bioenergética, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70–600, México 04510, D.F. México.

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synthesis of pyrophosphate with the energy derived from photosynthetic electron transport (Baltscheffsky, 1978; Baccarini-Melandri and Melandri, 1978).

Most inorganic pyrophosphatases, either cytoplasmic or membrane-bound, use the complex Mg–PPi as substrate for hydrolysis (Lahti, 1983); however, recent data (Randahl, 1979) suggest that Mg^{2+} ions also exert a regulatory action on the catalytic properties of the membrane-bound enzyme. Indeed, it has been shown (Celis *et al.*, 1985) that the Pi–PPi exchange reaction and the pyrophosphate hydrolysis as catalyzed by membrane-bound pyrophosphatase have a markedly different Mg^{2+} requirement for activity, i.e., optimal rates of hydrolysis are attained at 1 mM Mg^{2+}, while the exchange reaction at this concentration is very low, and rises to a maximum at 10 mM Mg^{2+}. These observations suggest that in the membrane-bound pyrophosphatase of chromatophores, Mg^{2+} concentrations are critical in the reversal of pyrophosphate hydrolysis.

The cytoplasmic pyrophosphatase of *Rhodospirillum rubrum* requires Zn^{2+} for both its stability and activity, while Mg^{2+} are required to form the Mg–PPi, the substrate for the enzyme (Klemme and Gest, 1971; Klemme *et al.*, 1971). In the membrane-bound pyrophosphatase of the same organism, Co^{2+} at high concentrations may substitute for Mg^{2+} in the reversal of electron transport as induced by pyrophosphate hydrolysis, i.e., about 10% of the response observed with Mg^{2+} is observed with Co^{2+} (Baltscheffsky, 1969b). Zn^{2+} and Mn^{2+} are even poorer substitutes for Mg^{2+}, while Ca^{2+} inhibited the reversal of electron transport detected in the presence of Mg^{2+}. Other reports indicate that Mn^{2+} and Co^{2+} do not support the PPi-driven ATP synthesis (Keister and Minton, 1971), although at the concentration used, substantial hydrolysis of PPi took place. It has also been reported that Mn^{2+} inhibits the synthesis of PPi in the presence of Mg^{2+}, although it is unable to support PPi synthesis (Nishikawa, *et al.*, 1973).

This set of observations suggests that divalent metal ions regulate the catalytic properties of the membrane-bound pyrophosphatase of chromatophores. In this work the effect of pH and divalent cations other than Mg^{2+} on the Pi–PPi exchange reaction and PPi hydrolysis as catalyzed by the membrane-bound pyrophosphatase of *Rs. rubrum* has been explored, both from the ability of the cations to complex PPi thus forming the substrate for the enzyme, and from their possible regulatory effects on the catalytic cycle of the enzyme.

Experimental

Materials

^{32}Pi was obtained from New England Nuclear (Boston, Massachusetts) and was purified as described elsewhere (de Meis *et al.*, 1980). All salts and buffers were of the highest purity available.

Bacterial Growth and Preparation of Chromatophores from Wild Type

Rhodospirillum rubrum were grown anaerobically in the light (tungsten lamps of 40 W at 30 cm) at 30°C in a medium described by Cohen-Bazire *et al.* (1957). Bacteria cells were harvested in the early exponential phase, late exponential phase, and stationary phase. It was found that the Pi–PPi exchange reaction was optimal in the late exponential phase; however, the hydrolytic reaction did not vary with the phase of growth (to be published). Accordingly bacteria harvested in the late exponential phase were used in this work.

The cells were washed with 50 mM MOPS,[2] pH 7.0, and 50 mM KCl, and chromatophores were prepared by sonication for 2 min in an MSE sonicator at full power, in 10 mM Tris-Cl, pH 7.5. After centrifugation at 26,000 × *g* for 20 min, the supernatant was collected and centrifuged at 105,000 × *g* for 90 min. For the experiments with divalent cations, the residual Mg^{2+} was eliminated from the chromatophores by washing with 5 mM EDTA, pH 7.5, 5 mM EGTA, pH 7.5, 10 mM Tris-Cl, pH 7.5, followed by a second washing with 10 mM Tris-Cl, pH 7.5. The final pellet was resuspended in 10 mM Tris-Cl, pH 7.5, and adjusted to a bacteriochlorophyll concentration of 1 mg/ml equivalent to 16.5 mg/ml of protein. Our conditions of growth and chromatophore preparation were standardized in such a way that the concentration of protein and Bchl varied by 12% and 6% respectively. The chromatophore preparation was kept at 4°C and used within the next 3 days. No change in the hydrolytic or Pi–PPi exchange reaction was detected within this time. Bacteriochlorophyll was determined as described (Clayton, 1963), and protein was determined by the method of Lowry *et al.* (1951).

Hydrolysis of Pyrophosphate and Pi–PPi Exchange Reactions

The hydrolytic and exchange reaction were determined in the dark with a Green safety light in the conditions described under Results. The reactions were arrested by 6% trichloroacetic acid (final concentration). Phosphate was determined in the supernatant as described (Fiske and Subbarow, 1925). The Pi–PPi exchange reaction was assayed by measuring the formation of [^{32}Pi]PPi from PPi and [^{32}Pi]; in the trichloroacetic acid supernatants, orthophosphate was removed by isobutanol/benzene (1 : 1 v/v) subsequently to the formation of the phosphomolybdate complex as described elsewhere (de Meis *et al.*, 1983). The water phase was washed 5 times with isobutanol–benzene to ensure removal of [^{32}Pi]. Aliquots of the water phase were withdrawn to assay incorporation of [^{32}Pi] into pyrophosphate by Cerenkov

[2] Abbreviations: Bchl, bacteriochlorophyll; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PPiase, membrane-bound pyrophosphatase (EC 3.6.1.1).

radiation in the ^{3}H-channel of a Beckman scintillation counter. In our conditions no uptake of [^{32}Pi] took place when PPi was omitted from the incubation mixture. This indicates that the uptake of [^{32}Pi] occurs as a consequence of the Pi–PPi exchange reaction and not to net synthesis.

Measurement of the Precipitation of Complexes

To our knowledge there are no data on the solubility constants of complexes of PPi with the divalent cations studied here. Therefore attempts to measure precipitation of complexes in various conditions were made.

Precipitate formation was determined by assay of the light scattering of the solution at 340 nm in an Aminco DW-2a spectrophotometer by positioning the cuvettes far from the photomultiplier to enhance light-scattering effects. Measurements were made 3 min after the addition of divalent cations.

Calculation of Free Metal Ions, Ligands, and Complex Concentrations

These were calculated with a program made available to us by Dr. Jaime Mas-Oliva of this University. The program resolves the simultaneous equations that describe the multiple equilibria that exist in solution, using the $\log_{10}$ of the association constants. These values were taken from Martell and Sillen (1971). The program was run in a Digital PDP11/34 minicomputer. The program is available upon request. The value of the various constants in our exact experimental conditions have not been described. Therefore we have used values that have been determined in solutions that resemble our experimental conditions. Of particular importance to our results are the data with Zn^{2+}, and the calculations of free Zn^{2+} closely agreed with results on conductivity measurements in our incubation conditions (see Results).

Conductivity Measurements

These were made with a conductivity meter CDM3 radiometer (Copenhagen) with an appropriate microcell.

Measurement of Electrochromic Carotenoid Band Shift

The measurements were carried out in an Aminco DW-2a spectrophotometer equipped with a side illumination attachment at 530 nm maximum and 508 nm as isosbestic (Baltscheffsky, 1969a). The illumination was made through a near-infrared filter (Kodak gratten 87C), and the photomultiplier tube was protected from the stimulating near infrared light by a blue filter (Corning No. 9782).

Results

Effect of pH on the Pi–PPi Exchange and Hydrolytic Reaction

It has been found (Celis *et al.*, 1985) that in the presence of 0.67 mM PPi, pyrophosphate hydrolysis as a function of Mg^{2+} at pH 8.0 is maximal at 1 mM Mg^{2+}, while under similar conditions the Pi–PPi exchange reaction reaches its highest rate at 10 mM Mg^{2+}. When the Pi–PPi exchange reaction and pyrophosphate hydrolysis were assayed at 10 mM Mg^{2+} (optimal concentration for exchange) and various pH, the results of Fig. 1 were obtained. Hydrolysis was optimal between pH 6–7, while the Pi–PPi exchange was low at this pH and reached its highest value at pH 7.5; at higher pH the exchange reaction showed a decline. At the concentration of chromatophores employed, approximately 50–60% of the pyrophosphate introduced into the mixture is hydrolyzed in a 5-min period. This coincides with the highest uptake of ^{32}Pi into PPi (Celis *et al.*, 1985). A plot of the amount of pyrophosphate hydrolyzed to that which underwent exchange (Fig. 1B) indicates that there is a marked change in the kinetic properties of the enzyme. At pH 5.0 the ratio is around 100, while it is about 20 at pH 7.5 and 8.0. Thus it would seem in agreement with previous data (Randahl, 1979; Celis *et al.*, 1985) that the kinetic characteristics of the enzyme are modulated by Mg^{2+} ions and by the pH in the incubation mixture.

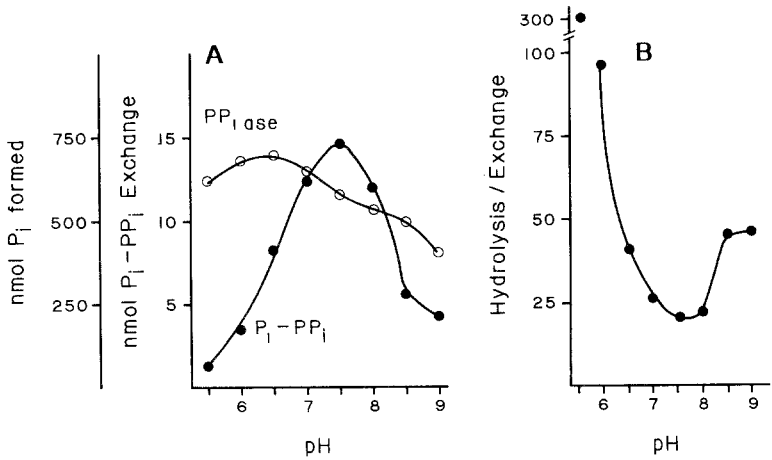

Fig. 1. Effect of pH on the hydrolytic activity and Pi–PPi exchange reactions of chromatophores. In panel A the hydrolysis of PPi (○) was measured under the following conditions: 50 mM Tris adjusted with maleic acid to the pH's indicated in the abscissa, 0.67 mM sodium pyrophosphate, 10 mM $MgCl_2$, and 100 μg Bchl equivalent to 1.65 mg protein. The Pi–PPi exchange reaction (●) was measured under the same conditions except that the media also contained 6.7 mM of Pi–Tris adjusted to the pH indicated in the abscissa (10^6 cpm). The incubation time was 5 min at 25°C. Panel B shows the hydrolysis/exchange ratio at the various pH.

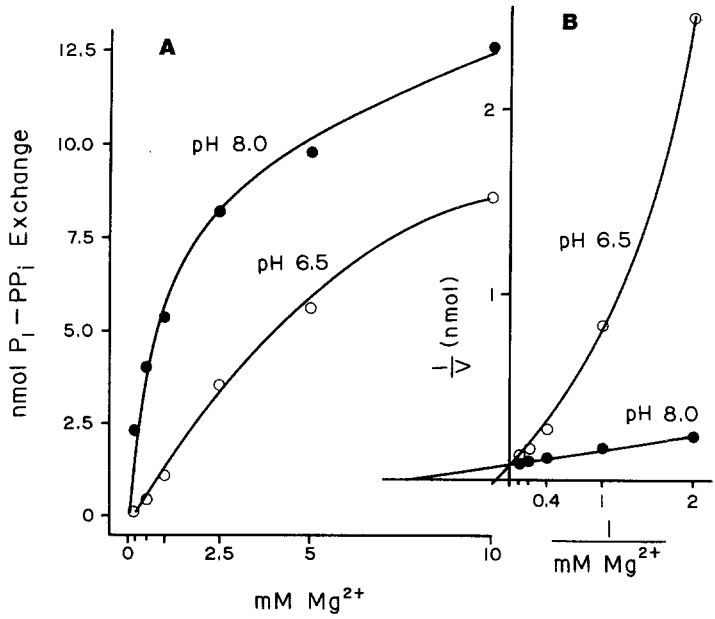

Fig. 2. Effect of $MgCl_2$ on the Pi–PPi exchange reaction at pH 6.5 and 8.0. In panel A the activity of the Pi–PPi exchange reaction is plotted against the concentration of $MgCl_2$ added. The conditions of reaction were the same as in panel A of Fig. 1. Panel B shows the Lineweaver–Burk plot of the data in panel A. Under these conditions about 60% of the PPi has been hydrolyzed.

In an attempt to determine the mechanism through which the kinetic characteristics of the enzyme are modulated by pH, the experiment of Figs. 2 and 3 were carried out. The exchange reaction was higher at pH 8.0 than at pH 6.5 at all concentrations of Mg^{2+} added (Fig. 2A). This finding would seem to indicate that Mg^{2+} is required for optimal rates of the Pi–PPi exchange reaction, but that its function is impaired by low pH even though hydrolysis is higher at pH 6.5 than at pH 8.0. In reference to previous experiments (Celis *et al.*, 1985), this could imply that Mg^{2+} binding to the enzyme is facilitated as the pH is raised. A Lineweaver–Burk plot of the exchange reaction at various concentrations of phosphate added at pH 6.5 and 8.0 (Fig. 3) showed that pH affects mainly the K_m of the reaction without appreciable change in the maximal velocity. The K_m for phosphate at pH 6.5 and 8.0 was 40 and 6.6 mM respectively. (The values shown in the figure have been corrected for dilution of ^{32}Pi that occurs during PPi hydrolysis.)

Effect of Divalent Metal Ions on the Pi–PPi Exchange Reaction

Previous findings (Celis *et al.*, 1985) indicate that the true substrate of the Pi–PPi exchange reaction is the Mg–Pi complex. In the experiment shown

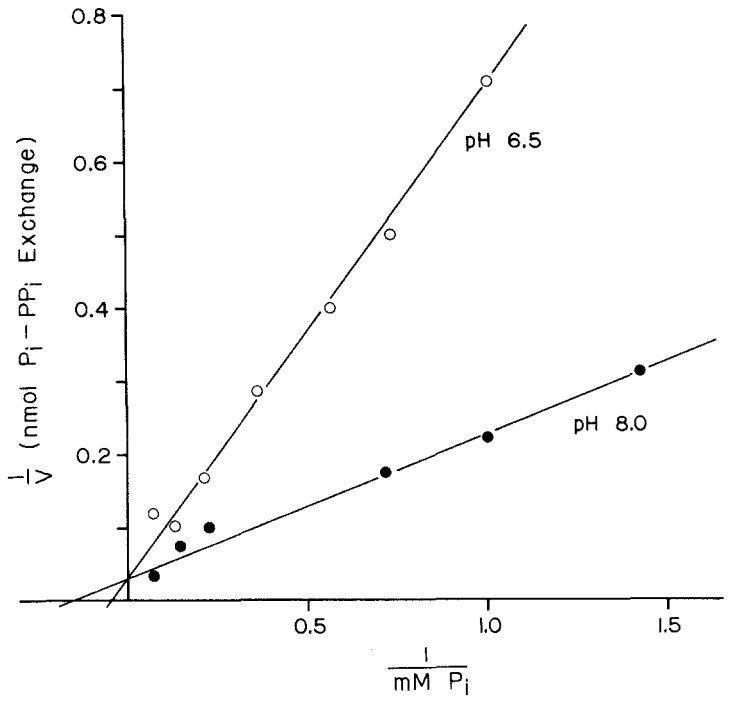

Fig. 3. Effect of different concentrations of Pi on the kinetics of the Pi–PPi exchange reaction. A Lineweaver–Burk plot of the Pi–PPi exchange reaction as a function of phosphate concentration at the indicated pH. The conditions were the same as in Fig. 1. The data of this experiment were corrected for the dilution effect of PPi hydrolysis. This was 60% at pH 6.5 and 30% at pH 8.

in Fig. 4 the effect of several divalent cations on the exchange reaction was explored at pH 8.0, at a concentration of 1 mM. At a concentration of 1 mM of the metal ions studied, precipitation was monitored nephelometrically (see Experimental), and no precipitation was detected. In agreement with previous findings (Keister and Raveed, 1974; Celis *et al.*, 1985) Mg^{2+} induced exchange of ^{32}Pi into PPi. After a maximal level was reached, a progressive diminution of labeled pyrophosphate was observed due to hydrolysis of pyrophosphate (Fig. 4); Mn^{2+} and Co^{2+} support the Pi–PPi exchange reaction, but it is noteworthy that with these cations, the exchange of ^{32}Pi is slower, i.e., approximately 50% of that observed with Mg^{2+}. Keister and Raveed (1974) did not find activity with Co^{2+} and Mn^{2+} under the same conditions that the Pi–PPi exchange was measured in this work; this is probably due to the slow reaction with Mn^{2+} and Co^{2+}, although Keister and Raveed did not report the time of measurement of the exchange with these cations. Also in Fig. 4, it is shown that Ca^{2+}, Zn^{2+}, and other divalent cations support a very limited exchange of ^{32}Pi into PPi. The effect of Zn^{2+}

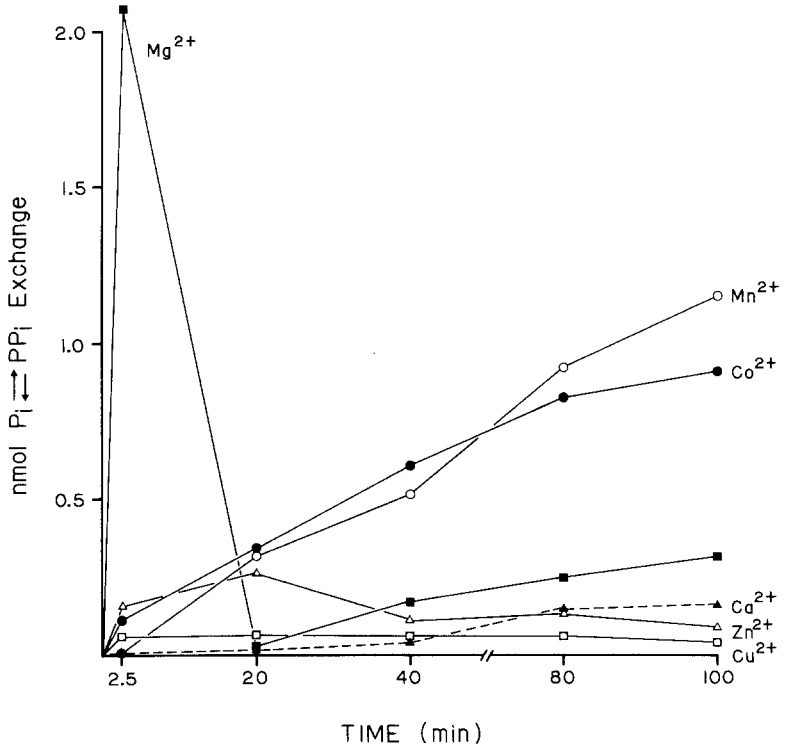

Fig. 4. Effect of divalent cations on the Pi–PPi exchange reaction of membrane-bound PPiase. The incubation media contained 50 mM Tris-maleate at pH 8.0, 0.67 mM sodium pyrophosphate, 1 mM Pi-Tris at pH 8.0 (10^6 cpm), 1 mM of the indicated divalent cation, and 100 μg Bchl. At 1 mM of Co^{2+} or Mn^{2+}, 20% of the PPi was hydrolyzed in 30 min.

was studied in concentrations that ranged between 0.1 and 10 mM and no important Pi–PPi exchange was observed. The Pi–PPi exchange reaction depends on the existence of electrochemical H^+ gradients (Keister and Raveed, 1974). thus it was considered that the presence of Zn^{2+} in which the exchange reaction was very low (Fig. 4) could lead to an impairment in the formation of the gradient. However, Zn^{2+}-dependent pyrophosphate hydrolysis induced an electrochromical carotenoid band shift of a magnitude slightly higher than that observed for Mg^{2+} (Fig. 5). The signal observed with Zn^{2+} was abolished by CCCP. This suggests that the low Pi–PPi exchange observed in the presence of Zn^{2+} is due to an effect on membrane-bound pyrophosphatase.

Effect of Divalent Cations on the Hydrolytic Reaction of the Membrane-Bound Pyrophosphatase

In previous studies the action of divalent cations on different functions that depend on PPi hydrolysis was tested at a single concentration

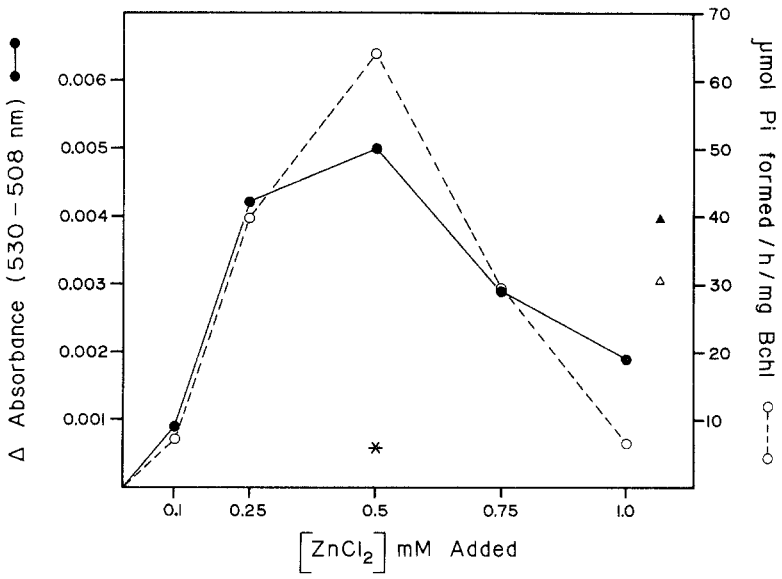

Fig. 5. Comparison between the electrochemical potential and the hydrolytic activity in the presence of different concentrations of Zn^{2+} in chromatophores. The experiments were conducted as follows: In a spectrophotometer cuvette was added 2.5 ml of 50 mM Tris-maleate, pH 6.5, and 36.2 μg of Bchl of chromatophores. In a double-beam spectrophotometer the carotenoid band shift was measured at wavelengths of 530–508 nm. The reaction was initiated with 0.67 mM of PPi, and the change in absorbance was recorded. After 2.5 min an aliquot was withdrawn, the reaction was stopped with 8.5% of TCA (final concentration), and the liberated Pi was measured as in Experimental. The results of the extent of the electrochromical carotenoid band shift (●) and the PPi hydrolysis (○) were plotted in the figure against $ZnCl_2$. (*) 40 μM of CCCP was added prior to addition of PPi at the indicated concentration of $ZnCl_2$. (△) Extent of carotenoid band shift produced by the addition of 10 mM $MgCl_2$ instead of $ZnCl_2$. (▲) amount of PPi hydrolyzed by 10 mM $MgCl_2$ instead of $ZnCl_2$. The carotenoid band shift was corrected for artifact produced by dilution of addition of PPi. The carotenoid band shift was checked by illumination of the preparation in the absence of PPi and divalent cations.

(Baltscheffsky, 1969b; Keister and Minton, 1971; Nishikawa *et al.*, 1973), and it was found that certain divalent cations (Co^{2+}, Zn^{2+}, and Mn^{2+}) substituted poorly for Mg^{2+}. Therefore, the effect of various cations on pyrophosphate hydrolysis at various concentrations at pH 6.5 was explored. Zn^{2+} at low concentration supports hydrolysis of PPi at a rate comparable to that observed with a high concentration of Mg^{2+}. Co^{2+} supports hydrolysis but at a rate which is about 50% of that attained with Mg^{2+}. Other cations (Ca^{2+}, Cu^{2+}, Fe^{2+}) do not produce appreciable hydrolysis of PPi at any of the concentrations tested. It is interesting to note that there is a definite concentration for Zn^{2+} and Co^{2+} at which maximal rates of hydrolysis are observed; the hydrolytic activity decreases above and below this concentration. At pH 8.0 Zn^{2+} at concentrations of 0.1–10 mM supports hydrolysis of PPi (data not shown), but at a level of 10% of that observed at pH 6.5.

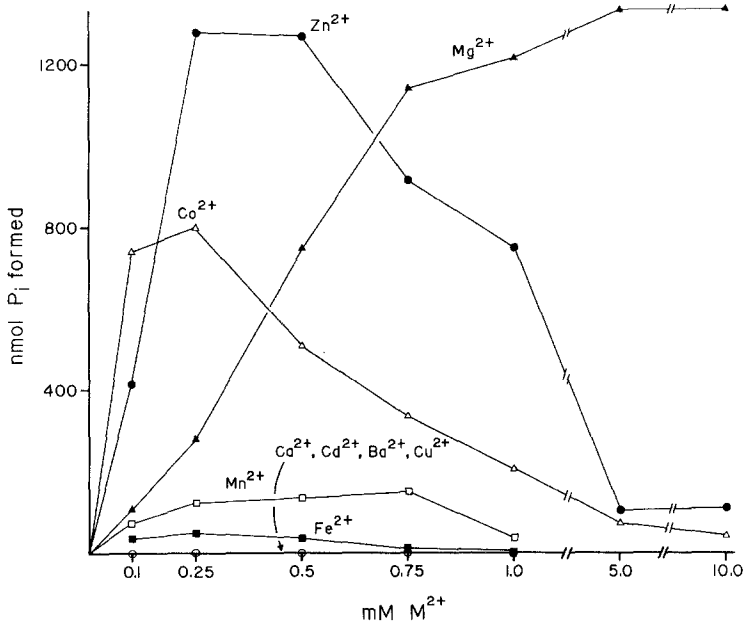

Fig. 6. Effect of different concentrations of divalent cations on the hydrolytic reaction of membrane-bound PPiase. The experimental condition were as follows: 50 mM Tris-maleate, pH 6.5, and 0.67 mM sodium pyrophosphate; the indicated concentration of divalent cations were added 15 sec before initiation of the reaction with 100 μg Bchl chromatophores. The incubation time was 5 min at 25°C.

The role of Zn^{2+} and Ca^{2+} on PPi hydrolysis was studied in more detail. Zn^{2+} is apparently the substrate for hydrolysis by complexing with PPi (see above), but at high concentrations it lowers the rate of hydrolysis. Ca^{2+} does not act as the substrate for hydrolysis at any concentration.

With respect to the biphasic action of Zn^{2+} on the membrane-bound pyrophosphatase, several possibilities arise: (a) The complex Zn–PPi is the substrate of the hydrolysis; (b) free Zn^{2+} is an inhibitor; and (c) the inhibitory species is the complex Zn–PPi–Zn. To differentiate between these possibilities, the hydrolytic activity, the calculated concentrations of the Zn–PPi complex, and free Zn^{2+} were plotted against the concentration of $ZnCl_2$ added to an incubation mixture that contained chromatophores and pyrophosphate at 0.67 mM (Fig. 7). Until equimolecular concentration of Zn^{2+} and PPi are reached (0.67 mM), practically all the added Zn^{2+} complexes with PPi due to its high association constant with PPi (Martell and Sillen, 1971). The hydrolytic activity at these concentrations of Zn–PPi complex is maximal. However, as the free Zn^{2+} concentration rises, the hydrolytic activity decreases. This suggests that free Zn^{2+} is an inhibitory species. Figure 7 also

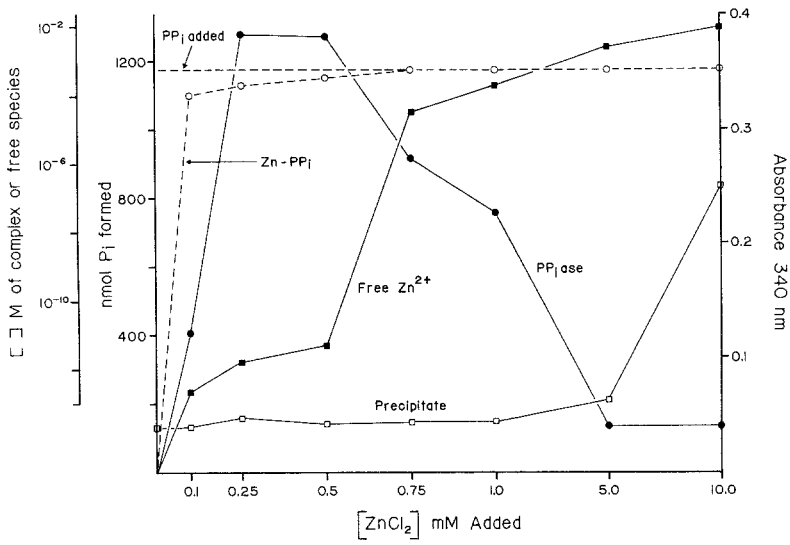

Fig. 7. Dependence of the hydrolytic reaction of membrane-bound PPiase on Zn–PPi and free Zn^{2+} concentrations. The concentration of added $ZnCl_2$ in the incubation mixture (conditions as in Fig. 6) is plotted against the hydrolytic activity (●). The calculated concentrations of Zn–PPi (○) and free Zn^{2+} (■) and the formation of precipitate (□) measured as absorbance at 340 nm under the same conditions as the hydrolytic reaction are also plotted.

shows that there is no precipitation of Zn–PPi until very high concentrations of $ZnCl_2$ are added (10 mM). This could explain the inhibition at these high concentrations, but it is important to note that at 5 mM $ZnCl_2$ precipitation of Zn PPi occurs, but only after 5–7 min of incubation has taken place. For this reason the experiments were made by adding the divalent cation at the incubation mixture 15 sec before the addition of chromatophores, and the time of incubation limited to 3 min.

With the analysis employed, it is not possible to know if the complex Zn–PPi–Zn forms at high concentration of $ZnCl_2$, since the association constant for the formation of this particular complex has not been reported. Accordingly, the conductivity experiments shown in Fig. 8 were carried out. As the concentration of $ZnCl_2$ in the mixture is increased, a direct increase in conductivity takes place. In the presence of 0.67 mM PPi an initial higher conductivity is obtained without $ZnCl_2$; when $ZnCl_2$ is added, the conductivity does not rise until a concentration of $ZnCl_2$ higher than 0.75 mM is attained. Apparently below this concentration Zn^{2+} is chelated by PPi. Above the aforementioned concentration of $ZnCl_2$, conductivity increases in proportion to added $ZnCl_2$. This latter increase in conductivity is not parallel to that observed in the absence of PPi, which suggests that $2[Zn^{2+}]$ complex with pyrophosphate. This indicates that the Zn^{2+}-induced

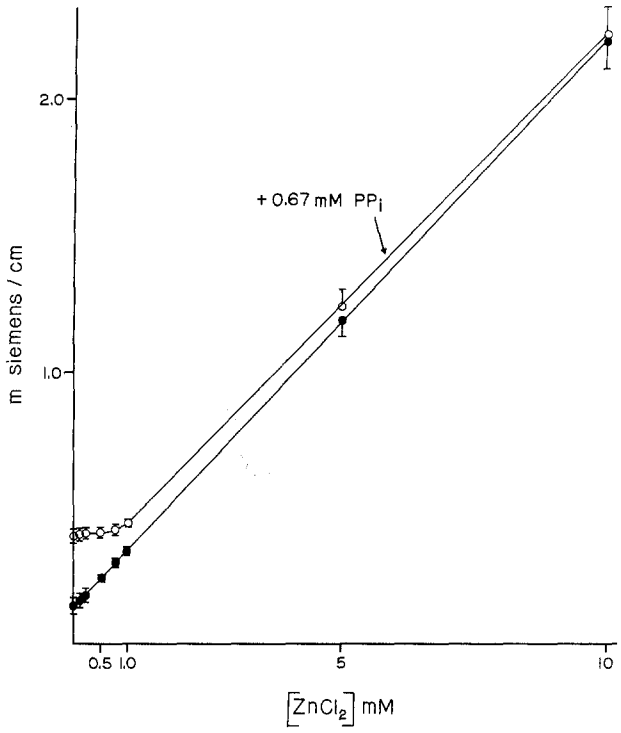

Fig. 8. Conductivity changes produced by $ZnCl_2$ in the presence of PPi. The mixture contained 2.5 mM Tris-maleate, pH 6.5, and the indicated concentrations of $ZnCl_2$. In the upper trace (O) the mixture contained 0.67 mM of sodium pyrophosphate.

inhibition of PPi hydrolysis is due to free Zn^{2+} or to very low concentrations of 2ZnPPi.

In the case of Co^{2+}, which behaves similarly to Zn^{2+} in the sense that low concentrations of Co^{2+} produce a maximum of activity although in lower magnitudes than that obtained with Zn^{2+} and at higher concentrations of Co^{2+}, an inhibition of activity was obtained (Fig. 6); analysis of the Co–PPi complex and free Co^{2+} also suggests that either free Co^{2+} or low concentrations of 2CoPPi induce inhibition of hydrolysis (data not shown).

In the case of Ca^{2+}, which does not support hydrolysis, several possibilities also arise: (a) Ca^{2+} forms complexes with PPi, but these are not substrates, (b) free Ca^{2+} is inhibitory; (c) PP^{4-} is the inhibitory species. To differentiate between these possibilities, the analysis in Fig. 9 was made. Ca^{2+} can complex with PPi in significant amounts; however, the concentrations of these complexes are lower than those obtained with Mg^{2+},

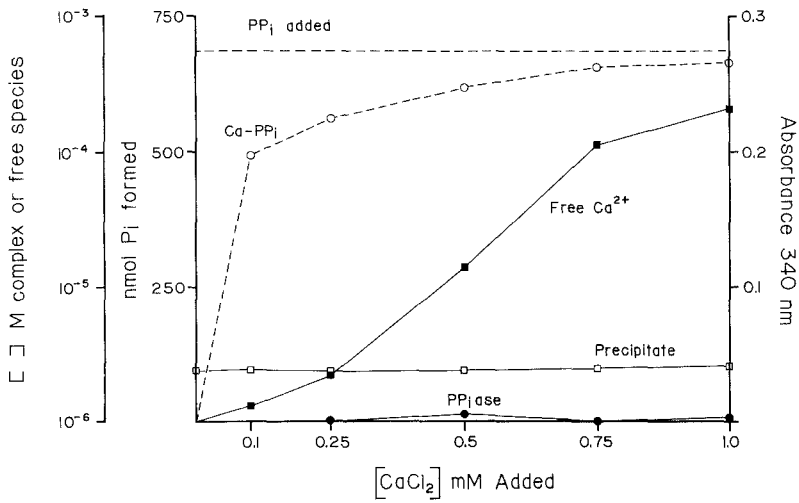

Fig. 9. Effect of the concentration of Ca–PPi complex and free Ca^{2+} on the hydrolysis of PPi by the membrane-bound PPiase. The experimental conditions for measurement of PPi hydrolysis were the same as Fig. 6. The appropriate association constant for Ca^{2+} was used for calculations of the complex and the free species.

due to a lower association constant. Thus, at all concentrations of $CaCl_2$ added, a substantial amount of free Ca^{2+} remains in the mixture. This suggests that the complex Ca–PPi is not recognized by the enzyme, and that free Ca^{2+}, Ca–PPi, or the remaining PPi^{4-} are the inhibitory species.

To decide between the latter possibilities, hydrolysis was measured at pH 8.0 in the presence of 10 mM $MgCl_2$ and 0.67 mM PPi which should provide enough substrate for the functioning of the enzyme; in the same mixture divalent cations were included at increasing concentrations. An inhibitory effect of hydrolysis was observed with the different divalent cations, but this was of a different magnitude. Zn^{2+} and Mn^{2+} are good inhibitors (80–95%), Ca^{2+} and Co^{2+} inhibit around 60%, and Cu^{2+} and Fe^{2+} inhibit between 20–40% (Fig. 10). The analysis of the experiment for the case of Zn^{2+} is shown in Fig. 11; Zn^{2+} displaces Mg^{2+} from the Mg–PPi complex and forms Zn–PPi which, as shown before (Figs. 6 and 7), is also an effective substrate for the enzyme. However, the activity is inhibited, which indicates that the inhibitor of the reaction is free Zn^{2+}. Ca^{2+} does not displace Mg^{2+} from the Mg–PPi complex (Fig. 12), but nevertheless it inhibits PPiase activity through a process that correlates with the concentrations of free Ca^{2+} and Ca–PPi complex. Cu^{2+}, which has an association constant similar to that of Mg^{2+}, forms complexes with PPi, but this complex is not recognized by the enzyme; moreover, it does not inhibit the hydrolytic activity (Fig. 10).

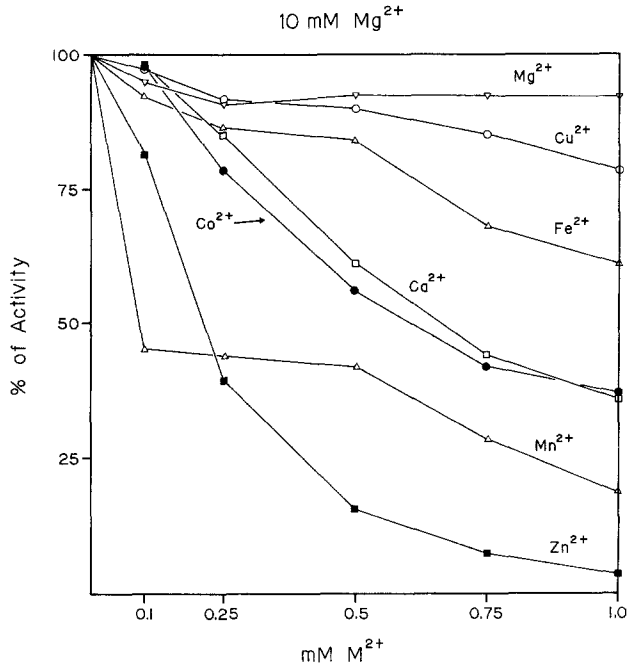

Fig. 10. Effect of different concentrations of divalent cations on the hydrolysis of the membrane-bound PPiase at high $MgCl_2$ concentration. The experimental conditions were 50 mM Tris-Cl, pH 8.0, 0.67 mM PPi–Na, pH 8.0, 10 mM $MgCl_2$, 100 μg Bchl of chromatophores, and the indicated concentrations of divalent cations. The incubation time was 5 min at 30°C.

In the conditions of the experiments reported in Figs. 8–12 the observed inhibition cannot be attributed to the presence of PP^{4-} since at the concentrations at which free divalent cations inhibit the hydrolytic reaction, all the PPi is in the form of complexes with either Mg^{2+} or Zn^{2+}.

Discussion

The results of this work indicate that the kinetic characteristics of the membrane-bound H^+-pyrophosphatase of *Rhodospirillum rubrum* are modulated by H^+ and divalent cations. The medium pH affects in different form the hydrolytic reaction and the Pi–PPi exchange reaction. The K_m for Mg^{2+} in the Pi–PPi exchange reaction is significantly lowered by raising the pH. This suggests, as proposed previously (Celis *et al.*, 1985), that the enzyme has a site for free Mg^{2+}, and the present data suggest that the association of Mg^{2+} for this site is favored by alkaline pH.

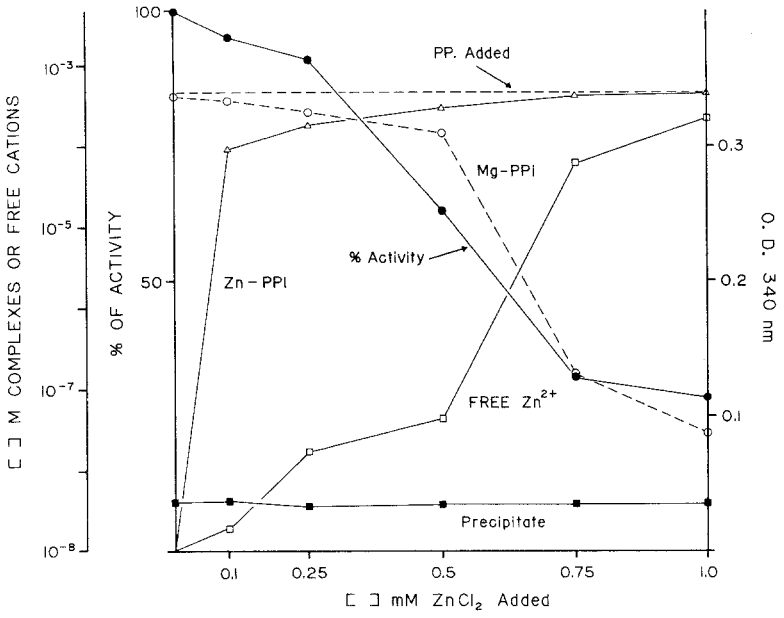

Fig. 11. Dependence of the hydrolytic reaction of membrane-bound PPiase on the concentrations of Zn–PPi complex and free Zn^{2+} at high concentrations of Mg^{2+}. The conditions were as in Fig. 10. Hydrolytic activity, % (●), Mg–PPi complex (○), Zn–PPi complex (△), free Zn^{2+} (□), precipitate (■), and PPi added (– – –) were plotted against concentration of $ZnCl_2$ added.

In addition, the influence of divalent cations other than Mg^{2+} has been studied for their effect on both reactions, as substitutes for Mg^{2+} in complex with PPi or Pi, and in their free form as inhibitors or activators.

It was found that only Mn^{2+} and Co^{2+} substitute, although only partially, for Mg^{2+} in the Pi–PPi exchange reaction.

The complex PPi–metal formed with different divalent cations was studied as the substrate in the hydrolytic reaction. Zn^{2+} at low concentrations is an excellent substrate in substitution for Mg^{2+}, while Co^{2+} and Mn^{2+} substitute for Mg^{2+} only partially. Although there is a similarity with yeast and *E. coli* pyrophosphatase (Butler and Sperow, 1977; Josse, 1966) where Zn^{2+} also supports PPi hydrolysis, this effect of Zn^{2+} has not been noted previously in the membrane-bound PPiase of *Rs. rubrum*. The cytoplasmic pyrophosphatase of *Rs. rubrum* (Klemme and Gest, 1971; Klemme *et al.*, 1971) is stabilized by Zn^{2+}, but Zn^{2+} alone is not a substrate in complex with PPi. This suggests that the complex Zn–PPi is recognized as a substrate for hydrolysis by the membrane-bound H^+-pyrophosphatase, but not by the cytoplasmic enzyme. In this sense it is interesting to note that the complexes of Cu–PPi and Ca–PPi can be formed at about the same concentration

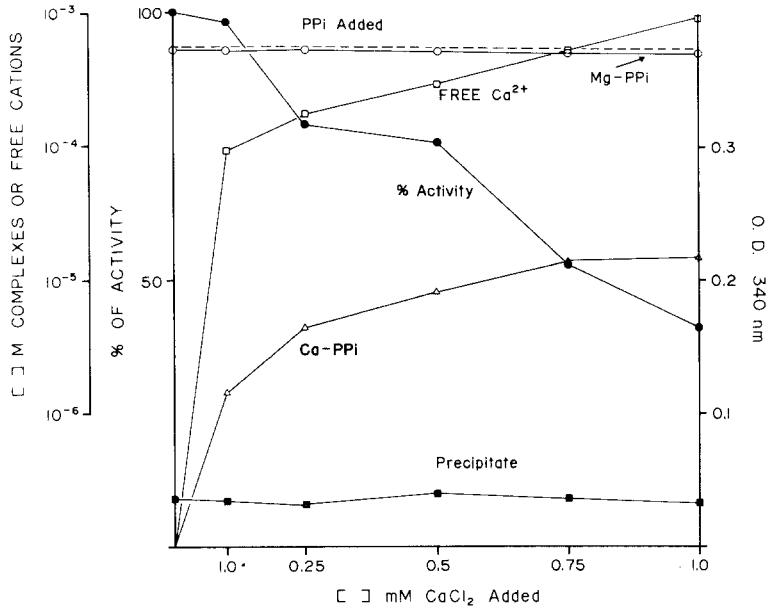

Fig. 12. Dependence of the hydrolytic reaction of membrane-bound PPiase on the concentration of Ca–PPi and Free Ca^{2+} at high concentrations of $MgCl_2$. The experimental conditions were as in Fig. 10. Activity, % (●), Ca–PPi (△), free Ca^{2+} (□), Mg–PPi complex (○), precipitate (■), and PPi added (---) were plotted against the concentration of $CaCl_2$ added.

as that for the Mg–PPi complex, but they are not recognized as substrates for hydrolysis by the particulate pyrophosphatase.

At high concentrations the Zn^{2+}, Co^{2+}, and Mn^{2+} do not function as substrates in the hydrolytic reaction, but instead induce inhibition of hydrolysis. It is likely that the inhibition is due to an effect of the free species of these divalent cations on the enzyme. However, the possibility that the complex Zn–PPi–Zn is the true inhibitor cannot be ruled out. There is still a possibility that the influence of pH and metal ions could represent indirect influences on catalytic activity as a result of primary influence on the membrane or with a component interfaced between the enzyme and the membrane.

Studies in yeast cytoplasmic PPiase (Moe *et al.*, 1979) show that the complex Zn–PPi can act as substrate, but in contrast with the membrane-bound PPiase of *Rs. rubrum*, the free Zn^{2+} acts as an activator and Co^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+} act as inhibitors through a competition with Mg^{2+} to form the complex with PPi (Moe *et al.*, 1985). It has also been demonstrated in yeast PPiase (Cooperman and Chiu, 1973; Knight *et al.*, 1984) that there are metal-ion binding sites; this kind of experiment is desirable to make in the purified membrane-bound PPiase of *Rs. rubrum*.

It is interesting that in the H^+-ATPase of mitochondria, high concentrations of free Mg^{2+} induce an increase in the ratio of ATP hydrolyzed to that which undergoes exchange (Senior, 1981), indicating that the H^+-ATPase has two binding sites for Mg^{2+}. Also Younis *et al.* (1983) reported a direct measurement of a binding site for Mg^{2+} in the H^+-ATPase of chloroplasts which is involved in the processes of energy transduction. Accordingly we propose that a general feature of the H^+-dependent energy-transducing enzymes that utilize the electrochemical proton gradient for the synthesis of pyrophosphate bonds have a requirement for divalent metal ions complexed with ATP or PPi as substrate, as shown in this work, in the particulate pyrophosphatase, when the divalent cations are in their free form they induce drastic kinetic changes in the enzyme. The role of this effect on the mechanism of energy transduction requires further studies.

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References

Baccarini-Melandri, A., and Melandri, B. A. (1978). In *The Photosynthetic Bacteria* (Clayton, R. K., and Sistrom, W. R., eds.), Plenum Press, New York and London, pp. 615–628.
Baltscheffsky, M. (1969a). *Arch. Biochem. Biophys.* **130**, 646–652.
Baltscheffsky, M. (1969b). *Arch. Biochem. Biophys.* **133**, 46–53.
Baltscheffsky, M. (1978). In *The Photosynthetic Bacteria* (Clayton, R. K., and Sistrom, W. R., eds.), Plenum Press, New York and London, pp. 598–613.
Butler, L. G., and Sperow, J. W. (1977). *Bioinorg. Chem.* **7**, 141–150.
Celis, H., Romero, I., and Gómez-Puyou, A. (1985). *Arch. Biochem. Biophys.* **236**, 766–774.
Clayton, R. W. (1963). *Biochem. Biophys. Acta* **75**, 312–323.
Cohen-Bazire, G., Sistrom, W. R., and Stainer, R. Y. (1957). *J. Cell. Comp. Physiol.* **49**, 25–68.
Cooperman, B. S., and Chiu, N. Y. (1973). *Biochemistry* **12**, 1670–1676.
de Meis, L., Martins O. B., and Alves, E. W. (1980). *Biochemistry* **19**, 4252–4261.
de Meis, L., Tuena de Gómez Puyou, M., and Gómez-Puyou, A. (1983). *Biochem. Biophys. Res. Commun.* **111**, 274–279.
Fisher, R. R., and Guillory, R. J. (1969). *FEBS Lett.* **3**, 27–30.
Fiske, C. A., and Subbarow, Y. (1925). *J. Biol. Chem.* **177**, 751–766.
Josse, J. (1966). *J. Biol. Chem.* **241**, 1948–1957.
Keister, D. L., and Minton, N. J. (1971). *Arch. Biochem. Biophys.* **147**, 330–338.
Keister, D. L., and Raveed, N. J. (1974). *J. Biol. Chem.* **249**, 6454–6458.
Klemme, J. H., and Gest, H. (1971). *Eur. J. Biochem.* **22**, 529–537.
Klemme, J. H., Klemme, B., and Gest, H. (1971). *J. Bacteriol.* **108**, 1122–1128.
Knight, W. B., Dunaway-Mariano, D., Ransom, S. C., and Villafranca, J. J. (1984). *J. Biol. Chem.* **259**, 2886–2895.
Lahti, R. (1983). *Microbiol. Rev.* **47**, 169–179.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J., (1951). *J. Biol. Chem.* **193**, 265–275

Martell, A., and Sillen, L. G. (1971). *Stability Constants of Metal-Ion Complexes*, Supplement No. 1, Special Publication No. 25, The Chemical Society, London.

Moe, O. A., Pham, S., Dang, T., and Styler, L. (1979). *Arch. Biochem. Biophys.* **196**, 73–78.

Moe, O. A., Pham, S., Selinsky, B., and Dang, T. (1985). *Biochem. Biophys. Acta* **827**, 207–214.

Nishikawa, K., Hosoi, K., Suzuki, J., Yoshimura, S., and Horio, T. (1973). *J. Biochem. (Tokyo)* **73**, 537–553.

Randahl, H. (1979). *Eur. J. Biochem.* **102**, 251–256.

Senior, A. E. (1981). *J. Biol. Chem.* **256**, 4763–4767.

Younis, H. M., Weber, G., and Boyer, I. S. (1983). *Biochemistry* **22**, 2505–2511.