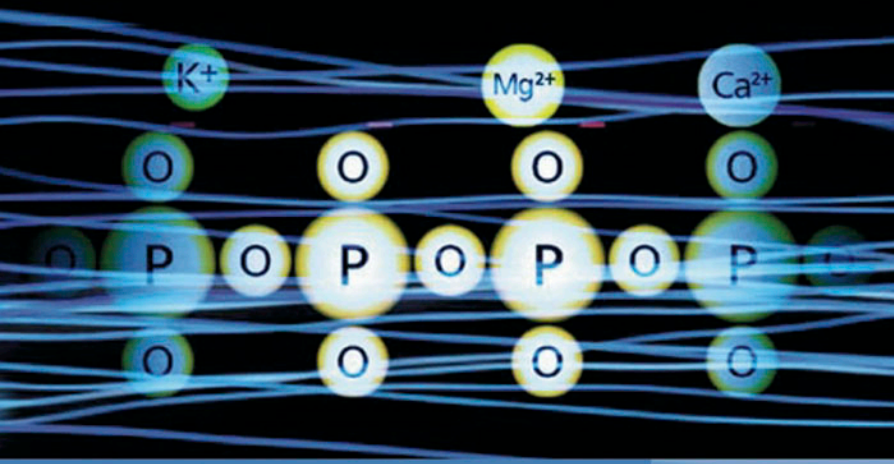
I S Kulaev
V M Vagabov
T V Kulakovskaya

THE BIOCHEMISTRY OF INORGANIC POLYPHOSPHATES

SECOND EDITION



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The Biochemistry of Inorganic Polyphosphates

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Second Edition

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in which at least two trees are planted for each one used for paper production.

To the respected memory of
Andrei Nikolaevich Belozersky,
an outstanding scientist,
teacher and man

CONTENTS

FOREWORD TO THE FIRST EDITION

The presence of high-molecular-weight polyphosphates in many microorganisms such as yeast, fungi and bacteria, has been known for a long time, but studies on the biochemical functions of these substances are of much more recent origin and still in a rudimentary state. Professor Igor S. Kulaev, one of the most eminent pupils of the late Professor Andrei N. Belozersky, who was an internationally known authority on nucleic acids, has dedicated in his laboratory at the University of Moscow, in conjunction with a large team of collaborators, intensive studies over many years to the somewhat neglected subject of the biochemical functions of polyphosphates. His group has studied the enzymes involved in the synthesis and breakdown of these compounds. There is no doubt that in some cases they can take over the phosphorylation functions of adenosine 5′-triphosphate (ATP), as the phosphate residues are linked together to form energy-rich phosphate bonds.

Professor Kulaev has taken the not inconsiderable trouble of collecting and critically reviewing the large amount of literature now available on the subject in one monograph, at present the only one in existence on this important field of study. With this onerous and time-consuming task, he has rendered a signal service to the international biochemical community, which owes him a large debt of gratitude for this work.

Professor Kulaev has shown that the study of the biochemical functions of the high-molecular-weight polyphosphates is still a very active field of research, offering a great challenge to the enterprising young biochemist in which many discoveries of general importance can still be made.

Professor Emeritus Ernst Chain, FRS
Imperial College of Science and Technology
London
1979

PREFACE

This book is devoted to the current problems of biochemistry of inorganic polyphosphates (PolyPs), linear polymers of orthophosphoric acid, which are important regulatory biopolymers widespread in living organisms. The great progress in the field of PolyP biochemistry over the last 15 to 20 years has contributed much to the appearance of this second edition.

The topics of this text include the following:

- Data on the chemical structure and properties of condensed inorganic phosphates.
- Comparative analysis of the methods of PolyP investigation in biological materials.
- Data on PolyP distribution in living organisms.
- Localization and forms of PolyPs in prokaryotic and eukaryotic cells.
- Characteristics of the known enzymes of PolyP metabolism.
- Description of the functions of PolyPs and PolyP-dependent enzymes, in particular, such important functions as phosphate and energy reservation, sequestration and storage of cations, formation of membrane channels, involvement in cell envelope formation and function, gene activity control, regulation of enzyme activities, participation in stress response, and stationary phase adaptation.

In addition, some chapters will be devoted to such problems as the peculiarities of PolyP metabolism in different organisms, applied aspects of PolyP biochemistry, and a discussion of the possible place of inorganic PolyPs in chemical and biological evolution.

The originality of this present edition lies in a comprehensive presentation of the modern concepts of PolyP biochemistry, including a comparative description of PolyP metabolism in prokaryotes and eukaryotes, i.e. the role of these compounds in the cells of organisms at different stages of evolution, and offers a critical analysis of the methods of isolation and quantitative assessment of these compounds and methods of studying PolyP-dependent enzymes. The contemporary literature on these problems is presented to its maximal extent. The book may therefore serve as a manual for researchers in this field, and in particular, as a textbook.

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INTRODUCTION

More than one hundred years ago, L. Liberman (1890) found high-polymeric inorganic polyphosphates (PolyPs) in yeast. These compounds are linear polymers containing a few to several hundred residues of orthophosphate (P_i) linked by energy-rich phosphoanhydride bonds.

Taking into consideration their significance for all living organisms, inorganic polyphosphates may be separated into two groups, namely pyrophosphate and high-molecular-weight PolyPs, which contain three to several hundred phosphate residues in one molecule. The functions of pyrophosphate and the enzymes of its metabolism are well distinguished from those of high-molecular-weight PolyPs and to date have been studied quite thoroughly. However, the same does not apply to the high-molecular-weight PolyPs. These mysterious cell components have so far been ignored in most biochemistry manuals. At the same time, a number of reviews (Harold, 1966; Kulaev and Vagabov, 1983; Wood and Clark, 1988; Kornberg, 1995; Kulaev, 1994; Kulaev *et al*., 1999; Kornberg *et al*., 1999; Kulaev and Kulakovskaya, 2000), including the special issue of *Progress in Molecular and Subcellular Biology* (Schröder, H. B. and Müller, W. E. G. (Eds), Vol. 23, 1999), have covered many important aspects of the current research into PolyP biochemistry.

The studies of recent years have greatly changed our ideas of the PolyP function in living organisms. Previously, it was considered either as 'molecular fossil' or as only a phosphorus and energy source providing the survival of microorganisms under extreme conditions. After the obtaining of conclusive evidence that these compounds occur in representatives of all kingdoms of living organisms, including the higher animals, it became obvious that PolyPs are necessary for practically all living creatures from different stages of evolution. One would think that these compounds, in the first place, have a regulatory role, participating in metabolism correction and control on both genetic and enzymatic levels. This is why they have not disappeared in the course of evolution of living organisms on the Earth. In recent years, first of all by A. Kornberg and his co-workers (Rao and Kornberg, 1996; Kornberg *et al*., 1999), it has been established that PolyPs are directly related to the switching-over of the genetic programme characteristic of the logarithmic growth stage of bacteria to the programme of cell survival under stationary conditions – 'a life in the slow lane'.

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The discovery by R. Reusch (Reusch and Sadoff, 1988; Reusch, 1992; Reusch, 2000), which proved the involvement of PolyPs in the formation of channels across the cell membranes, extended our previous notions of the function of these compounds. Such channels formed by PolyPs and poly-β-hydroxybutyrate with Ca^{2+} are involved in the transport processes in organisms from different evolution stages.

Surely, the most important function of PolyPs in microorganisms – prokaryotes and the lower eukaryotes, which depend a lot on the changing environmental conditions – is phosphate and energy reservation. In this connection, under certain growth conditions these organisms are able to accumulate PolyPs in much greater amounts than the higher eukaryotes, the dependence of which on external factors is much less due to homeostasis, being strictly regulated by hormones.

The important achievement of recent years has become the finding of non-identical sets of enzymes of PolyP metabolism in different organelles of eukaryotic cells, obtained mainly for yeast (Kulaev and Kulakovskaya, 2000; Lichko *et al*., 2003a). This result is in favour of considerable distinctions in the physiological role of PolyPs in different compartments of eukaryotic cells.

One of the basic questions, which has only just begun to be investigated, concerns the ways of PolyP involvement in the regulation of gene expression. While there are appreciable achievements for bacterial cells in this direction, elucidation of the role of PolyPs in nuclei is still an important prospective problem for eukaryotes and particularly for the higher representatives of this kingdom.

At the present time, the significance of PolyP investigations for biochemistry in general is now clear. In particular, an effective biotechnology approach as a tool for phosphorus removal from wastewater using polyphosphate-accumulating microorganisms has been developed (Kortstee *et al*., 1994; Ohtake *et al*., 1999; Mino, 2000; Keasling *et al*., 2000). The intense attention of researchers has also been drawn to the solution of several important medical and biological problems associated with polyphosphate biochemistry. First of all, there is a question about the involvement of PolyPs in the mechanisms of pathogenesis of a number of pathogenic microorganisms and the creation of novel drugs. In the opinion of A. Kornberg (1999), one of the targets of novel antimicrobial drugs may be polyphosphate kinase – an enzyme of PolyP biosynthesis in bacteria. Studies of the participation of PolyPs and the enzymes of their metabolism in the regulation of bone tissue development also seem to be promising (Schröder *et al*., 2000).

Thus, further studies in the field of PolyP biochemistry offer great prospects, which will more than once give unexpected results for elucidating the most important regulatory mechanisms of the living cell.

1

THE CHEMICAL STRUCTURES AND PROPERTIES OF CONDENSED INORGANIC PHOSPHATES

For a proper understanding of the processes which take place in living organisms, a precise knowledge of the chemical structures of the compounds that participate in these processes is required. It is therefore deemed essential to present, even if only briefly, an account of present-day ideas of the chemical structures of condensed phosphates, hitherto often known by the long-obsolete terms 'metaphosphates' and 'hexametaphosphates'.

1.1 The Structures of Condensed Phosphates

The first mention of condensed inorganic phosphates dates back to 1816, when Berzelius showed that the vitreous product formed by the ignition of orthophosphoric acid was able to precipitate proteins (Van Wazer, 1958). Graham (1833) described a vitreous phosphate which he obtained by fusion of NaH_2PO_4. Believing that he had isolated a pure compound with the formula $NaPO_3$, Graham named this as a 'metaphosphate'. Shortly afterwards, however, Fleitmann and Hennenberg (1848), working in Liebig's laboratory, demonstrated that the 'metaphosphates' having the general formula MPO_3 (where M is hydrogen or a monovalent metal) were mixtures of closely related compounds which differed mainly in their degree of polymerization. The numerous investigations which were carried out over the next 100 years (for reviews, see: Ebel, 1951; Karbe and Jander, 1942; Teichert and Rinnmann, 1948; Topley, 1949; Van Wazer, 1958), although they provided a wealth of new

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data which shed much light on the structures and properties of this group of compounds, threw into perhaps even greater confusion both the chemical basis of the nomenclature of these compounds, and the names of the compounds themselves. This is perhaps hardly surprising, since these investigations were carried out with compounds of inadequate purity, using rather crude investigation methods. It was thanks to the work of Thilo (1950, 1955, 1956, 1959, 1962), Van Wazer (1950, 1958), Ebel (1951, 1952a–d, 1953a,b) and Boulle (1965) that the chemical structures and properties of this group of compounds were finally established, thus making it possible to bring order into their classification (Van Wazer and Griffith, 1955; Thilo and Sonntag, 1957).

According to the current classification, condensed phosphates are divided into cyclophosphates, polyphosphates and branched inorganic phosphates (or 'ultraphosphates').

1.1.1 Cyclophosphates

The true cyclophosphates (metaphosphates) have the composition which, since the time of Graham, has been incorrectly assigned to the whole group of condensed phosphates, i.e. MPO_3. These compounds are built up from cyclic anions. Only two representatives of this group have so far been investigated in detail – the cyclotriphosphate, $M_3P_3O_9$, and the cyclotetraphosphate, $M_4P_4O_{12}$, shown in Figure 1.1.

The existence of mono- and dimetaphosphates has not been demonstrated in practice, and is theoretically unlikely (Ebel, 1951; Thilo, 1959; Van Wazer, 1958). The possible presence of cyclopentaphosphates and cyclohexaphosphates in a mixture of condensed sodium phosphates was shown by Van Wazer and Karl-Kroupa (1956), followed by Thilo and Schülke (1965). In addition, more highly polymerized cyclic phosphates containing as many as 10 to 15 orthophosphoric acid residues have been observed in some samples of the condensed phosphates prepared by Van Wazer (1958). Furthermore, cyclooctaphosphate (Schülke, 1968; Palkina *et al*., 1979) and cyclododecaphosphate (Murashova and Chudinova, 1999) have been obtained in the crystalline state.

It should be pointed out that the term 'hexametaphosphate', which is frequently encountered in the literature, refers in fact to the compound known as *Graham's salt*, which

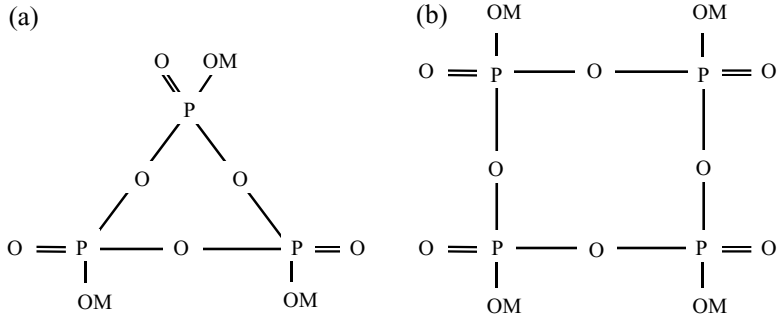

Figure 1.1 Structures of (a) cyclotriphosphate and (b) cyclotetraphosphate.

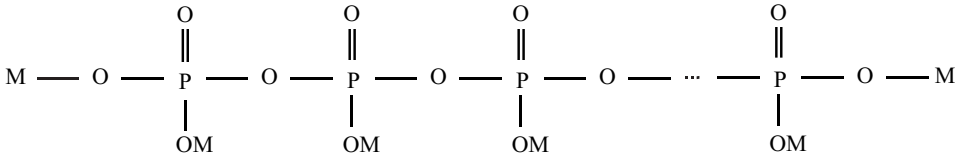

Figure 1.2 Structure of a linear condensed phosphate (PolyP), where M is H^+ or a monovalent metal cation.

is a mixture of condensed sodium phosphates containing cyclic phosphates (including cyclohexaphosphate), but which is mainly composed of highly polymerized linear polyphosphates (Van Wazer and Griffith, 1955; Thilo and Sonntag, 1957).

1.1.2 Polyphosphates

Polyphosphates (PolyPs) have the general formula $M_{(n+2)}P_nO_{(3n+1)}$. Their anions are composed of chains in which each phosphorus atom is linked to its neighbours through two oxygen atoms, thus forming a linear, unbranched structure which may be represented schematically as shown in Figure 1.2. The degree of polymerization, n, can take values from 2 to 10^6, and as the value of n increases, the composition of the polyphosphates, i.e. the cation-to-phosphorus ratio, approximates to that of the cyclophosphates, which explains the belief which prevailed until recently that 'polyphosphate' and 'metaphosphate' were equivalent terms. Polyphosphates in which $n = 2$–5 can be obtained in the pure, crystalline state (Van Wazer, 1958), but members of this series in which n has higher values have been obtained in appreciable amounts only in admixtures with each other.

In contrast to the cyclophosphates, they are designated as 'tripolyphosphates', 'tetrapolyphosphates', etc., although the mono- and dimeric compounds are still called by their old names of 'orthophosphate' (P_i) and 'pyrophosphate' (PP_i), respectively. In addition, the highly polymeric, water-insoluble potassium polyphosphate ($n \sim 2 \times 10^4$), which has a fibrous structure of the asbestos type, is still called *Kurrol's salt*. We may mention in passing that the facile preparation of Kurrol's salt (by fusion of KH_2PO_4 at 260 °C), and the ease with which it is converted into the water-soluble sodium form by means of cation-exchange materials, has led to its frequent preparation and use in chemical and biochemical work as an inorganic polyphosphate.

Even better known is Graham's salt, the vitreous sodium polyphosphate ($n \sim 10^2$) obtained by fusion of NaH_2PO_4 at 700–800 °C for several hours, followed by rapid cooling. Graham's salt is a mixture of linear polyphosphates with different chain lengths. Fractional precipitation from aqueous solution by means of acetone (Van Wazer, 1958) affords less heterogeneous fractions with different molecular weights. For example, a sample of Graham's salt, in which the chains on average have 193 phosphorus atoms (i.e. $n \sim 193$), can be separated by this method, as shown in Figure 1.3.

As can be seen from this Figure, the sample contains molecules of different sizes. The fraction of highest molecular weight has $n \sim 500$, i.e. its molecular weight is of the order of

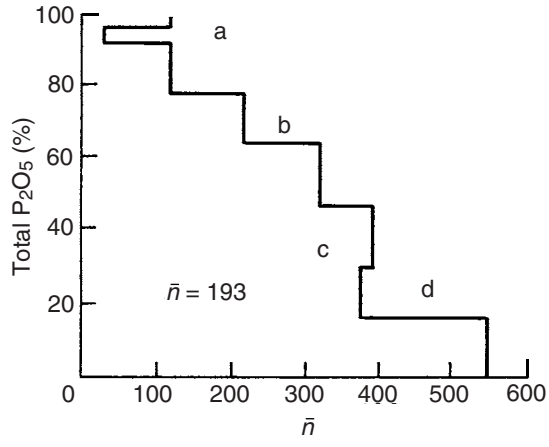

Figure 1.3 Distribution curve (by size) obtained for sodium polyphosphate molecules (Graham's salt, $n \sim 193$) after fractional precipitation, after Van Wazer (1958): (a) cyclic phosphates; (b), (c) and (d) linear polyphosphates.

40 000. It is interesting to note that the reason for the failure of Graham's salt to crystallize is that it consists of a mixture of homologous chains differing only in their lengths. Since all of the components of the homologous series of polyphosphates closely resemble each other, crystallization cannot take place with ease because molecules of different dimensions seek to displace each other on the growing crystal, thereby bringing its growth to a stop. When the chains are very long (such as is the case in Kurrol's salt), this does not occur, since the individual chains pass through many elementary cells of the crystal, and the chain length is not an important factor in determining the lattice parameters of the crystal (Van Wazer, 1958).

A second factor which determines the maximum chain lengths of the polyphosphates which are able to crystallize is the increase in polarity of the molecules which takes place as the degree of polymerization increases.

Two factors thus appear to be responsible for the failure so far to obtain linear polyphosphates containing 6–200 phosphorus atoms in a crystalline state: (1) the difficulty of crystallization from a mixture of similar compounds, and (2) the effect of polar groups on the molecules.

In addition to linear polyphosphates, Graham's salt usually contains very small amounts of cyclophosphates (see Figure 1.3). For example, a sample of Graham's salt with $n \sim$ 100–125 was shown by Van Wazer (1958) to contain 4 % of cyclotriphosphate, 2.5 % of cyclotetraphosphate, 0.8 % of cyclopentaphosphate, 0.5 % of cyclohexaphosphate, and fractional percentages of higher polymeric cyclophosphates. The compositions of two samples of Graham's salt obtained by Dirheimer (1964) are shown in Table 1.1.

The conformations of polyphosphate chains in the crystals depend on the nature of the metal cations. The period of the recurring unit changes depending on the charge, shape and electronic envelope structure of the metal cations. The structures of some crystalline

Table 1.1 Compositions of synthetic samples of Graham's salt (Dirheimer, 1964). The phosphorus contents of the poly- and cyclophosphates are expressed as a percentage of the total phosphorus contents of the compounds.

Polyphosphates and cyclophosphates	Sample 1	Sample 2
High-molecular-weight polyphosphate	68.1	75.1
Polyphosphates ($n \sim 5$–10)	17.3	13.6
Tetrapolyphosphate plus cyclotriphosphate	7.8	7.0
Tripolyphosphate	4.5	2.8
Pyrophosphate	2.3	1.5

polyphosphates with recurrence periods from 2 to 24 phosphate residues are shown in Figure 1.4.

1.1.3 Branched Inorganic Phosphates, or 'Ultraphosphates'

High-molecular-weight condensed phosphates which, unlike the linear polyphosphates, contain 'branching points', i.e. phosphorus atoms which are linked to three rather than two neighbouring phosphorus atoms, are known as branched phosphates (or 'ultraphosphates'). Such phosphates have a branched structure, a fragment of which is shown in Figure 1.5. In this type of structure, the individual polyphosphate chains are linked to form a 'network', which is the reason for the name given to this type of condensed phosphates. The existence of this group of phosphorus compounds was observed in some samples of both Kurrol's and Graham's salt, as identified by chemical methods (Van Wazer and Holst, 1950; Strauss and Smith, 1953; Strauss *et al*., 1953; Strauss and Treitler, 1955a,b; Thilo, 1956, 1959; Van Wazer, 1958). In samples of Graham's salt with very long chains (of the order of several hundred phosphorus atoms), approximately one in every thousand phosphorus atoms is a branching point (Strauss and Smith, 1953; Strauss *et al*., 1953; Strauss and Treitler, 1955a,b). The presence of branching in polyphosphate chains, or in other words, the presence of a *reticular structure*, can be detected by the decrease in the viscosity of aqueous solutions which occurs following dissolving the compounds in water (owing to the rapid hydrolysis of the lateral bonds, which are very unstable). Figure 1.6 shows how the proportion of lateral bonds in Graham's salt increases as the chain length is increased.

Although branched phosphates have not yet been found in living organisms (perhaps as a consequence of their unusually rapid hydrolysis in aqueous solution, irrespective of pH, even at room temperature), it is believed that their presence in biological materials cannot be excluded.

Information on the chemical compositions of the condensed inorganic phosphates, together with descriptions of their chemical and physico-chemical properties, can be found in several papers, reviews and monographs (Thilo, 1950, 1955, 1956, 1959; Van Wazer, 1950, 1958; Ebel, 1951; Griffith *et al*., 1973; Ohashi, 1975; Corbridge, 1980). We shall

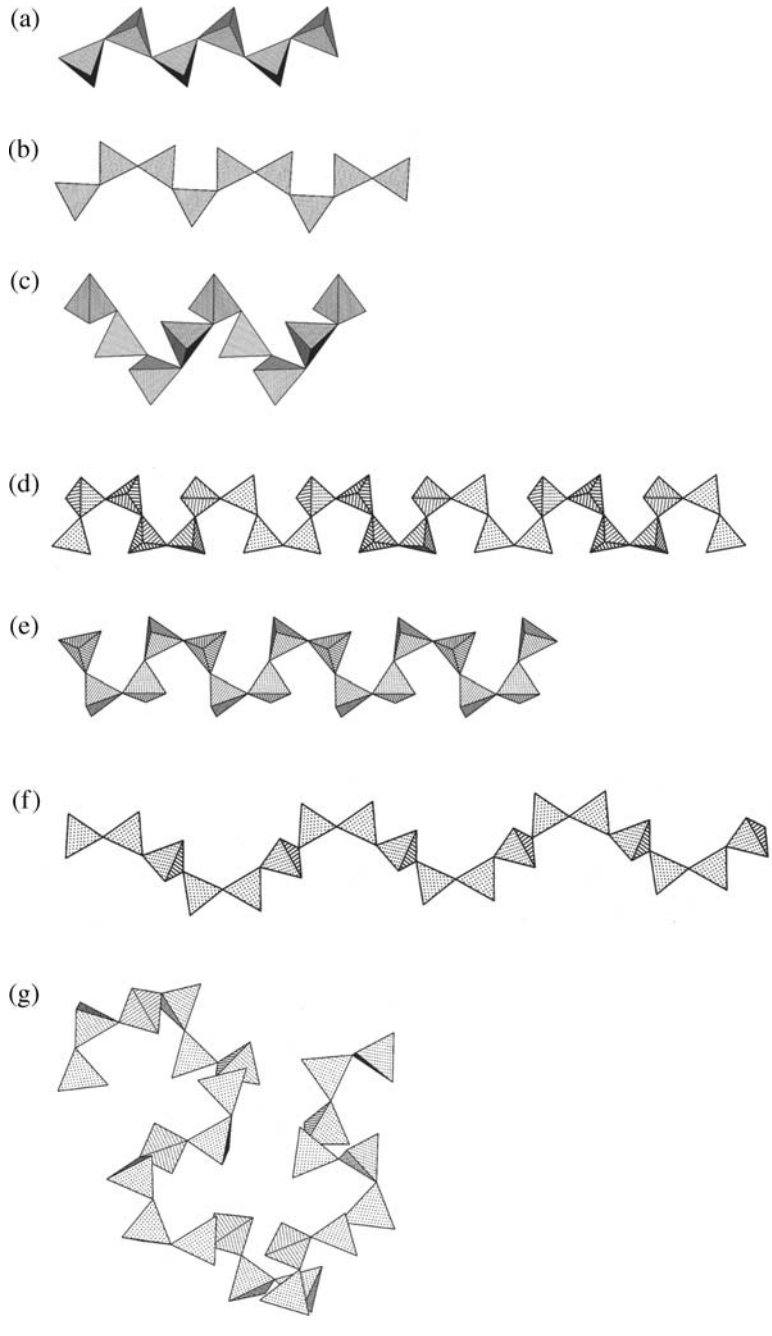

Figure 1.4 Structures of various crystalline polyphosphates: (a) $(Na_2HP_3O_9)_n$ (Jost, 1962); (b) $[Na_3H(PO_3)_4]_n$ (Jost,1968); (c) $(NaPO_3)_n$ (Immirzi and Porzio, 1982); (d) $(KPO_3)_n$ (Jost and Schulze, 1969); (e) $[Ca_2(PO_4)_3]_n$ (Schneider *et al*., 1985); (f) $[(NH_4)Cu(PO_3)_3]_n$ (Tranqui *et al*., 1969); (g) $[NaMn(PO_3)_3]_n$ (Murashova and Chudinova, 1997).

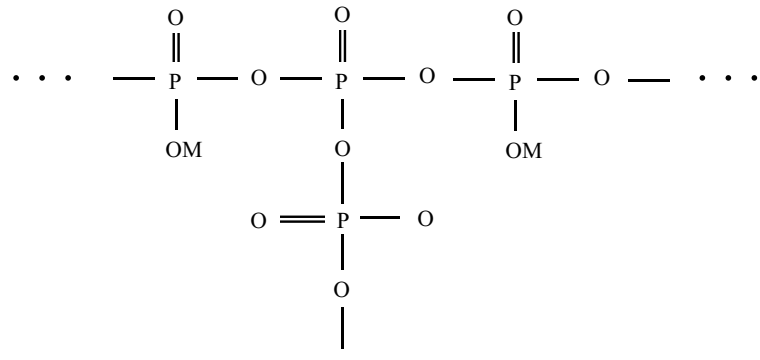

Figure 1.5 Structure of branched phosphate.

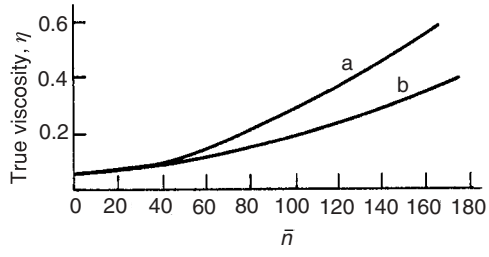

Figure 1.6 Changes in the viscosities of solutions of polyphosphates of different chain lengths on keeping for 12 h, where the abscissa represents the mean chain length as determined by end-group titration: (a) immediately after solution; (b) after keeping for 12 h (Strauss and Treitler, 1955b).

dwell here very briefly on those properties of condensed phosphates that are useful for their identification and chemical determination in living organisms.

1.2 Some Chemical Properties of Condensed Inorganic Polyphosphates

Polyphosphates are salts of acids that, in solution, contain two types of hydroxyl groups that differ in their tendency to dissociate. The terminal hydroxyl groups (two per molecule of polyphosphoric acid) are weakly acidic, whereas the intermediate hydroxyl groups, of which there are a number equal to the number of phosphorus atoms in the molecule, are strongly acidic (Van Wazer, 1958). Cyclophosphates do not contain terminal hydroxyl groups and, for this reason, the corresponding acids possess only strongly acidic groups which in solution are dissociated to approximately the same extent. Thus, titration of weakly and strongly acidic groups is a convenient means of determining whether a given condensed phosphate is a cyclo- or a polyphosphate. Moreover, this method provides a means of determining the average chain length of linear polyphosphates (Wan Wazer, 1950; Ebel, 1951; Samuelson, 1955; Langen and Liss, 1958a,b; Chernysheva *et al.*, 1971) It is interesting that this was

the method used by Samuelson (1955) in showing for the first time that Graham's salt was not a cyclophosphate – as had been believed for almost 100 years – but a mixture of linear polyphosphates.

All alkali metal salts of condensed polyphosphoric acids are soluble in water. Potassium pyrophosphate is especially soluble, with, for example, 100 g of water dissolving 187.4 g of $K_4P_2O_7$ at 25 °C, 207 g at 50 °C, and 240 g at 75 °C. Exceptions to this rule are the water-insoluble Kurrol's salt (a macromolecular crystalline potassium polyphosphate), and the compounds known as Maddrell's salts (crystalline sodium polyphosphates of very high molecular weight). Kurrol's salt is readily soluble in dilute solutions of salts containing cations of univalent metals (but not K^+), for example, 0.2 M NaCl. It is worth mentioning that Graham's salt dissolves in water only when it is stirred rapidly. Without stirring, the compound forms a glue-like mass in water. Polyphosphates of divalent metals such as Ba^{2+}, Pb^{2+} and Mg^{2+} are either completely insoluble or dissolve to only a very limited extent in aqueous solutions. The polyphosphates of certain organic bases such as guanidine are also sparingly soluble in water (Singh, 1964). Other solvents (liquid ammonia, anhydrous formic acid, and organic solvents such as ethanol and acetone) dissolve only trace amounts of sodium and ammonium polyphosphates. Low-molecular-weight polyphosphates dissolve readily in very dilute aqueous alcoholic solutions, but addition of alcohol to these solutions rapidly reduces their solubility. Figure 1.7 shows that an ethanol–water mixture containing 40 % of ethanol is a very poor solvent for both potassium pyrophosphate and potassium tripolyphosphate (1.5 g per 100 g of solution).

Condensed phosphates, other than branched phosphates, are stable in neutral aqueous solution at room temperature. The hydrolysis of the P–O–P bond in linear polyphosphates such as Graham's salt liberates energy equivalent to approximately 10 kcal/mol (Yoshida, 1955a,b; Van Wazer, 1958), i.e. the same amount of energy as is liberated in the hydrolysis of the terminal phosphoric anhydride bonds in the adenosine 5′-triphosphate (ATP) molecule. Hydrolysis of the cyclotriphosphate also liberates this same amount of energy (Meyerhof *et al*., 1953).

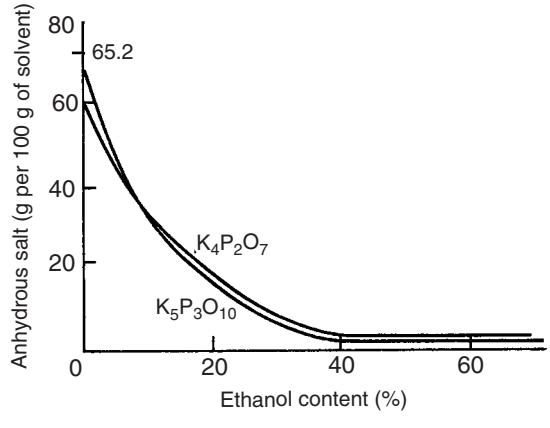

Figure 1.7 Solubility curves for potassium pyrophosphate and potassium tripolyphosphate in ethanol–water mixtures at 25 °C (Van Wazer, 1958).

The branching points in branched phosphates, in which one atom is bonded through oxygen to three other phosphorus atoms, are extremely labile. The rate of hydrolysis of the branching points in the reticular phosphates in aqueous solution at 25 °C, resulting in the formation of linear polyphosphates, is about 1000 times greater than that of the P–O–P bonds in the linear polyphosphates. Hydrolysis of the branching points liberates 28 kcal mol^{-1} (Van Wazer, 1958), which is much more than that liberated in the hydrolysis of the 'central' phosphoric anhydride bonds.

The linear polyphosphates and cyclophosphates are hydrolysed extremely slowly at neutral pH and room temperature in comparison with other polyacids such as polyarsenates and polyvanadates, and are unique in this respect. The 'half-hydrolysis time' for the P–O–P bonds in linear polyphosphates at pH 7 and 25 °C is several years (Van Wazer, 1958). The rate of hydrolysis of these bonds is increased by raising the temperature, reducing the pH, and by the presence in the solution of colloidal gels and complex cations. The hydrolysis of these bonds is dependent on the ionic strength of the solutions (Van Wazer, 1958).

When neutral solutions of polyphosphates are heated at 60–70 °C for 1 h, they are broken down quantitatively to cyclotriphosphate and orthophosphate. It has been shown that this hydrolysis does not occur randomly, but rather from the end of the polyphosphate chain (Thilo and Wieker, 1957). Thilo (1962) related the formation of cyclotriphosphates during the hydrolysis of linear polyphosphates in neutral solution (and even in non-aqueous solution) to the presence of a particular type of spiral secondary structure which makes it sterically possible for a rearrangement of the bonds to occur within the molecule with the formation of small closed chains (Figure 1.8).

In alkaline solutions, cyclophosphates undergo ring fission, even on gentle warming, to form linear polyphosphates with corresponding chain lengths (Ebel, 1951). Linear polyphosphates also undergo hydrolysis under alkaline conditions (Niemeyer and Richter, 1969, 1972), but more particularly under acidic conditions (pH, 3.5–4.0). Under these conditions, significant hydrolysis of the P–O–P bonds takes place even at room temperature, and here breakdown occurs along the length of the chains rather than from the ends of the chains,

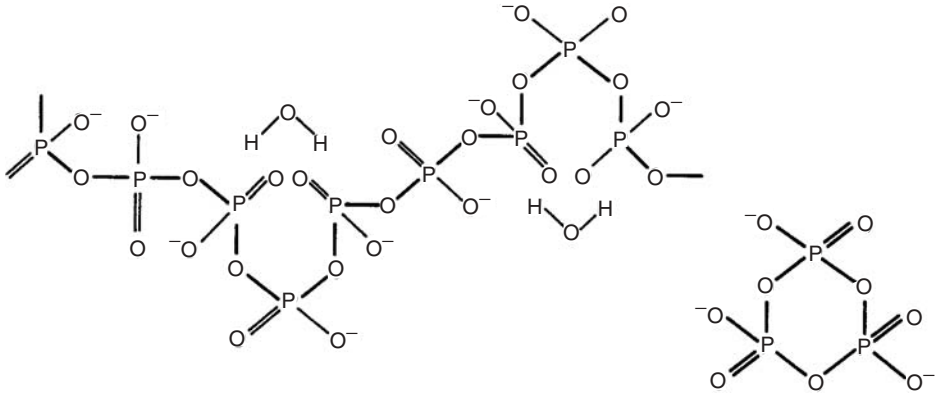

Figure 1.8 Illustration of the incomplete hydrolysis of linear high-molecular-weight polyphosphates to cyclotriphosphate and orthophosphate (Thilo, 1956, 1962).

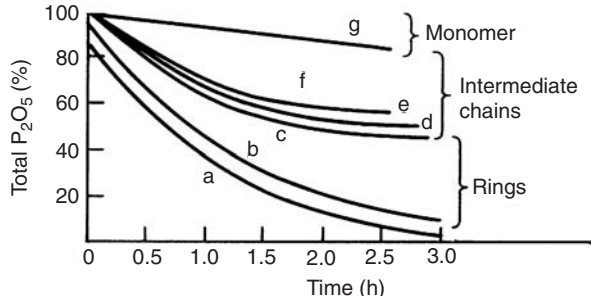

Figure 1.9 Results of a chromatographic examination of the hydrolysis products of Graham's salt at pH 4 and 90 °C: (a) high-molecular-weight polyphosphates; (b) cyclic phosphates containing four to six phosphorus atoms; (c) cyclotriphosphate; (d) pyrophosphate; (e) tripolyphosphate; (f) linear polyphosphates containing four to 15 phosphorus atoms; (g) orthophosphate (Van Wazer, 1958).

to form polymers with increasingly lower molecular weights, down to orthophosphate. The results of an investigation of the hydrolysis products of Graham's salt at pH 4.0 and 90 °C are shown in Figure 1.9. It can be seen from this figure that the proportions of the hydrolysis products (linear polyphosphates, cyclophosphates and orthophosphate) are very dependent on the duration of hydrolysis. When the reaction time is increased to 3 h, the higher polymeric polyphosphates disappear altogether, with the mixture consisting entirely of low-molecular-weight poly- and cyclophosphates and orthophosphate. When the pH of the solution is reduced to 1 and below, the extent of hydrolysis of polyphosphates to orthophosphate increases rapidly. Linear polyphosphates such as Graham's salt are completely hydrolysed after 7-15 min at 100 °C in 1 N HCI (Van Wazer, 1958).

1.3 Physico-Chemical Properties of Condensed Inorganic Polyphosphates

Apart from the low-molecular-weight polyphosphates and cyclophosphates, condensed inorganic phosphates are macromolecular compounds, and this affects their properties and behaviour in solution.

Aqueous solutions of polyphosphates of low ionic strength and pH values near neutral are very viscous, with the viscosity increasing with increasing mean chain length (Malmgren, 1948; Ingelman and Malmgren, 1950; Van Wazer, 1950). The presence of branched phosphates in any given sample of condensed phosphates results, as we have seen, in a very high initial viscosity which decreases rapidly following dissolution in water, even at room temperature (see Figure 1.6).

Polyphosphates in aqueous solutions of low ionic strength are capable of forming complexes with other polymers, especially proteins (Katchman and Van Wazer, 1954), *basic polypeptides* (Singh, 1964), *and nucleic acids* (Kulaev and Belozersky, 1958; Ebel *et al*., 1958c). This ability increases as the chain length of the polyphosphate molecule increases. In acidic solution, these complexes separate as precipitates. The ability of condensed

phosphates to precipitate proteins from acidic solutions has been known from a very early date (Perlmann, 1938; Ebel, 1951; Van Wazer, 1958; Wiame, 1958). It has been shown that this property of polyphosphates is due to a simple total charge, which is dependent on the pH. Katchman and Van Wazer (1954) showed that the higher the molecular weight of a water-soluble protein, then the less polyphosphate is required.

A similar polycation–polyanion interaction is found in the metachromatic reaction, in which high-molecular-weight polyphosphates cause a shift in the absorption maximum of cationic dyes, such as toluidine blue, towards shorter wavelengths (Ebel, 1951; Bergeron and Singer, 1958). This reaction essentially involves polymerization of the dye on the macromolecular anion (Wiame and Lefebvre, 1946; Wiame, 1947a,b). In the case of toluidine blue, addition of polyphosphate to the solution results in a change in colour from blue to violet–red, and a shift in the position of the absorption maximum from 630 nm (which is characteristic of solutions of the monomeric form of toluidine blue) to 530 nm (typical of the complex of polyphosphate and the polymerized dye) (Arloing and Richard, 1921; Damle and Krishnan, 1954; Tewari and Krishnan, 1959; Correll and Tolbert, 1964). However, only comparatively high-molecular-weight polyphosphates are capable of undergoing the metachromatic reaction, either in solution or on paper (Ebel and Muller, 1958; Tewari and Krishnan, 1959; Correll and Tolbert, 1964) Tripoly- and cyclotriphosphates, for example, do not react with toluidine blue (Kornberg, 1956).

Linear polyphosphates possess properties very similar to those of cross-linked, solid ion-exchange agents (Thilo, 1955). The behavior of polyphosphates as dissolved ion-exchange agents is yet further evidence of their ability to form complexes with counter-ions. Polyphosphates are known to be very good complexing agents for many metal ions (Van Wazer, 1958). This property is widely exploited in the fractionation of polyphosphates, and for other analytical purposes.

The information given above concerning the chemical and physico-chemical properties of the inorganic polyphosphates will assist in the better understanding and prediction of the behaviour of these compounds during their extraction from cells and their subsequent fractionation. Knowledge of these properties will facilitate the development and use of efficient and reliable biochemical procedures for the isolation, purification, identification and determination of polyphosphates.

2

METHODS OF POLYPHOSPHATE ASSAY IN BIOLOGICAL MATERIALS

2.1 Methods of Extraction from Biological Materials

The earlier work on the isolation of PolyPs from the cells of living organisms usually employed the same methods as those used for the extraction of nucleic acids. It was not until 1936 that MacFarlane (MacFarlane, 1936) proposed a specific method for the extraction and fractionation of condensed phosphates present in cells. It was found that these phosphates could be divided into two main fractions, i.e. one soluble in 5 % trichloroacetic acid (TCA) and the other insoluble, and ever since then cellular condensed polyphosphates have been divided into acid-soluble and acid-insoluble fractions.

Although most workers have used the same extractant, 5 % TCA (or occasionally 0.5 M $HClO_4$), to obtain the acid-soluble fraction, a variety of methods have been used to isolate acid-insoluble condensed phosphates. The most common method of extraction of acid-insoluble PolyPs from cells involves the use of dilute sodium hydroxide solution, pH 9–12 (MacFarlane, 1936; Belozersky, 1955; Belozersky and Kulaev, 1957; Mudd *et al*., 1958; Krasheninnikov *et al*., 1968). Widely used methods for the extraction of acid-insoluble PolyPs from biological materials are several variants of the method of Schmidt and Thannhauser, i.e. the use of 1 M potassium hydroxide at 37 °C for various periods of time (Schmidt and Thannhauser, 1945; Chaloupka and Babicky, 1957, 1958; Zaitseva *et al*., 1959; Griffin *et al*., 1965; Griffin and Penniall, 1966).

In addition, some PolyP fractions can be extracted by hot solutions of acids, either 5 % TCA (Wiame, 1947a; Belozersky, 1955; Belozersky and Kulaev, 1957; Bukhovich and

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Belozersky, 1958, 1959; Konovalov, 1960) or 10 % perchloric acid, at 80–100 °C (Krishnan *et al*., 1957; Drews, 1960b; Fedorov, 1961; Harold, 1960, 1962ab; James and Casida, 1964). When this method of extraction was employed, the condensed phosphates are hydrolysed to orthophosphate, the amount of which indicates the amount of condensed phosphates present in the acid-insoluble fraction. Hughes and co-workers (Hughes *et al*., 1963) used a prolonged (5 h) extraction of acid-insoluble PolyPs with 10 % TCA at 20–22 °C. In the author's opinion, this method ensures an almost complete extraction of acid-insoluble PolyPs from the cells of bacteria and other microorganisms.

Later investigations showed that it was possible to carry out further fractionation of the PolyPs present in biological material depending on the chain length. Such fractionation has been carried out by Langen and Liss in the laboratory of Lohmann (Langen and Liss, 1958ab, 1959; Liss and Langen, 1960, 1962). Their method consists of successive extraction of cells in the cold with 1 % TCA, a saturated solution of a salt such as $NaClO_4$, dilute NaOH solution (pH 10), and a more concentrated solution of alkali (0.05 N NaOH). This method, either in its original version or modified in various ways, has been used extensively for the fractionation of PolyPs from different organisms. Its advantage is that the fractions obtained were localized at different intracellular sites and showed different physiological activity.

Another, and apparently successful, method for the fractionation of PolyPs present in biological material is that developed by Miyachi and co-workers (Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi and Miyachi, 1961; Miyachi *et al*., 1964). These researchers successively extracted the PolyPs present in the cells of *Chlorella* and other organisms with 8 % TCA in the cold (fraction A), then with a solution of NaOH, pH 9, in the cold (fraction B), and finally with a 2 N solution of KOH at 37 °C for 18 h. The PolyPs extracted with 2 N KOH by the method of Schmidt and Thannhauser were further separated by Miyachi into two fractions, i.e. one precipitated by neutralization with $HClO_4$ in the presence of $KClO_4$ (fraction C) and that which was not precipitated under these conditions (fraction D). The work of Miyachi showed that this mode of fractionation of *Chlorella* PolyPs yielded fractions which differed in their physiological activity and also apparently cellular location.

It should be pointed out that neither of the methods described above was aimed at obtaining completely unmodified preparations of cellular condensed polyphosphates. Other, much milder, methods of extraction from cells have been developed in order to obtain samples of condensed phosphates, which are as little modified as possible to completely avoid the use of strong acids and alkali.

The mildest methods for the extraction of condensed phosphates are as follows: (i) extraction with hot water (Kornberg and Kornberg, 1954; Chayen *et al*., 1955; Lohmann and Langen, 1956; Dirheimer and Ebel, 1957; Dirheimer, 1964); (ii) extraction with dilute sodium carbonate solution (Ingelman and Malmgren, 1950; Ebel, 1952a,b); (iii) extraction with hot 2 M sodium chloride solution (Kaltwasser and Schlegel, 1959; Kaltwasser, 1962; Kaltwasser *et al*., 1962); (iv) extraction with cold distilled water following a preliminary treatment of the material with alcohol and ether (Schmidt *et al*., 1946; Malmgren, 1949; Ebel, 1952a; Lohmann and Langen, 1956; Dirheimer and Ebel, 1957; Chaloupka and Babicky, 1958; Dirheimer, 1964); (v) extraction with sodium hypochlorite (Harold, 1963b).

On the basis of the work carried out at our laboratory, we consider the extraction method of Langen and Liss (1959) with the modification of Kulaev *et al*. (1966a) to be one of the best available for the separation and quantitative determination of different PolyP fractions localized at different intracellular sites and apparently displaying

specific physiological roles. PolyPs were most completely extracted from yeast cells by this method (Table 2.1), whereas the method employed by Chernyshova *et al*. (1971) and Clark *et al*. (1986) permitted the extraction of only about 80 % of PolyPs from biomass. The sequential treatment of yeast cells with cold diluted perchloric acid, salt and weak alkali allowed the isolation of PolyPs with degrees of polymerization from as low as 2 to 8 to as high as 200. Moreover, the former method (Kulaev *et al*., 1966a) made it possible (see table 2.1) to isolate five PolyP fractions from yeast cells, whose synthesis and degradation are closely related to metabolic processes in individual cell compartments. The extraction of PolyP fractions depends rather on their state or localization in the cell than on the degree of polymerization.

Clark *et al*. (1986) found that two different extractions were required to isolate all PolyPs from *Propionibacterium shermanii,* yet these two fractions contained PolyPs of identical size. These authors concluded that one fraction was soluble PolyP, while the second fraction was more tightly complexed in granules. The most frequently used procedure, i.e. extraction with ice-cold trichloroacetic acid (TCA), followed by extraction with alkali, and also the Langen and Liss variation of this procedure, do not extract all PolyPs from *P. shermanii* or from several other organisms. To demonstrate that the chains were not shortened by the extraction procedure developed for *P. shermanii,* Clark *et al*. (1986) included ^{32}P-labelled PolyP during the extraction and then analysed the radioactive PolyP before and after the procedure by using gel electrophoresis. They found that their procedure did not cause PolyP hydrolysis, in contrast to the procedures of Langen and Liss (1959) and Harold (1966). Other procedures that are apparently mild include extraction with hot water, while sodium dodecylsulfate, carbon tetrachloride and phenol/chloroform have been used to extract PolyPs which are apparently located in granules.

For the analysis of PolyPs in activated sludge, some modifications of the extraction method have been developed. Müssig-Zufika *et al*. (1994) compared various chemical fractionation methods to retrieve intact PolyPs from activated sludge and pure cultures. They concluded that the degree of PolyP hydrolysis during the treatment was strongly dependent on the extraction method being employed. Using the method of Mino *et al*. (1985) (with cold TCA extraction), 24 % of the PolyPs were hydrolysed to P_i, while only 5 % and 1 % hydrolysis occurred by the methods of Psenner *et al*. (1984) and Clark *et al*. (1986), respectively. The 'Clark method', which included a cold TCA–acetone extraction step, was not suitable for Gram-positive bacteria and for bacteria from activated sludge due to their cell wall structures. Müssig-Zufika *et al*. (1994) described a modified extraction method applicable to pure cultures, mixed cultures and activated sludge. This technique was essentially a combination of various extraction methods (including mechanical agitation) and did not result in PolyP hydrolysis, producing intact PolyP chains that could be analysed further.

It should be concluded that PolyP extraction from 'new' organisms, where PolyP metabolism has been little studied, needs careful verification of the fullness and intactness of the PolyP chains.

2.2 Chromatographic Methods

Reliable identification of polyphosphates often includes the 'Thilo and Wicker method' of chromatographic analysis of the products of partial hydrolysis (Thilo and Wicker, 1957). In this approach, the hydrolysis of condensed phosphates in neutral solution at 60 °C yields

Table 2.1 Content (μg P_i/g dry biomass) and degree of polymerization (n) of PolyPs isolated from *S. cerevisiae* cells.[a,b]

	Method of Langen and Liss (1959), with modification of Kulaev *et al.* (1960)				Method of Langen and Liss (1959), with modification of Chernychova *et al.* (1971)				Method of Clark *et al.* (1986)			
Fraction[c]	P_i	PolyP	%	n	P_i	PolyP	%	n	P_i	PolyP	%	n
0.5 N $HClO_4$; 0 °C; 30 min; PolyP(I)	2890	5857	28.7	8–26	2890	5857	28.9	8–26	—	—	—	—
2 % Trichloroacetic acid–acetone; 20 °C; 3 min	—	—	—	—	—	—	—	—	3166	406	2.4	8–12
$NaClO_4$; 0 °C; 60 min; PolyP(II)	—	3131	15.3	20–28	—	3131	15.5	20–28	—	—	—	—
2 mM EDTA; pH 7–8; 20 °C; 3 min	—	—	—	—	—	—	—	—	96	7940	47.6	29–35
NaOH; pH 9–10; 0 °C, 30 min; PolyP III	—	5323	26.0	32–37	—	—	—	—	—	—	—	—
2 mM EDTA; pH 7–8; 20 °C; phenol–chloroform; 5 min	—	—	—	—	—	—	—	—	—	5265	31.6	37–46
NaOH; pH 12; 0 °C; 30 min; PolyP(IV)	—	4759	23.3	50–55	—	—	—	—	—	—	—	—
1 % SDS; pH 7.4; 0 °C, 30 min	—	—	—	—	—	7057	34.8	50–62	—	—	—	—
0.5 N $HClO_4$; 90 °C; 40 min; PolyP(V)	—	1358	6.7	—	—	4203	20.8	—	—	3062	18.4	—
Total PolyP		20 428	100			20 248	100			16 673	100	
Total P_i		33 980				33 980				33 980		

[a] The data presented are taken from triplicate experiments.
[b] '—' indicates not detected.
[c] EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

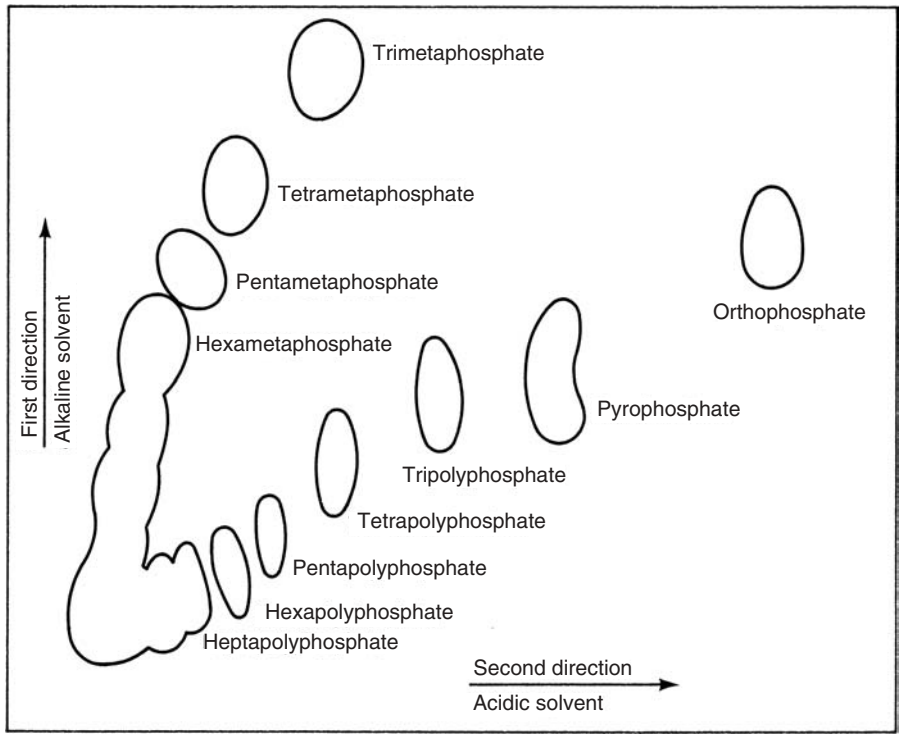

Figure 2.1 Separation of PolyPs and cyclophosphates by two-dimensional paper chromatography. The basic solvent is isopropanol–isobutanol–water–25 % ammonia (40:20:39:1), while the acidic solvent is isopropanol–water–25 % TCA–25 % ammonia (70:30:20:0.3) (Dirheimer, 1964).

cyclotriphosphate and orthophosphate, which are readily identified chromatographically, especially when 'Ebel's basic solvent' is used (Ebel, 1952a).

Ebel and co-workers have developed both paper chromatography (Ebel, 1949, 1951, 1952bc, 1953ab, 1954, 1958; Ebel and Dirheimer, 1957) and column chromatography (Ebel and Bush, 1956; Ebel *et al.*, 1962) techniques for the analysis of PolyPs. Paper chromatographic separation of condensed phosphates only permits the separation and determination of comparatively low-molecular-weight PolyPs and cyclophosphates ($n = 2$–9). Using Ebel's method of two-dimensional paper chromatography (Ebel, 1952b, 1953ab), it is possible to separate the oligomeric PolyPs and cyclophosphates. An example of such a separation, carried out by Dirheimer (1964), is shown in Figure 2.1.

In addition to paper chromatography, chromatography using ion-exchange resins has been successly employed. Using this method, it is possible to separate polyphosphates with values of n of 2 to 12 (Figure 2.2). Paper electrophoresis has also been employed to separate oligomeric PolyPs and cyclophosphates, together with thin-layer chromatography (Wade and Morgan 1955; Kulaev and Rozhanets, 1973; Kulaev *et al.*, 1974a,c).

However, these methods are only capable of separating polyphosphates of fairly low molecular weight. Two methods are currently available for the separation of

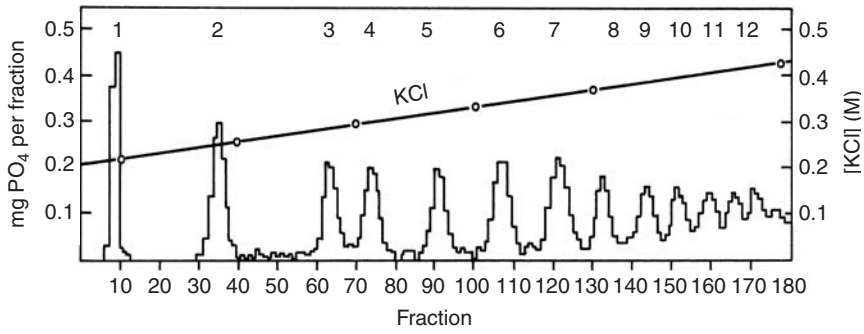

Figure 2.2 Separation of PolyPs of low molecular weight by ion-exchange chromatography on Dowex 1 XI0 in a KCl gradient. The numbers 1–12 represent the number of phosphorus atoms in the PolyP molecules constituting the various fractions (Matsuhashi, 1963).

high-molecular-weight PolyPs. The first of these is Van Wazer's method for the fractional precipitation of PolyPs of different chain lengths from aqueous solutions with acetone (Van Wazer, 1958). This technique yields a great number of fractions, which differ from each other in the lengths of the PolyP chains. The second method, also developed in the laboratory of Van Wazer (Ohashi and Van Wazer, 1964), gives similar results to those obtained by paper chromatography. A method for the separation of inorganic PolyPs on Sephadex columns has also been developed in Ebel's laboratory (Felter *et al*., 1968). Gel filtration on Sephadex G-10 is a suitable method for the purification of PolyP from P_i and PP_i (Andreeva and Okorokov, 1993).

Some methods for fast chromatographic separation and detection of PolyPs in food, biological samples or water have been proposed (Halliwell *et al*., 1996; Baluyot and Hartford, 1996; Svoboda and Schmidt, 1997; Bewsler *et al*., 2001). A single-column chromatographic system with indirect UV detection was elaborated, and the dependencies of PolyP retention on the concentrations of pyromellitic acid and ethylenediaminetetraacetic acid (EDTA) in the mobile phase and on the pH of the eluent were determined (Svoboda and Schmidt, 1997). A high performance liquid chromatography (HPLC) method has also been used for the separation of PolyPs (Lorenz and Schröder, 1999).

2.3 Colorimetric and Fluorimetric Methods

One of the simplest methods of estimation of PolyPs in extracts is based on the assay of P_i, which is released from the PolyPs by hydrolysis with 1 M HCl at 90 °C for 10 min. The P_i released under these conditions is defined as 'labile phosphorus'. If the compounds containing organic labile phosphorus (i.e. nucleotide phosphates, sugar phosphates, etc.) were removed from the extracts by adsorption on Norit charcoal, the increase in P_i content after hydrolysis can be attributed to PolyP and pyrophosphate (PP_i). Estimation of the PP_i content (Mansurova, 1989) before hydrolysis may be needed in some cases for more precise calculations of the PolyP content. P_i may be determined by one of the well-known chemical methods (Fiske and Subarrow, 1925; Weil-Malerbe and Green, 1951).

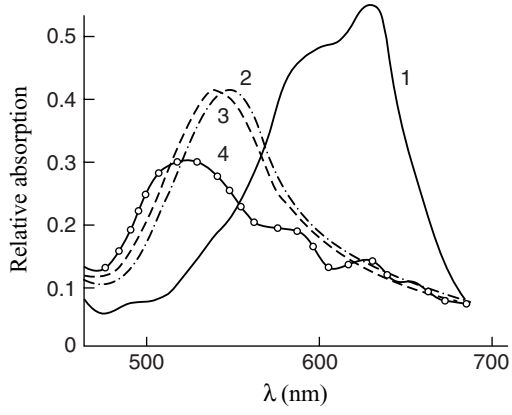

Figure 2.3 Absorption spectra of (1) toluidine blue (3.3×10^{-5} M), and dye with three PolyP fractions isolated from *Neurospora crassa* by consecutive treatment with (2) 0.5 N $HClO_4$ (PolyP(I), 3.3×10^{-3} M), (3) saturated $NaClO_4$ solution (PolyP(II), 3.3×10^{-3} M) and (4) 1% SDS, pH 7.4–7.6 (PolyP(III), 3.3×10^{-3} M) (see Table 2.1) (Chernysheva *et al*., 1971).

By precipitating barium salts at different pH values (e.g., 2.5, 4.5 and 7), it is possible to distinguish between the condensed phosphates, which differ in their polymerization degrees or are bound with different compounds in the cell. It is also possible to use organic bases such as guanidine to precipitate polyphosphates selectively from their aqueous solutions. However, a PolyP assay according to the labile phosphorus of barium and other water-insoluble salts is possible but not fully reliable, because not all condensed phosphates are precipitated as barium salts. Oligomeric poly- and cyclophosphates, in particular tripolyphosphate and cyclotriphosphate, are not precipitated by barium at any pH. Again, precipitation by barium salts may result in some degradation of the PolyPs.

The content of long-chain PolyPs may be estimated by measuring the metachromatic effect in the absorption spectrum of toluidine blue (Chernysheva *et al*., 1971; Leitao *et al*., 1995; Lorenz and Schröder, 1999). Toluidine blue in an aqueous solution exhibits a concentration-dependent absorption spectrum due to a monomer (λ_{max}, 632 nm)–dimer (λ_{max}, 590 nm) equilibrium. The PolyP induced the maximal shift of the absorption spectrum to 545 nm. Nucleic acids also induce metachromasia, but with a shift of about 570 nm with DNA and 590 nm with RNA. Figure 2.3 demonstrates the typical absorption spectra of toluidine blue and toluidine blue with different preparations of PolyPs (Chernysheva *et al*., 1971).

The PolyP concentrations in the samples were determined from a calibration graph of the 530/630 nm absorption ratio for standard PolyP solutions (Leitao *et al*., 1995; Mullan *et al*., 2002). Samples containing PolyPs were added to the dye solution (6 mg l^{-1} in 40 mM acetic acid) and the absorption values at 530 nm and 630 nm were determined. The change in the 530/630 nm absorption ratio is roughly proportional to the PolyP concentration over the range 25–75 μM (expressed in P_i). Some disadvantages of the method result from the fact that PolyPs of a chain length less that 10 give only weak or no metachromatic reaction and the interaction between the dye and the PolyP is affected by many compounds, such as different polyanions, cations and proteins, and depends on the ionic strength and pH (Lorenz and Schröder, 1999).

The method used for determination of PolyP, which is based on the Mn^{2+}-induced quenching of the fluorescence of the calcium indicator Fura-2, has been described (Lorenz *et al*., 1997a). The effect of Mn^{2+} ions on the Fura-2 fluorescence is gradually removed in the presence of increasing PolyPs concentrations; this allows the quantification of PolyPs isolated from tissues or cells. The described method has some advantages when compared with the conventional detection procedures based on the metachromatic effect. It can be applied to the determination of pyrophosphate, tripolyphosphate and other short-chain PolyPs not detectable by toluidine blue (Lorenz *et al*., 1997a).

2.4 Cytochemical Methods

The oldest and most extensively used method for determination of PolyPs in biological materials, although of course the least accurate, is based on the staining of cells and tissues by certain basic dyes such as toluidine blue, neutral red and methylene blue. The presence of condensed phosphates in the organisms is judged by the appearance in the cells of metachromatically stained granules, or volutin granules.

Two basic principles are involved in metachromasia: first, the interaction between dye and substrate molecules, and secondly, the interaction between adjusted dye molecules aggregated to the substrate. A striking change in the absorption spectrum of a metachromatic dye in the presence of polyelectrolyte is generally characteristic of the specific nature of the polymer. For example, such changes have been related to the chain length, conformation and the functional group of an individual polymer. The interaction between the dye and the polymer is influenced by experimental conditions such as pH, temperature, ionic strength, and the molar ratio of the polymer residues to the dye molecules.

When 'Loeffler's methylene blue' is used, the PolyP-containing granules appear pink–violet on the blue background of the cells (Murray *et al*., 1994), while 'Neisser staining method' gives purple–black granules on the yellowish-brown background of the counter-stained cells (Bartholomew, 1981). Neisser staining is more suitable for determining PolyP accumulation than Loeffler's method because of its higher contrast between the granule and the cell (Serafim *et al*., 2002). Toluidine blue, which shares the same metachromatic properties as methylene blue, can also stain PolyP granules.

The staining by basic dyes such as methylene blue, toluidine blue and neutral red has been used for the detection of PolyPs in living organisms for a long time (Wiame, 1946, 1947a,b, 1948, 1949, 1958; Macary, 1951; Widra, 1959; Drews, 1960 a,b; Ebel, 1952d; Ebel *et al*., 1955, 1958a,b; Ebel and Mehr, 1957; Ebel and Muller, 1958; Tewari and Krishnan, 1959; Prokof'eva-Bel'govskaya and Kats, 1960; Dmitrieva and Bekker, 1962; Voelz *et al*., 1966; Tijssen *et al*., 1982; Lopez-Revilla and Gomez-Dominiguez, 1985; Suresh *et al*., 1985) and up to date has been one of the simplest and cheapest PolyP visualizing methods (Rees *et al*., 1992; Leitao *et al*., 1995; Imsiecke *et al*., 1996; Serafim *et al*., 2002). PolyP staining first showed the presence of characteristic granule clusters in activated sludge and suggested the existence in them of PolyP-accumulating bacteria (Fuhs and Chen, 1975). The staining method of PolyP detection is often used in the study of polyphosphate-accumulating microorganisms of activated sludge (Suresh *et al*., 1985; Rees *et al*., 1992; Serafim *et al*., 2002).

It should be noted, however, that, despite the fact that in most cases the cytochemical detection of metachromatic granules is associated with the actual presence of PolyPs in the organism, such methods must nevertheless be carried out with great caution. This is primarily due to the fact that basic dyes are also capable of staining other polymeric compounds present in the cells.

Some methods for the differential staining of PolyPs and polyhydroxyalkanoate-containing granules in cells have been developed (Rees *et al*., 1992) and critically analysed in a recent review (Serafim *et al*., 2002). The staining by Nile blue or Sudan black, which do not stain PolyP granules, or sequential staining with Nile blue and methylene blue allows a differentiation of the two types of granules in some cases. Thus, the cytochemical distinguishing of cell inclusions is still an interesting, but not simple, experimental task.

The more sensitive and convenient method of PolyP detection *in situ* is fluorescence microscopy using fluorochromes of the type 4′,6′-diamino-2-phenylindole.2HCl (DAPI), which is commonly used for DNA detection. At a high concentration (50 mg ml^{-1}), it also stains PolyP granules and lipid inclusions (Allan and Miller, 1980; Tijssen *et al*., 1982; Streichan *et al*., 1990). DAPI–DNA fluorescence is blue–white, while DAPI–PolyP and DAPI–lipid fluorescence is yellow. The lipid fluorescence is weak and fades in a few seconds, while the PolyP granules appear bright yellow, thus allowing discrimination of the above types of cell inclusions (Streichan *et al*., 1990).

The excitation wavelength for DAPI is 330–385 nm. The emission maximum of DAPI is 456 nm; different polyaniones, such as DNA or poly(glutamic acid), induced a strong increase in the fluorescence intensity depending on the concentration. PolyPs showed, however, a shift of the fluorescence maximum to 525 nm (Figure 2.4).

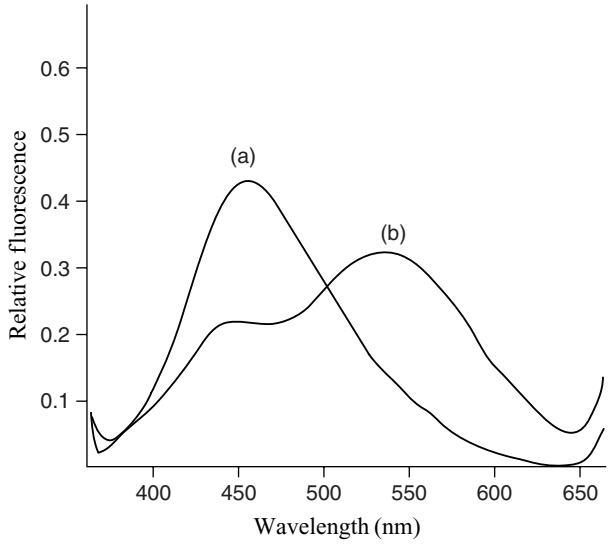

Figure 2.4 Fluorescence spectra of DAPI (4′,6′-diamino-2-phenylindole) in 3 mM tris-maleate, pH 5.0: (a) 0.2 μg ml^{-1} DAPI; (b) 0.2 μg ml^{-1} DAPI plus 10 μg ml^{-1} PolyP$_{200}$ (Tijssen *et al*., 1982).

DAPI staining was used for identification of vacuolar PolyPs (Allan and Miller, 1980) and cell-surface PolyPs (Tijssen *et al*., 1982) of yeast. This method is commonly used in the identification of PolyP-accumulating microorganisms from activated sludge during the study of *Enhanced Biological Phosphorus Removal* (EBPR) (Serafim *et al*., 2002). The DAPI–PolyP staining has often been used in studies of EBPR because of the possibility to combine this procedure with *in situ* molecular identification (FISH analysis). In the latter analysis, the 16S rRNA fluorescent probes specifically bind with the target bacteria (Wagner, *et al*., 1994; Bond and Rees, 1999; Bond *et al*., 1999; Kawaharasaki *et al*., 1999; Crocetti *et al*., 2000). They appear as fluorescent cells, and bacteria belonging to a specific taxonomic group may be identified in mixed biomass such as activated sludge. Procedures that combine FISH with methylene blue staining (Crocetti *et al*., 2000) or with DAPI staining (Kawaharasaki *et al*., 1999) allow visualization of PolyP granules in taxonomicaly identified cells. The sequential FISH, DAPI and polyhydroxyalcanoates (PHAs) staining methods have been described (Liu *et al*., 2001). It should be noted that further studies of various samples and adequate conditions are required to check the reliability of these new cytochemical approaches.

2.5 X-Ray Energy Dispersive Analysis

PolyP-containing deposits in cells are also visualized by electron microscopy as electron-dense regions, and when such microscopy is combined with X-ray energy dispersive analysis, it is possible to detect the phosphorus, presumably present as PolyPs, and metal cations such as Na, K, Ca, Mg, Mn, Zn, Ba and Al. Such analyses are useful to identify the metal composition of PolyP granules and to obtain evidence of PolyP involvement in cation chelation. In a number of studies, this method was successfully used for the detection and chemical analysis of PolyP granules in various organisms (Ashford *et al*., 1975; Callow *et al*., 1978; Adamec *et al*., 1979; Doonan *et al*., 1979; Baxter and Jensen, 1980a; Scherer and Bochem, 1983; Vořišek and Zahleder, 1984; Pettersson *et al*., 1985; Ogawa and Amano, 1987; Väre, 1990; Ashford *et al*., 1999; Ramesh *et al*., 2000; Schonborn *et al*., 2001).

Interesting data on the structure and formation of PolyP granules in cyanobacteria were obtained by electron microscopic and cytochemical methods (Jensen, 1968, 1969; Jensen and Sicko, 1974; Sicko-Goad *et al*., 1975; Jensen *et al*., 1982).

The study of microbial cell granules by X-ray dispersive analysis revealed that the composition of the granules changed markedly depending on the chemical and ionic composition of the culture medium. For example, the quantitative ratios of Ca, Mg and K in PolyP granules of bacteria in wastewaters varied depending on the influence concentrations of these metal cations (Schonborn *et al*., 2001). The quantitative X-ray analysis of laboratory grown cyanobacterium *Plectonema boryanum* and bacterium *Staphylococcus aureus* revealed that typical *in vivo* PolyP bodies contain (in μg): O (4.3×10^{-8}), C (1.2×10^{-8}), P (6.7×10^{-9}), Mg (1.3×10^{-9}), Ca (6.7×10^{-10}), K (6.7×10^{-10}), Fe (6.0×10^{-10}), S (5.4×10^{-10}) and Al (5.9×10^{-10}) (Goldberg *et al*., 2001).

This method has often been used in the investigation of PolyPs in mycorrhiza fungi (Ashford *et al*., 1975; Callow *et al*., 1978; Orlovich and Ashford, 1993; Bucking *et al*., 1998; Ashford *et al*., 1999). For example, an energy dispersive X-ray spectrum from a

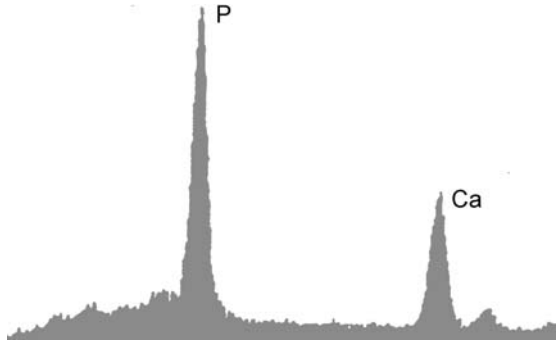

Figure 2.5 Energy dispersive X-ray spectrum optained from a spherical electron-opaque granule of the fungus *Pisolithus tinctorius*, showing peaks for P and Ca (Orlovich and Ashford, 1993).

'electron-opaque' granule of the fungus *Pisolithus tinctorius* is shown in Figure 2.5 (Orlovich and Ashford, 1993).

The elemental compositions of vacuolar granules in different ectomycorrhizal fungi, *Pisolithus tinctorius, Suillus bovinus* and *Xerocomus badius* (Bucking *et al*., 1998), were determined by electron energy loss spectroscopy (EELS) and energy dispersive X-ray spectroscopy (EDXS). The investigations dealt with the advantages and limitations of the EDXS and EELS techniques with respect to the determination of elemental compositions of vacuolar granules and the effect of different specimen preparation techniques. Axenic cultures of these fungi, as well as field mycorrhizae, were used for the analysis. The results, after conventional chemical fixation and dehydration of the material, were compared with those obtained after cryofixation followed by freeze-drying of the samples. Light microscopic studies were also carried out to control the occurrence of vacuolar granules in living hyphae. The results showed that vacuolar granules existed in the living hyphae of different ectomycorrhizal fungi and were not an artifact of the fixation or other specimen preparation procedures of the cells. EDXS and EELS differed in their ability to detect the elemental compositions of these granules. Both analytical techniques found phosphorus in the vacuolar bodies, which indicates a deposition of polyphosphates. PolyP granules are strongly negative polyanions, which contain different cations to balance the negative charge. These cations were often difficult to determine by EELS and could only be shown by EDXS, but the cations varied considerably depending on the technique used for specimen preparation. In chemically fixed and dehydrated material, Mg, K and Ca, in particular, were detected in the granules. However, measurements of cryofixed and freeze-dried specimens showed that the most abundant cations in PolyP granules were K and Mg and the incorporation of Ca has to be interpreted as a result of the chemical specimen preparation (Bucking *et al*., 1998).

All known work revealed that the compositions of PolyP granules changed markedly depending on the chemical, and in the first place, ionic composition of the culture medium. However, strictly speaking, this method of PolyP identification is not universally appropriate. First, it identifies the presence in granules of phosphate but *not* phosphoryl groups and secondly, it does not detect any PolyP if its concentration is not high enough. It should be noted that the phosphate-containing granules might consist not only of PolyPs but also of

other phosphorus compounds. For example, the sulfate-reducing bacterium *Desulfovibrio gigas* forms electron-dense granules in the cells. Energy dispersive X-ray analysis of the granules in the cells showed that they contain large amounts of P, Mg and K. Gel electrophoresis, ^{31}P nuclear magnetic resonance (NMR) spectroscopy and chromatographic analyses of isolated granules revealed that they contained, instead of PolyPs, a novel metabolite, which was identified as alpha-glucose 1,2,3,4,6-pentakis(diphosphate) (Hensgens *et al*., 1996).

Therefore, the identification of PolyPs by X-ray techniques in some cases needs confirmation by using other physico-chemical methods.

2.6 ^{31}P Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a well-established method in the study of phosphorus metabolism (Glonek *et al*., 1971; Salhany *et al*., 1975; Burt *et al*., 1977; Ugurbil *et al*., 1978; Navon *et al*., 1977a,b, 1979; Ferguson *et al*., 1978; Ostrovsky *et al*., 1980; Gadian, 1982; Sianoudis *et al*., 1986; Roberts, 1987; Shanks and Bailey, 1988; Chen, 1999). *In vivo* ^{31}P NMR spectroscopy remains unique, being the least disruptive and quantitative method (Gadian, 1982; Roberts, 1987; Chen, 1999).

The basic principle of the nuclear magnetic resonance (NMR) spectroscopic technique involves measurement of the ratio frequency (rf) of the energy adsorbed by magnetic nuclei (Roberts, 1987; Chen, 1999). NMR spectroscopy is a useful tool in analytical chemistry for the detection, identification and structure elucidation of compounds. Phosphorus compounds of living cells include phosphates, phosphonates and various esters of phosphates and phosphonates. The chemical shift of ^{31}P atoms in these compounds can span over a 30 ppm range, thus making ^{31}P-NMR spectroscopy an attractive tool for examining phosphorus metabolites in microorganisms, plants and animal tissues. In addition, the method has no problem of solvent suppression, since no water signal appears in the ^{31}P resonance region. The common chemical shifts of biological phosphorus compounds are shown in Figure 2.6. The simplicity of the ^{31}P NMR spectrum, usually containing 8–12 resonances,

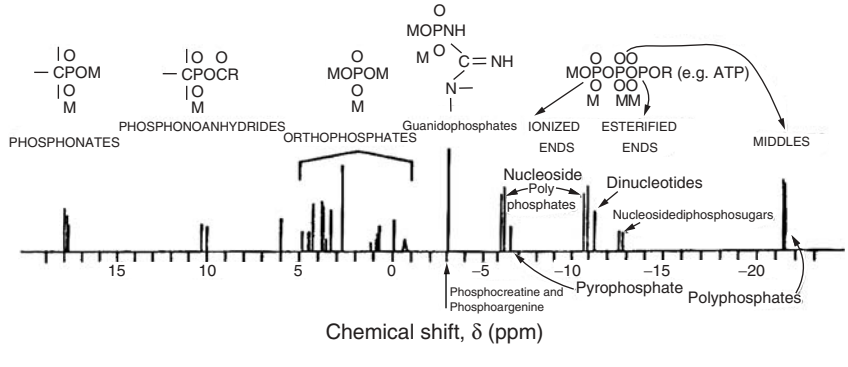

Figure 2.6 Chemical shifts of biological phosphorus compounds at pH 10.0 (Van Wazer and Ditchfeld, 1987).

is due to the fact that narrow signals are generated only from relatively mobile compounds. Insoluble or immobilized compounds, such as membrane phospholipids, usually give very broad signals that are 'NMR-invisible' or appear as broad components underlying the narrow metabolite signals.

NMR spectroscopy can provide information about cellular compartmentalization of metabolites. For instance, compartmentalization of P_i in yeast is reflected by a split signal for P_i in the NMR spectrum, presumably the consequence of pH difference between the compartments (Navon *et al*., 1979; Gillies *et al*., 1981; Nicolay *et al*., 1982, 1983). These pH-induced shifts of the NMR signal, which reflect changes in the environment of the nucleus, are determined by the pK_a of the compound, because each of the ionized forms of P_i has a unique electron structure and therefore a unique chemical shift (Gillies *et al*., 1981). So, NMR spectroscopy can be used to study the structure of phosphate compounds and to examine their metabolism and native environment in biological samples, including intact cells. Thus, intracellular pH homeostasis may be studied by ^{31}P NMR spectroscopy (Gillies *et al*., 1981; Nicolay *et al*., 1982, 1983; Hesse *et al*., 2002)

PolyP NMR spectra have been widely investigated (Glonek *et al*., 1971; Salhany *et al*., 1975; Burt *et al*., 1977, Navon *et al*., 1977a,b; Ugurbil *et al*., 1978; Ferguson *et al*., 1978; Ostrovsky *et al*., 1980; Tijssen and Van Steveninck , 1984; Roberts, 1987; Chen, 1999). This polymeric species gives three resonance peaks: terminal P (PP1), at about −6.62 to −7 ppm; penultimate P (PP2–PP3) at about −20.17 to −21.7 ppm; middle P (PP4) at about −22.37 to −22.5 ppm.

Figure 2.7 shows a typical ^{31}P NMR spectrum of *S. cerevisiae* cells assigned from previous work in the literature (Nicolay *et al*., 1982; Shanks and Bailey, 1988; Beauvoit *et al*., 1989, 1991; Gonzalez *et al*., 2000). The resonances from 4.5 to 3 ppm are in the sugar phosphate region. Cytoplasmic (P^{cyt}) and extracellular (P^{ex}) P_i give resonances at 1.62 and 0.68 ppm, respectively. The left shoulder on the extracellular P_i peak could be due to P_i in the vacuoles. The resonance at −1.34 ppm is from phosphomannan. The resonance at −5.39 ppm was assigned to the γ-phosphate groups of nucleoside triphosphate and the β-phosphate groups of nucleoside diphosphate, at −10.05 ppm to the α-phosphate groups of nucleoside phosphates, and at −18.90 ppm to the β-phosphate groups of nucleoside triphosphate, respectively. The point at −10.66 ppm includes both NAD and NAD(H). The peak PP1 (−6.62 ppm) is the sum of PP_i and the terminal phosphate groups of PolyP. The points PP2 (−18.00 ppm) and PP3 (−20.17 ppm represent) penultimate phosphates from PolyP, while PP4 (−22.37 ppm) represents the middle phosphate from longer PolyPs. Using the employed integrated NMR bioreactor system, an excellent *in vivo* ^{31}P NMR spectrum was obtained, and a clear phosphoenolpyruvate signal could be detected in addition to the commonly observed peaks in *S. cerevisiae* (Gonzalez *et al*., 2000).

The peak intensity can be used to calculate the concentration or chain length of linear PolyPs. With regard to the quantitative determination of PolyPs by using the NMR spectroscopic method, it was shown (Krupyanko *et al*., 1998) that the total intensity of core phosphate groups is proportional to the concentration of each individual PolyP with a certain chain length, but no proportional correlation was observed during the transitions between PolyPs with a small number of phosphate groups and PolyPs with a large number of these groups.

From the signal intensities of the terminal and middle phosphate groups, the chain lengths of the PolyPs can be determined according to Equation (2.1), where n represents the average

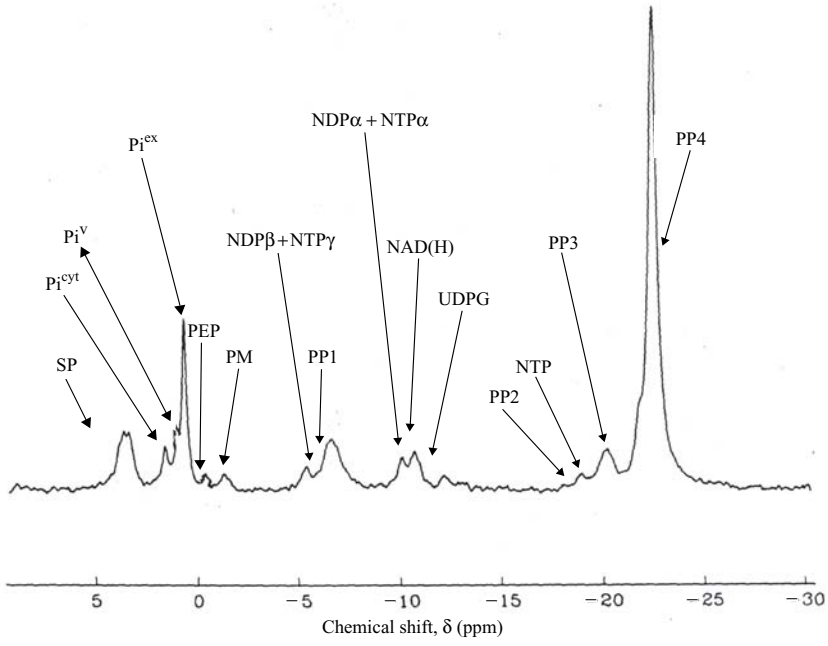

Figure 2.7 The ^{31}P NMR spectrum at 202.46 MHz of *S. cerevisiae* (adapted from Nicolay *et al*., 1982; Shanks and Bailey, 1988; Beauvoit *et al*., 1991; Gonzales *et al*., 2000): SP, sugar phosphate; Pi ^{cyt}, cytoplasmic Pi; Pi^{ex}, extracellular Pi; Pi^{v}, vacuolar Pi; PM, phosphomannan; NTPα and NDPα, α-phosphate groups of nucleoside triphosphates and nucleoside diphosphates, respectively; NTPβ and NDPβ, β-phosphate groups of nucleoside triphosphates and nucleoside diphosphates, respectively; NTPγ, γ-phosphate group of nucleoside triphosphates; PP1, pyrophosphate and terminal phosphate of PolyP; NAD(H), nicotinamide adenine dinucleotide; UDPG, uridinediphosphoglucose; PEP, phosphoenolpyruvate; PP2 and PP3, penultimate phosphates of PolyP; PP4, middle phosphates of PolyP.

chain length, [PP1] the terminal phosphate groups signal intensity and [PP4] the middle phosphate groups signal intensity, respectively (Pilatus *et al*., 1989):

$$n = 2\left(\frac{3 \times [\mathrm{PP1}] + [\mathrm{PP4}]}{[\mathrm{PP1}]}\right) \tag{2.1}$$

The reported data (Nicolay *et al*., 1982; Pilatus *et al*., 1989) show that the signal intensity of the middle phosphate groups is proportional to the PolyP concentration. However, there is no proportionality in the signals from PolyPs with different chain lengths: the contribution of the middle phosphate groups to the total signal intensity in ^{31}P NMR spectra decreases with the increasing length of the PolyP chain.

A special investigation was carried out to check the correlation between the decrease in the ^{31}P NMR signal intensity of the middle phosphate groups and the chain length, and to obtain an equation describing this dependence, thus making it possible to take this effect

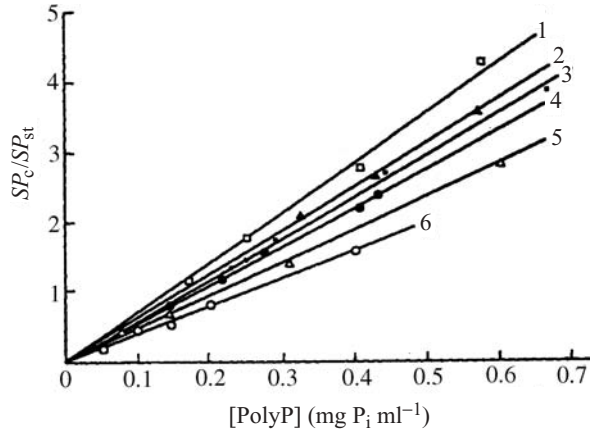

Figure 2.8 Concentration-dependence of the signal intensity ratio of the chemical shifts of core phosphate groups of linear PolyPs relative to the signal intensity of a standard P_i sample. PolyP samples with the following numbers of middle phosphate groups (n_c) in the molecules were used: (1) 204; (2) 115; (3) 58; (4) 35; (5) 11; (6) 3. The concentrations of PolyP in the samples were estimated from the amount of P_i liberated following hydrolysis in 2 N HCl after 10 min at 100 °C (Krupyanko *et al*., 1998).

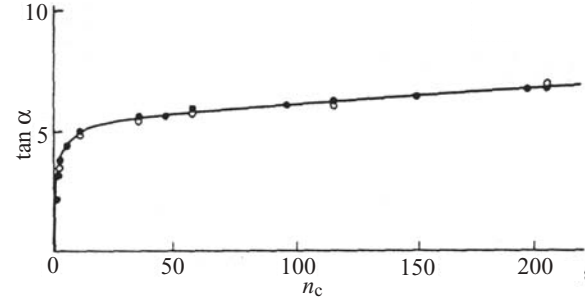

Figure 2.9 Changes in the slope angles of the experimental plots shown in Figure 2.8 ($tg(\alpha_{exp})$) and theoretically from Equation (2.2) ($tg(\alpha_{calc})$) as a function of the number of middle phosphate groups in PolyP molecules (n_c): (o) experimental data; (•) theoretical data obtained from Equation (2.2) (Krupyanko *et al*., 1998).

into account in PolyPs assays of biological samples (Krupyanko *et al*., 1998). Synthetic linear PolyPs were used for this study after chromatographic purification on Sephadex G-75. The experimental results and theoretical data are compared in Figures 2.8 and 2.9. The concentration-dependence of the ratio of the signal intensity of the middle phosphate groups (SP_c) of PolyPs to the signal intensity of a standard P_i sample (SP_{st}) is shown in Figure 2.8. It can be seen that the contribution of the middle (n_c) groups to the total intensity of the P peaks decreases with an increase in the PolyP chain length. This is manifested by a disproportionately greater increase in the slope angle ($tg(\alpha)$) of the experimental plots with

an increase in the chain length of the PolyPs (Figure 2.9):

$$tg(\alpha) = \frac{\left(\frac{SP_c}{SP_{st}}\right)}{C} \tag{2.2}$$

In the above equation, SP_c is the total intensity (peak area) of signals from the middle phosphate groups, SP_{st} is the standard P_i signal intensity, and C is the PolyP concentration, expressed in mg P_i per ml. The effect of reduction of the contribution of inner phosphate groups to the total intensity of the P_c signals is obviously associated with the primary and secondary structures of PolyPs.

From the experimental data (Figure 2.9), the following equation was derived (where n_c is the number of monomeric phosphate groups in the PolyP molecule):

$$tg(\alpha) = \frac{5.838n_c}{1.664 + n_c} + 0.005n_c \tag{2.3}$$

The experimental data correspond well to the theoretical calculation of $tg(\alpha)$ obtained from Equation (2.3). With regard to the quantitative determination of PolyP and PolyP chain length by using NMR spectroscopy, it must be taken into account that the total intensity of the middle phosphate groups is proportional to the concentration of each individual PolyPs with a certain chain length, but no proportional correlation is observed during the transitions between PolyPs with a small number of phosphate groups and PolyPs with a large number of these groups. The contribution of PP4 groups to the total intensity of the peak decreases with an increase in the PolyP chain length. By using Equations (2.2) and (2.3), the chain lengths of PolyP samples may be defined more accurately.

The ^{31}P NMR spectroscopic technique was used for the detection and study of PolyPs in different organisms, including bacteria (Navon *et al*., 1977b; Ferguson *et al*., 1978; Rao *et al*., 1985; Suresh *et al*., 1985; Kjeldstad and Johnson, 1987; Kjeldstad *et al*., 1988; Lawrence *et al*., 1998), yeast (Den Hollander *et al*., 1981; Greenfeld *et al*., 1987; Holahan *et al*., 1988; Bourne, 1990; Loureiro-Dias and Santos, 1990; Beauvoit *et al*., 1991; Castro *et al*., 1995, 1999; Vagabov *et al*., 1998, 2000; Gonzalez *et al*., 2000; Trilisenko *et al*., 2002), fungi (Yang *et al*., 1993; Pilatus *et al*., 1989; Hesse *et al*., 2002), algae (Elgavish and Elgavish, 1980; Elgavish *et al*., 1980; Sianoudis *et al*., 1986; Lundberg *et al*., 1989; Bental *et al*., 1990), and protozoa (Moreno *et al*., 2000). PolyPs were also observed by this method in soils (Adams and Byrne, 1989). One of the advantages of NMR spectroscopy is the possibility of observing changes in the PolyP signals of living cells. Such an approach is widely used and gives important information about the PolyP dynamics under different conditions (Suresh *et al*., 1985; Zhang and Majidi, 1994).

PolyPs, which can be detected by NMR spectroscopy are called 'NMR-visible', and represent a more mobile fraction of the total PolyP content. Lack of an 'NMR-visible' PolyP signal does not indicate the absence of PolyPs in a sample. Accurate values of the chemical shifts of these signals depend on the pH and residual concentrations of divalent cations in the extract (Pilatus *et al*., 1989).

The intensities of the signals in the study of PolyPs by using ^{31}P NMR spectroscopy directly depend on the degree of PolyP binding with other structures and compounds of

the cell, in particular, with metal ions. Therefore, while the detection of a PolyP signal indicates the presence of these compounds in the cell, the absence of this signal still cannot be considered as direct evidence for the absence for PolyPs, as was well described in the work using *Chlorella fusca* (Sianoudis *et al*., 1986).

In some microorganisms of aerobically activated sludge, the NMR resonance characteristics of PolyPs were only observed when the cell structure was disrupted by treating with a strong alkali (Pereira *et al*., 1996).

The interaction with cations lays the basis for the ^{31}P NMR spectroscopic method, allowing one to distinguish between extracellular and intracellular pools of PolyPs. Ethylenediaminetetraacetic acid (EDTA) can be used to complex the divalent cations bound to PolyPs and to produce a new ^{31}P NMR shift. However, because the cell membrane is impermeabile to EDTA, only extracellular PolyPs is affected. This method was used successfully on a cell suspension of *Propionibacterium* sp. (Serafim *et al*., 2002).

Thus, authors often apply the term 'visible' PolyPs when discussing the results obtained by this method (Loureiro-Dias and Santos,1990). It can be considered that the most reliable approach is the combination of PolyP chain length determination by NMR spectroscopy with the method of chemical extraction from cells and their quantitative analysis.

The above approach allowed determination of the content and polymerization degree of PolyPs in volutin granules (Jacobson *et al*., 1982), in vacuoles (Trilisenko *et al*., 2002), and in different PolyP fractions obtained from yeast cells (Vagabov *et al*., 1998, 2000). As an example, Figure 2.10 shows the ^{31}P NRP spectra of the acid-soluble PolyPs from isolated yeast vacuoles.

Thus, the NMR approach gives a most precise picture of the PolyP content and polymerization degree in different cell compartments by a combination of NMR spectroscopy with the methods of sub-cellular fractionation and chemical extraction of PolyPs.

2.7 Other Physical Methods

Infrared spectroscopy has been rarely used for PolyP characterization (Datema *et al*., 1977). Electrospray ionization mass spectrometry (ESI–MS) has been applied to the characterization of phosphates (P_i, PP_i, $PolyP_3$, $PolyP_4$, and tricyclophosphate). The high selectivity of ESI–MS allowed the detection of these compounds without any pre-separation by ion chromatography or capillary electrophoresis. The limits of detection for ESI–MS were estimated to be in the range from approximately 1 to 10 ng ml^{-1} (Choi *et al*., 2000).

2.8 Gel Electrophoresis

One of the most effective and widely used methods for PolyP separation is gel electrophoresis. This technique was adapted for PolyPs by Robinson *et al*. (1984, 1987) and Pepin *et al*., 1986. Electrophoresis in 15–20 % polyacrylamide gel allows PolyP resolution up to a chain length of 100–200 phosphate residues. A mixture of 90 mM trisborate (pH 8.3) with 2.7 mM

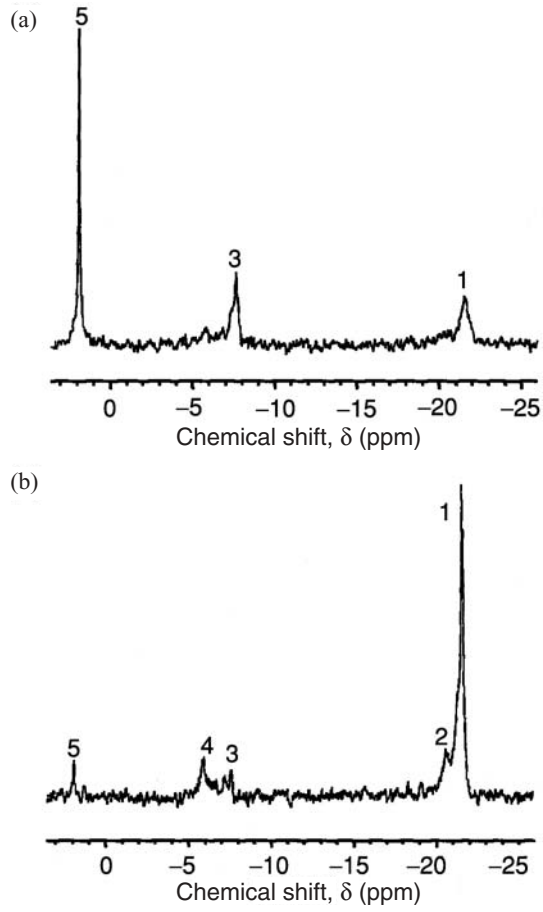

Figure 2.10 NMR spectra of acid-soluble PolyPs extracted from vacuoles of yeast (*Saccharomyces cerevisiae*): (a) PolyP precipitated from the extract with Ba^{2+} at pH 8.2; (b) PolyP precipitated from the extract with Ba^{2+} at pH 4.5: 1, middle phosphate groups; 2, 'pre-terminal' phosphate groups; 3, terminal phosphate groups; 4, γ-phosphate groups of nucleoside triphosphates; 5, P_i (Trilisenko *et al*., 2002).

EDTA and 7 M urea is normally used (Robinson *et al*., 1984, 1987; Clark and Wood, 1987; Lorenz *et al*., 1994b). The gels were stained with 0.05 % toluidine blue, 25 % methanol and 1 % glycerol, followed by 'de-staining' in 25 % methanol and 5 % glycerol (Pepin and Wood, 1986), or radioactive PolyPs were detected by autoradiography. A typical example of the gel electrophoresis of a number of commercial PolyP preparations (Monsanto and Sigma) in 15 % polyacrylamide gel is shown on Figure 2.11.

PolyPs with longer chains can be separated by using low-percentage polyacrylamide gels for PolyPs of 800 residues or agarose gels for PolyPs with chain lengths of 500–1700 residues (Clark and Wood, 1987). A preparative procedure to obtain PolyPs of limited chain lengths by using electrophoresis was developed by Pepin and Wood (1986).

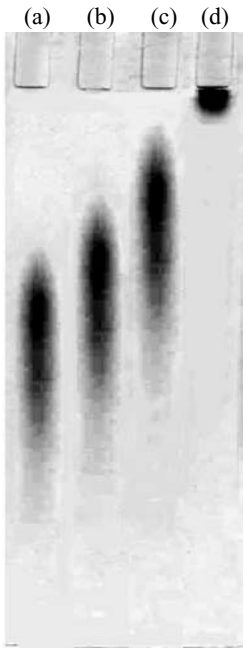

Figure 2.11 Electrophoresis of PolyPs with average chain lengths of (a) 15, (b) 25, (c) 45 (Sigma) and (d) 188 (Monsanto) phosphate residues in 20 % polyacrylamide gel (PAAG) with 7 M urea; staining was achieved by using toluidine blue (Pestov *et al*., 2003).

Elaboration of new electrophoretic methods for PolyP separation is continuing. For example, capillary electrophoretic separations of sodium PolyPs with chain lengths of 5 to 44 has been reported. In this work, a buffer containing pyromellitic acid, triethanolamine and hexamethonium hydroxide gives high-resolution separation of linear and cyclic PolyPs (Stover, 1997; Wang and Li, 1998).

Because of its efficiency, the electrophoretic method is now widely used in studies of PolyPs. It should be noted that for electrophoretic evaluation, the PolyPs must be extracted from biological material, while nucleic acids, proteins and other interfering compounds must be eliminated.

2.9 Enzymatic Methods

The greatest advantage of enzymatic methods for PolyP determination is their high specificity to PolyPs. Their wide application in recent years results from the development of adequate methods of obtaining PolyP-dependent enzymes in sufficient quantities.

The first method of enzymatic PolyP assay was proposed by Clark *et al*. (1986). In this technique, PolyPs were determined by polyphosphate glucokinase obtained from *Propionibacterium shermanii*. Glucose-6-phosphate dehydrogenase reduced NADP through utilization of the formed glucose-6-phosphate, and the increase in NADPH concentration was measured.

At the present time, many methods using PolyP-dependent enzymes for the assay of their substrates have now been developed. Polyphosphate kinase (PPK) catalyses the reversible

transfer of the terminal γ-phosphate of ATP to PolyP (Kornberg *et al*., 1956) The *Escherichia coli* polyphosphate kinase gene has been cloned, sequenced (Akiyama *et al*., 1992) and overexpressed (Crooke *et al*., 1994). Thus, the recombinant PPK is available for enzymatic analysis.

Exopolyphosphatase (PPX) catalyses the hydrolysis of the PolyP terminal residues to P_i almost completely (Akiyama *et al*., 1993). Bacterial PPX does have preference for longer PolyPs, while the major *Saccharomyces cerevisiae* exopolyphosphatase PPX1 can act on PolyP chains of 3 to 1000 residues. Cloning the gene for PPX1 (Wurst *et al*., 1995) and overproducing it in *E. coli* enabled the use of this enzyme as a powerful analytical reagent. It is $\sim$ 100-fold more active as the specific exopolyphosphatase of *E. coli*. In *S. cerevisiae*, another highly active exopolyphosphatase was found, which had preference for longer PolyPs, similar to the bacterial form (Andreeva *et al*., 2001). Therefore, several enzymes may be used for the development of enzymatic analysis of PolyPs with different chain lengths.

Two enzyme-based methods for the estimation of PolyPs in biological samples have been described (Ault-Riché and Kornberg, 1999). The first of these (Rao *et al*., 1998) requires prior labelling of the culture with ^{32}P. PolyPs are extracted from the cells by treatment with a solution containing formic acid, urea, sodium dodecyl sulfate (SDS), EDTA and carrier $PolyP_{65}$. The suspension is sonicated, and the PolyPs are bound to DE81 ion-exchange filter discs. The latter are washed, and the PolyPs are eluted with KCl. ATP and other phosphorus-containing organic compounds are removed from the eluate by using Norit charcoal. The PolyPs are concentrated by re-adsorption onto DE81 discs. After washing, the discs are treated with the purified recombinant yeast spPPX1. Decrease in the ^{32}P content on the filters or an increase in the ^{32}P content released from the filter corresponds to the amount of PolyP. Limitations of the radioactive method are the requirement for prior culture labelling and the inconsistent extraction of PolyPs.

There is an example of using the recombinant yeast spPPX1 for PolyP analysis without the need for prior labelling of the culture (Ruiz *et al*., 2001a). Aliquots of PolyP extracts ($\sim$ 1.5 nmol) were incubated for 15 min at 37 °C with 60 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$ and 3000–5000 units of purified spPPX1 in a final volume of 0.075 ml. One unit corresponded to the release of 1 pmol of P_i per min at 37 °C. The release of P_i was monitored by various well-known chemical methods.

The second modern enzymatic method of PolyP assay (Ault-Riché *et al*., 1998; Ault-Riché and Kornberg, 1999) involves the rapid isolation of PolyP by using powdered glass or glass filters, followed by its conversion to ATP under the action of PPK and an estimation of the generated ATP using a luciferin–luciferase system. The cells were lyzed with 4 M guanidinium isothiocyanate, and PolyP was adsorbed onto glass or a glass filter. SDS was added during the binding step to prevent protein binding. PolyP was eluted by hot water for a buffer with low ionic strength. Then, the PolyP was converted to ATP by purified polyphosphate kinase in the presence of a 10-fold excess of ADP. The ATP generated was measured by a luminometer.

Therefore, the availability of purified enzymes specific to PolyPs allowed the development of rapid, sensitive and definitive assays. It should be noted, however, that PolyPs in biological samples may not be effectively hydrolysed by exopolyphosphatase or be available for polyphosphate kinase (Sethuraman *et al*., 2001).

Thus, many effective methods for PolyP determination and characterization have now been developed. These allow PolyP assays not only in extracts, but also in cell homogenates or even in living cells. The most appropriate information about the PolyP content, polymerization degree and metabolism may be obtained by combining different methods, including the extraction of PolyPs from cells.

3

THE OCCURRENCE OF POLYPHOSPHATES IN LIVING ORGANISMS

The first report on the presence of condensed inorganic phosphates in living organisms dates back to 1888, when Liebermann (Liebermann, 1888) found them in yeast 'nuclein'. Soon after that, Kossel (Kossel, 1893) and Ascoli (Ascoli, 1899) showed that condensed phosphates formed a part of 'plasminic acids', obtained by the partial hydrolysis of yeast nucleic acids. Recent data have shown that PolyPs are widespread in various organisms and can be found in the cells of procaryotes and eucaryotes, especially fungi, plants and animals. Table 3.1 lists some organisms in which condensed phosphates have been identified. Only reviews are cited in this table concerning *Escherichia coli* and *Saccharomyces cerevisiae,* the microorganisms where PolyP metabolism has been most extensively studied. It should be noted, however, that new bacteria species from activated sludge accumulating PolyPs are very numerous and only some of them are presented in the table (see Chapter 9).

In addition, it should be mentioned that only certain methods for the detection of PolyPs may provide good evidence of their occurrence in the organisms under study. These are the chemical methods of extraction, along with the subsequent identification of cyclotriphosphate among the products of partial hydrolysis by chromatography, enzymatic methods, electophoretic techniques and ^{31}P NMR spectroscopy, which were described earlier in Chapter 2. The data obtained by cytochemical methods must now be regarded as being only preliminary.

On the other hand, it is also necessary to treat with caution any conclusions about the absence of PolyPs in any particular organism. Their content depends significantly on the development stage, growth conditions, tissues or cell compartments being analysed. Reports on the absence of PolyPs in various organisms, e.g. *Streptococcus faecalis* (Harold, 1966), several algae (Langen, 1958), some insects (Wiame and Lefebvre,1946), many *Actinomycetes* (Kokurina *et al*., 1961), lichens and crayfish (Kulaev, 1979), and cells and tissues

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Table 3.1 The occurence of polyphosphates in living organisms.

Species	Reference
Procaryotes	
Archae	
Halobacterium salinarium	Andreeva *et al*., 2000; Smirnov *et al*., 2002a,b
Methanosarcina frisia	Rudnick *et al*., 1990
Sulfolobus acidocaldarius	Skorko, 1989
Sulfolobus solfataricus	Cardona *et al*., 2002
Cyanobacteria	
Anabaena variabilis	Carr and Sandhu, 1966; Correl, 1965; Correl and Tolbert, 1962, 1964; Drews and Niklowitz, 1957; Kostlan, 1972
Anacystis nidulans	Niemeyer and Richter, 1969; Keyhani *et al*., 1996
Clocothece sp.	Fuhs, 1958
Cylindrosperum lichiniforme	Drews and Niklowitz, 1957
Lingbyaerogineo amplivaginata	Bringmann, 1950
Lingbyaerogineo coerula	Bringmann, 1950
Nostoc puriforme	Jensen, 1968
Oscillatoria amoena	Fuhs, 1958
Oscillatoria limosa	Drews and Niklowitz, 1957
Oscillatoria sp.	Ebel *et al*., 1958b; Keck and Stich, 1957
Phormidium ambiguum	Ebel, 1952a–c
Phormidium frigidum	Keck and Stich, 1957
Phormidium uncinatum	Keck and Stich, 1957
Plectonema boryanum	Jensen, 1969; Jensen and Sicko, 1974
Eubacteria	
Acetobacter suboxydans	Klungsöyr *et al*., 1957
Acinetobacter sp.	Deinema *et al*., 1985; Bonting *et al*., 1991, 1993a,b; Kortstee *et al*., 2000
Actinomyces (Streptomyces) aureofaciens	Guberniev *et al*., 1959; Prokof'eva-Bel′govskaya and Kats, 1960; Guberniev and Torbochkina, 1961; Belousova and Popova, 1961; Kokurina *et al*, 1961; Kulaev *et al*, 1976
Actinomyces (Streptomyces) eritreus	Guberniev *et al*., 1961
Actinomyces (Streptomyces) griseus	Ebel *et al*., 1958a,b; Hagedorn, 1959
Aerobacter (Klebsiella) aerogenes	Smith *et al*., 1954; Widra, 1959; Harold, 1963a,b, 1964, 1966; Ohtake *et al*., 1999; Kato *et al*., 1993b
Aerobacter cloaceae	Smith *et al*., 1954
Agrobacterium radiobacter	Tardieux-Roche, 1964
Azotobacter agile	Zaitseva and Belozersky, 1958; Zaitseva and Tszyun-in, 1961

Table 3.1 (*Continued*)

Species	Reference
Azotobacter vinelandii	Esposito and Wilson, 1956; Zaitseva and Belozersky, 1960; Zaitseva *et al*., 1959, 1960a,b, 1961; Zaitseva and Frolova, 1961
Bacillus brevis	Hughes and Muhammed, 1962
Bacillus cereus	Tardieux-Roche, 1964
Bacillus freidenreihii	Smith *et al*., 1954
Bacillus megaterium	Hughes and Muhammed, 1962
Bacillus prodigiosus	Hughes and Muhammed, 1962
Brevibacterium ammoniagenes	Kozel'tsev *et al*., 1969
Burkholderia cepacia	Mullan *et al*., 2002
Caulobacter vibroides	Grula and Hartsell, 1954
Chlorobium thiosulfatofillium	Cole and Hughes, 1965; Fedorov, 1959; Hughes *et al*., 1963
Chromatium okenii	Schlegel, 1962
Chromatium sp.	Fedorov, 1959
Clostridium sp.	Szulmajster and Gardiner, 1960
Clostridium sporogenes	Hughes and Muhammed, 1962
Corynebacterium diphteriae	Minck and Minck, 1949; Belozersky *et al*., 1950; Davis and Mudd, 1955, 1956; Ebel, 1949, 1951, 1952a–d; Korchagin, 1954; Sall *et al*., 1956, 1958; Szymona and Szymona, 1961
Corynebacterium xerosis	Dirheimer, 1964; Dirheimer and Ebel, 1964b; Hughes and Muhammed, 1962; Muhammed *et al*., 1959; Widra, 1959
Escherichia coli	Nesmeyanova *et al*., 1973; Nesmeyanova, 2000; Kornberg, 1995, 1999; Kornberg *et al*., 1999
Helicobacter pylori	Bode *et al*., 1993
Hydrogenomonas sp.	Kaltwasser and Schlegel, 1959; Kaltwasser, 1962; Schlegel, 1951; Schlegel and Kaltwasser, 1961
Lactobacillus casei	Hughes and Muhammed, 1962
Micrococcus denitrificans	Kaltwasser *et al*., 1962
Micrococcus flavus	Kawai *et al*., 2000
Micrococcus lysodeikticus	Friedberg and Avigad, 1968
Microlutatus phosphovorus	Nakamura *et al*., 1995
Microtrix parvicella	Erhart *et al*., 1997
Mycobacterium avium	Ruska *et al*., 1951/1952
Mycobacterium cheloni	Mudd *et al*., 1958
Mycobacterium phlei	Drews, 1958a,b, 1959a,b, 1960a,b; Glauert and Brieger, 1955; Hughes and Muhammed, 1962; Knappe *et al*., 1959; Winder and Denneny, 1954, 1957
Mycobacterium smegmatis	Hughes and Muhammed, 1962; Winder and Denneny, 1955, 1957; Winder and Roche, 1967
Mycobacterium thramnopheos	Mudd *et al*., 1958

(*continued*)

Table 3.1 (*Continued*)

Species	Reference
Mycobacterium tuberculosis	Hughes and Muhammed, 1962; Kölbel, 1958; Krüger-Thiemer and Lembke, 1954; Meissner, 1957; Meissner and Diller, 1953; Meissner and Kropp, 1953; Winder and Denneny, 1957
Myxococcus coralloides	Gonzales *et al*., 1990
Myxococcus xanthus	Voelz *et al*., 1966
Neisseria meningitidis	Tinsley *et al*., 1993
Nitrobacter sp.	Butt and Lees, 1960
Nitrobacter winogradsky	Ullrich and Bock, 1972; Van Gool *et al*., 1971
Nitrosomonas europaea	Terry and Hooper, 1970
Propionibacterium shermanii	Konovalova and Vorob'eva, 1972; Kulaev *et al*., 1973a; Clark *et al*., 1986
Pseudomonas aerugenosa	Kim *et al*., 1998; Ishige *et al*., 1998; Miyake *et al*., 1999
Rhodopseudomonas palustris	Fedorov, 1959, 1961
Rhodopseudomonas spheroides	Carr and Sandhu, 1966; Weber, 1965
Rhodospirillum rubrum	Baltcheffsky, 1967a,b,c, 1969; Kulaev *et al*., 1974a; Niklowitz and Drews, 1955; Weber, 1965
Serratia marcescens	Smith *et al*., 1954
Spirillum volutans	Belozersky, 1941, 1945
Staphylococcus albus	Hughes and Muhammed, 1962
Staphylococcus aureus	Kulyash, 1972
Streptococcus sl-1	Tanzer and Krichevsky, 1970
Streptomyces lividans	Chouayekh and Virolle, 2002
Tetrasphera australiensis	Maszenan *et al*., 2000
Tetrasphera elongata	Hanada *et al*., 2002
Tetrasphera japonica	Maszenan *et al*., 2000
Eucaryotes	
Algae	
Acetabularia crenulata	Kulaev *et al*., 1975; Niemeyer and Richter, 1972
Acetabularia mediterranea	Grunze and Thilo, 1955; Richter, 1966; Rubtsov *et al*., 1977; Rubtsov and Kulaev, 1977; Stich, 1953, 1955, 1956; Thilo *et al*., 1956
Ankistrodesmus braunii	Domanski-Kaden, and Simonis, 1972; Kanai and Simonis, 1968; Lysek and Simonis, 1968; Ullrich and Simonis, 1969; Ullrich, 1970
Ceramium sp.	Langen, 1958; Lohmann, 1958
Chara sp.	Keck and Stich, 1957
Chilomonas sp.	Ebel *et al*., 1958b
Chlamydomonas reinhardtii	Ruiz *et al*., 2001b
Chlamydomonas sp.	Keck and Stich, 1957

Table 3.1 (*Continued*)

Species	Reference
Chlorella ellipsoides	Hase *et al*., 1963; Kanai *et al*., 1963; Miyachi, 1961; Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi *et al*., 1964; Nihei, 1955, 1957; Tamiya *et al*., 1953
Chlorella pyrenoidosa	Baker and Schmidt, 1963, 1964a,b; Curnutt and Schmidt, 1964a,b; Hermann and Schmidt, 1965; Kandler, 1957; Okutsov and Grebennikov, 1977; Schmidt, 1966; Sommer and Booth, 1938; Wintermans, 1954, 1955; Wintermans and Tija, 1952
Chlorella sp.	Correll, 1965; Ebel *et al*., 1958; Holzer, 1951; Khomlyak and Grodzinskii, 1972; Wassink, 1957; Wassink *et al*., 1951
Chlorella vulgaris	Kuhl, 1960
Cladophora sp.	Langen, 1958
Cosmarium sp.	Keck and Stich, 1957
Dunaliella viridis	Vagabov and Serenkov, 1963
Dunaliella salina	Pick and Weiss, 1991
Enteromorpha sp.	Langen, 1958; Lohmann, 1958
Euglena gracilis	Albaum *et al*., 1950; Lohmann, 1958; Smillie and Krotkov, 1960
Fragillaria sp.	Keck and Stich, 1957
Hydrodiction reticulatum	Kuhl, 1960, 1962; Pirson and Kuhl, 1958
Mongeotia sp.	Keck and Stich, 1957
Navicula sp.	Keck and Stich, 1957
Oedogonium sp.	Keck and Stich, 1957
Phaerodactilum tricornutum	Leitao *et al*., 1995
Polytomela ceca	Wiame and Lefebvre, 1946
Rhodopalocystis gleifera	Täumer, 1959
Scenedesmus obliquus	Pakhomova *et al*., 1966; Kulaev and Vagabov, 1967; Vagabov and Serenkov, 1963
Scenedesmus quadricauda	Baslavskaya and Bystrova, 1964; Overbeck, 1961, 1962
Scenedesmus sp.	Goodman *et al*., 1955; Sundberg and Nilshammer-Holmvall, 1975
Spirogira sp.	Keck and Stich, 1957
Ulotrix sp.	Keck and Stich, 1957
Vaucheria sp.	Keck and Stich, 1957
Zygnema sp.	Keck and Stich, 1957
Fungi (including yeast)	
Agaricus bisporus	Kritsky and Kulaev, 1963; Kritsky *et al*., 1965a,b; Kulaev *et al*., 1960a,b
Aspergillus nidulans	Shepherd, 1957

(*continued*)

Table 3.1 (*Continued*)

Species	Reference
Aspergillus niger	Bajaj *et al*., 1954; Bajaj and Krishnan, 1953; Belozersky, 1958, 1959a,b; Belozersky and Kulaev, 1957; Ebel *et al*., 1958b; Ingelman, 1947, 1948; Ingelman and Malmgren, 1950; Kulaev, 1956, 1971; Kulaev and Belozersky, 1957, 1958, 1962a,b; Lindeberg and Malmgren, 1952; Mann, 1944a,b; Nishi, 1961; Krishnan and Bajaj, 1953a,b; Krishnan *et al*., 1957; Neuberg and Fischer, 1938; Mikhailovskaya, 1951
Aspergillus oryzae	Jordanov *et al*., 1962
Badihamia utricularis	Niklowitz, 1957
Botryotridium pililiferum	Ebel *et al*., 1958b
Brethanomyces animalis	Ebel *et al*., 1958b
Candida guillermondii	Grigor'eva *et al*., 1973; Levchuk *et al*., 1969; Naumova *et al*., 1968; Streshinskaya *et al*., 1970; Vorob'eva *et al*., 1973; Kulaev *et al*., 1974b
Candida humicola G-1	McGrath and Quinn, 2000
Candida tropicalis	Ebel *et al*., 1958b
Candida utilis	Bourne, 1990; Nunez and Callieri, 1989
Candida vulgaris	Ebel *et al*., 1958b
Chaetonium globosum	Ebel *et al*., 1958b
Choenephora cucurbitarumi	Dietrich, 1976
Claviceps paspali	Kulaev and Uryson, 1965
Claviceps purpurea	De Waart, 1961; De Waart and Taber, 1960
Cliocladium roseum	Ebel *et al*., 1958b; Muller and Ebel, 1958a,b
Colleotrichum lindenmuthianum	Dietrich, 1976; Rodrigues, 1993
Cryptococcus laurentii	Dietrich, 1976
Culvularia maculans	Dietrich, 1976
Cunigamella elegans	Dietrich, 1976
Dipliodia natalensis	Ebel *et al*., 1958b
Endomyces magnusii	Afanas'eva and Kulaev, 1973; Afanas'eva *et al*., 1968; Kulaev, 1956; Kulaev and Afanas'eva, 1969, 1970; Kulaev *et al*., 1967a,b, 1970c; Skryabin *et al*., 1973
Fusarium coccineum	Navashin *et al*., 1983
Fusarium coeruleum	Ebel *et al*., 1958; Muller and Ebel, 1958
Giromitra esculenta	Kulaev *et al*., 1960
Isaria gelina	Ebel *et al*., 1958b
Kloeckera mulleri	Ebel *et al*., 1958b
Lethinus tigrinus	Kritsky and Belozerskaya, 1968
Morchella hortansis	Ebel *et al*., 1958b
Mortierella alpina	Dietrich, 1976
Mucor javanicum	Dietrich, 1976
Mucor mucedo	Datema *et al*., 1977
Mucor racemosus	James and Casida, 1964

Table 3.1 (*Continued*)

Species	Reference
Mycelia steria	Ebel *et al*., 1958b
Mycothecium verrucaria	Ebel *et al*., 1958b; Muller and Ebel, 1958
Mycotorula eisenorosa	Ebel *et al*., 1958b
Nectria rubi	Ebel *et al*., 1958b
Neurospora crassa	Harold, 1959, 1960, 1962a,b; Harold and Miller, 1961; Houlahan and Mitchell, 1948; Krasheninnikov *et al*., 1967, 1968; Kritsky *et al*., 1970, 1972; Kulaev and Konoshenko, 1971a,b; Kulaev *et al*, 1966a,b, 1967, 1968, 1970a,b,c,d, 1972a,b,c; Kulaev and Mel'gunov; 1967, Kulaev and Urbanek, 1966; Mel'gunov and Kulaev, 1971; Chernycheva *et al*., 1971; Chernysheva, 1972; Kulaev and Bobyk, 1971; Afanas'eva and Kulaev, 1973
Penicillium chrysogenum	Belozersky, 1959; Dmitrieva and Bekker, 1962; Ebel *et al*., 1958b; Kulaev, 1956, 1971,1974; Kulaev *et al*., 1959, 1960b,c, 1961, 1964a,b, 1966b; Kulaev and Okorokov, 1967, 1968; Okorokov *et al*., 1973a,b; Gotovtseva, 1956
Phycomyces balkesleanus	Lohmann and Langen, 1956; Petras, 1958
Physarum polycephalum	Goodman *et al*., 1968; Guttes *et al*., 1961; Sauer *et al*., 1956; Pilatus *et al*., 1989
Physarum sp.	Keck and Stich, 1957
Pisolithus tinctorius	Ashford *et al*., 1999
Rhodotorula rubra	Ebel *et al*., 1958b
Saccharomyces carlsbergensis	Kulaev and Vagabov, 1983
Saccharomyces cerevisiae	Kulaev and Vagabov, 1983; Kornberg, 1995, 1999; Kulaev *et al*., 1999
Saccharomyces fragilis	Tijssen *et al*., 1982
Saccharomyces mellis	Weimberg, 1970; Weimberg and Orton, 1964, 1965
Schizosaccharomyces pombe	Kulaev *et al*., 1973
Staphylotrichum coccosporum	Ebel *et al*., 1958b
Torula sp.	Ebel, 1951, 1952
Torula spora roxi	Ebel *et al*., 1958
Torula utilis	Chayen *et al*., 1955; Rautanen and Mikkulainen, 1951
Vertiollium honigii	Ebel *et al*., 1958
Zygorhyncus exponens	Dietrich, 1976
Mosses	
Leptobrium, Polytrichum	Keck and Stich, 1957
Protozoa	
Amoeba chaos chaos	Mattenheimer, 1958
Amoeba sp.	Ebel *et al*., 1958

(*continued*)

Table 3.1 (*Continued*)

Species	Reference
Crithidia fasciculata	Janakidevi *et al*., 1965
Entamoeba sp.	Lopez-Peville and Gomez-Domingues, 1985
Leishmania major	Rodrigues *et al*., 2002a; Moreno *et al*., 2000
Tetrahymena pyriformis	Rosenberg, 1966
Trypanosoma brucei	Moreno *et al*., 2000
Trypanosoma cruzi	Ruiz *et al*., 2001a
Flowering plants	
Cotton, seeds	Asamov and Valikhanov, 1972; Valikhanov *et al*., 1980
Cuscuta reflexa	Tewari and Singh, 1964
Deschampsia flexiosa	Nassery, 1969
Banksia ornata, roots and stems	Jeffrey, 1964
Lemna minor	Inhülsen and Niemeyer, 1975; Niemeyer, 1975
Maize, roots	Vagabov and Kulaev, 1964
Malus domestica (apple), leaves	Schmidt, 1971, 1972; Schmidt and Buban, 1971
Solanum lycopersicum (tomato), leaves, hypocotils	Khomlyak and Grodzinskii, 1970, 1972; Klein, 1952
Spinacea oleracea (spinach), leaves	Miyachi, 1961
Triticum vulgare (wheat), leaves	Wang and Manchini, 1966
Urtica dioica	Nassery, 1969

of higher animals and plants (Ebel, 1952c; Korchagin, 1954; Lohmann, 1958) should *not* be regarded as evidence for their inability to synthesize and accumulate these compounds under suitable conditions of growth or in separate cell compartments.

With regard to animals, PolyPs were found in the freshwater sponge *Ephydatia muelleri* (Imsiecke *et al*., 1996) and in some insects, namely the imago stage of *Blaberus cranifera* (Kulaev *et al*., 1974c), larvae of *Deilephila euphorbiae* (Heller *et al*., 1950; Heller,1953, 1954) and *Galeria mellonella* (Niemerko, 1950, 1953; Niemierko and Niemierko,1950), excretions of *Achroca grissela* (Pierpoint, 1957c), *Celleria euphorbiae* (Heller, 1953),and *Galeria mellonella* (Niemierko, 1950, 1953, Niemierko and Niemierko,1950; Wojtezak, 1954; Pierpoint, 1957c).

PolyPs were also observed in embryos of frog *Rana japonica* (Shiokawa and Yamana, 1965) and different tissues of mammalians (Grossman and Lang, 1962; Penniall and Griffin, 1964; Griffin *et al*., 1965; Gabel, 1971; Gabel and Thomas, 1971; Mansurova *et al*., 1975a; Kumble and Kornberg, 1995; Lorenz *et al*., 1997b; Leyhausen *et al*., 1998; Kornberg, 1999; Schröder *et al*., 1999, 2000). Thus, it can be seen that PolyPs are very widespread in living organisms at different stages of evolution.

4

THE FORMS IN WHICH POLYPHOSPHATES ARE PRESENT IN CELLS

As mentioned in Chapter 2, PolyPs may be present in living cells both in the free and bound states. Modern methods, including ^{31}P NMR spectroscopy, give some evidence for this concept. Since the classical work of MacFarlane (1936), it has been considered that acid-soluble low-molecular-mass PolyPs are present in cells in the free state. However, the question as to the state of the more polymeric PolyPs within a cell cannot yet be considered as having been finally resolved.

4.1 Polyphosphate–Cation Complexes

Being polyanions, PolyPs can form complexes with different cations including biologically important Ca^{2+} and Mg^{2+} (Van Wazer and Campanella, 1950; Van Wazer, 1958; Corbridge, 1980; Bonting *et al.,* 1993a; Cini *et al.,* 2000). The dissociation constants for Mg^{2+}– and Ca^{2+}–polyphosphate complexes were 9.3×10^{-2} M and 1.5×10^{-2} M, respectively (Bonting *et al.,* 1993a).

Using electron microscopy and energy disperse X-ray microanalysis, it was shown that PolyP granules of cyanobacteria contain Mg^{2+}, Ca^{2+}, Mn^{2+} and other cations (Baxter and Jensen, 1980a,b; Jensen *et al.,* 1982). Large PolyP granules of *Acinetobacter* contain Mg^{2+}, Ca^{2+} and K^{+} in a ratio which depends on the extracellular concentrations of the above cations (Bonting *et al.,* 1993a). In bacteria, PolyP complexes with heavy metals were observed: with Sr^{2+} and Ba^{2+} in *Plectonema boryanum* (Baxter and Jensen, 1980 a,b), with Ni^{2+} in *Staphylococcus aureus* (Gonzales and Jensen, 1998), and with Cd^{2+} in *Anacystis nidulans* (Keyhani *et al.,* 1996) and *Escherichia coli* (Keasling and Hupf, 1996; Keasling, 1997; Keasling *et al.,* 2000).

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PolyPs can form complexes with arginine, spermidine, lysine, Mg^{2+}, Ca^{2+} and Mn^{2+} in vacuoles of yeast (Wiemken and Dürr, 1974; Dürr *et al.,* 1979; Okorokov *et al.,* 1980; Lichko *et al.,* 1982; Westenberg *et al.,* 1989; Dünn *et al.,* 1994) and *Neurospora crassa* (Cramer and Davis, 1984). The cells of protozoa (Docampo and Moreno, 2001; Rodrigues *et al.,* 2002a,b) and some algae (Ruiz *et al.,* 2001b) possess an acidic organelle, acidocalcisome, which contains pyrophosphate and PolyPs bound with Ca^{2+}, Mg^{2+}, Zn^{2+} and other cations. It should be noted that in these organelles, low-molecular-weight PolyPs, including $PolyP_3$, are present in the bound state.

4.2 Polyphosphate–Ca^{2+}–Polyhydroxybutyrate Complexes

Specific complexes containing polyhydroxybutyrate (PHB) and PolyPs have been found in membranes of many organisms (Reusch and Sadoff, 1988; Reusch, 1992, 1999a, 2000). When such components of *E. coli* membranes were isolated and analysed, Ca^{2+} was found to be the predominant neutralizing cation (Reusch and Sadoff, 1988). The polymer length was 130–150 residues for PHB ($\sim$ 12 kD), as measured by non-aqueous size-exclusion chromatography (Seebach *et al.,* 1994a), and 55–70 residues for PolyP ($\sim$ 5 kD), as determined by acrylamide gel electrophoresis (Castuma *et al*., 1995). The molecular weight of the complex was estimated as 17 ($\pm$ 4) kD by non-aqueous size-exclusion chromatography (Reusch *et al.,* 1995). These measurements indicate a 1:1 ratio between the two polymer strands and a 2:1 ratio of monomer residues for PHB:PolyP.

The detailed structure of PolyP–PHB complexes is still unknown. However, some assumptions were made on the basis of physical properties and sizes of the polymers, and the low dielectric environment they inhabit. It is clear that the highly polar polyanionic PolyPs must be shielded from the hydrophobic region of the bilayer by amphiphilic PHB. The models for membrane channel complexes were proposed by Reusch and co-workers (Reusch and Sadoff, 1988; Reusch *et al.,* 1995) and by Seebach and co-workers (Seebach *et al*., 1994b, 1996). The first model proposes that PHB has a coiled conformation such as it displays in solution (Figure 4.1), while the second one assumes that PHB maintains the folded helix form of its solid-state structure (Figure 4.2). A consequence of both arrangements is the formation of multiple parallel 'lanes' between the two polymers, with multiple cation-binding sites lining each of these lanes.

In the 'Reusch model', the complexes have the liquid properties of polymer electrolytes and this suggests a family of conformations rather than a single defined structure. In the 'Seebach model', several PHB molecules surround the PolyP unit. Individual PHB chains are free to adopt various positions in the phospholipid lattice; hence, a well-defined structure is again unlikely. Further studies may help us in choosing one of these two proposed models.

4.3 Complexes of Polyphosphates with Nucleic Acids

PolyP–ribonucleic acid complexes have been isolated from a variety of organisms (Belozersky, 1955, 1958, 1959a; Chayen *et al.,* 1955; Chaloupka and Babicky, 1957, 1958;

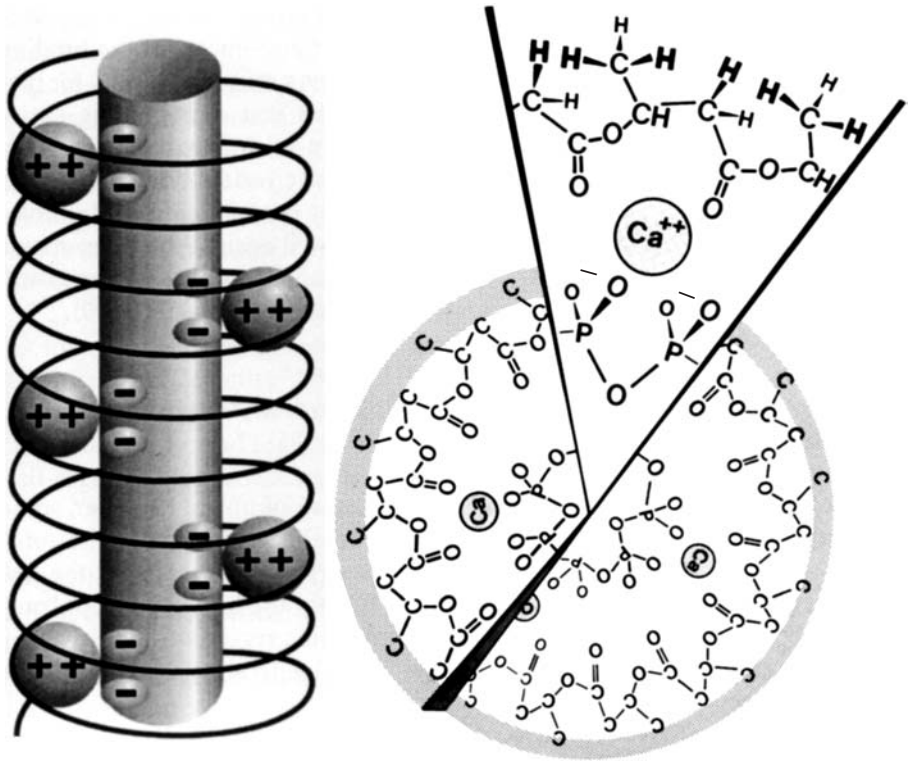

Figure 4.1 Model of the PolyP–PHB channel structure as proposed by Reusch and co-workers (from Reusch, 1999a). The central cylinder represents the PolyP helix with binding sites for Ca^{2+}, with the Ca^{2+}–PolyP complex being surrounded by the PHB helix (Reusch and Sadoff, 1988; Das *et al.,* 1997).

Kulaev and Belozersky, 1957, 1958; Ebel *et al.,* 1958c, 1962, 1963; Langen and Liss, 1958; Stahl and Ebel, 1963; Wang and Manchini, 1966).

All of these investigations sought to resolve the question of whether the RNA was combined with the PolyP or if they were simply co-precipitated during extraction and separation from the cells as a result of similarities in their chemical and physico-chemical properties. Solution of this problem has been found to cause great difficulties. It was found impossible to separate these compounds completely by precipitation and re-precipitation in the presence of Ba^{2+}, Mg^{2+}, Ca^{2+} and other metal ions. The use of different conditions for RNA separation (by sodium dodecyl sulfate (SDS) or phenol, or by a combination of the two) from yeast cells, which contained large amounts of PolyPs, failed to yield RNA fractions free from PolyPs. It proved especially difficult to separate PolyPs from such RNA fractions when they contained relatively small amounts of PolyPs. For example, the RNA–PolyP complex obtained from *Aspergillus niger* could not be separated by electrophoresis in a Tiselius apparatus (Kulaev and Belozersky, 1958). The same conclusion was drawn when paper chromatography was used in an attempt to separate the PolyP–RNA complex from yeast, in which RNA predominated (Chayen *et al.,* 1955). When fractions

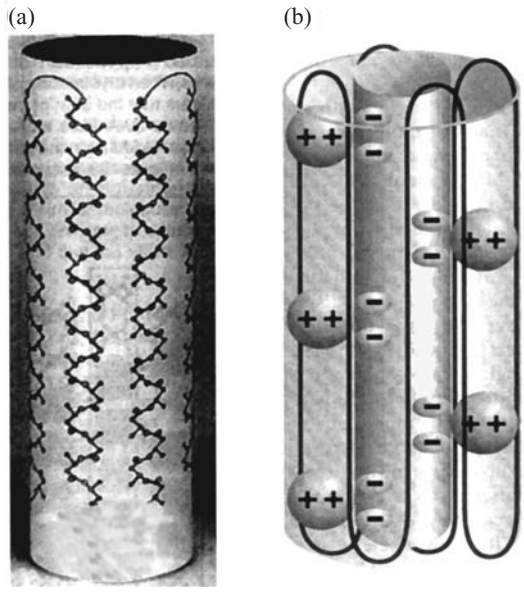

Figure 4.2 Models of the PolyP–PHB channel structure as proposed by Seebach and co-workers (from Reusch, 1999a). (a) The tube-like arrangement of PHB helices, where neighbouring helices form a cylinder surrounding the Ca^{2+}–PolyP complex (Seebach *et al.,* 1994a, 1996). (b) The central cylinder represents the PolyP helix, which contains binding sites for Ca^{2+}. The Ca^{2+}–PolyP complex is surrounded by and solvated by an outer cylinder composed of a β-like sheet of PHB (Das *et al.,* 1997).

containing large amounts of PolyP were examined, however, it was found possible to separate the latter from the RNA either by electrophoresis (Chayen *et al.,* 1955; Ebel *et al.,* 1962; Dirheimer *et al.,* 1963) or by paper chromatography (Ebel *et al.,* 1962; Dirheimer and Ebel, 1964a). In particular, electrophoretic examination in a Tiselius–Swensson apparatus of three PolyP–RNA complexes was undertaken (Belozersky and Kulaev, 1970). This series of experiments was carried out with the PolyP–RNA complex from brewer's yeast (PolyP/RNA ratio of 1:7), after preliminary purification by electrophoresis on a cellulose column, and with two fractions obtained from baker's yeast without any preliminary purification (PolyP/RNA ratios of 1:4 and 1:9).

Parallel experiments were carried out using artificial mixtures of PolyP and RNA with various ratios of the components. Electrophoresis was performed in acetate buffer: pH 4.5–4.7, ionic strength 0.04, temperature 2 °C, current 9 mA, and potential gradient 6–7 V cm^{-1}. In all of the experiments, the mean electrophoretic mobilities of the PolyP and RNA were calculated, together with the approximate ratios of PolyP to RNA, from the areas under the peaks on the electrophoregrams. The electrophoresis of the PolyP–RNA complexes, preliminarily purified by electrophoresis on a cellulose column, gave only a single symmetrical peak. This would appear to indicate the presence in this fraction of a homogenous PolyP–RNA complex, but this particular experiment had the disadvantages that the fraction was present in a very low concentration and only a small amount of PolyP was present therein.

Comparison of the peak areas on the electrophoregrams showed, however, that the PolyP component was much smaller than it should have been had it contained all of the PolyP present in the complex. Thus, in the less PolyP-rich fraction, instead of a PolyP/RNA ratio of 1:4, the electrophoregram showed a ratio of 1:11, and electophoregram examination of a fraction with a PolyP/RNA ratio of 9:1 gave peaks in the ratio of 4:1. This suggested that some part of the PolyP present in these fractions was combined with the RNA, while the other part was in the free state.

In order to establish whether the PolyP was bound to the RNA, even if only partially, by divalent metal cations, the PolyP–RNA fractions were dialysed before electrophoresis against a 10^{-3} M solution of the known complexing agent EDTA. This treatment resulted in a certain increase in the PolyP peaks, although it did not lead to complete separation of all PolyP from the RNA. These results suggested that divalent metal cations played some role in the formation of PolyP–RNA complexes. In order to confirm this assumption, a series of experiments on separation by electrophoresis of artificial mixtures of PolyP and RNA was carried out. In these studies, PolyP from a yeast acid-soluble fraction with an average chain length of 30 residues and synthetic sodium PolyP with an average chain length of 75 residues were used. The pure RNA material (Merck) was the same in both cases. Examination of the results showed that the mixtures, which contained yeast PolyP (with Ca^{2+}), displayed marked discrepancies in the PolyP to RNA ratios before and after separation in a Tiselius apparatus. The area under the early displayed peak was substantially smaller than it should have been with regard to the initial PolyP content in the material. On the other hand, the PolyP/RNA ratio remained nearly the same when the mixture of RNA with synthetic PolyP was analysed. These results demonstrated the absence of covalent bonds in the complexes isolated from biological material. It is very likely that PolyP and RNA are bound by divalent metal cations.

It should be noted that in the work of Ebel and co-workers (Ebel *et al.,* 1958c; 1962, 1963; Dirheimer and Ebel, 1964a; Dirheimer *et al.,* 1963) techniques for the preparative separation of PolyP–RNA complexes from yeast by using activated carbon (Ebel *et al.,* 1962; Muller-Felter and Ebel, 1962; Stahl and Ebel, 1963; Stahl *et al.,* 1964) and Sephadex G-200 (Dirheimer and Ebel, 1964a) were developed. In addition, these complexes can be separated into their components by precipitation of the PolyP in the presence of high concentration of barium salts (Belozersky and Kulaev, 1970).

Belozersky and Kulaev (1970) and Stahl and Ebel (1963) showed that Ca^{2+} and Mg^{2+} ions were responsible for the formation of very stable and 'difficult-to-separate' PolyP/RNA complexes. Investigations into the possible existence of covalent or hydrogen bonds in these complexes have shown that both forms of bonding are absent, while electrostatic interactions mediated by Ca^{2+}, Mg^{2+} and other metal ions are possibly present (Ebel *et al.,* 1962; Belozersky and Kulaev, 1964, 1970). It should be noted that there might be a certain similarity between RNA–PolyP and PHB–PolyP, in particular, participation of divalent cations in the linkage of the two polymers. The model of the linkage of the PolyP and RNA chains through such divalent cations is presented in Figure 4.3.

The question of the functions of RNA–PolyP complexes needs further investigation. It is probable that the complexing with PolyP enhances the RNA stability. Some evidence has been obtained that in *E. coli* PolyPs inhibit RNA degradation by degradosome (Blum *et al.,* 1997).

The possibility of PolyP interaction with DNA is now confirmed by data evidencing its participation in gene activity control (Kornberg, 1999; Kornberg *et al.,* 1999). Earlier,

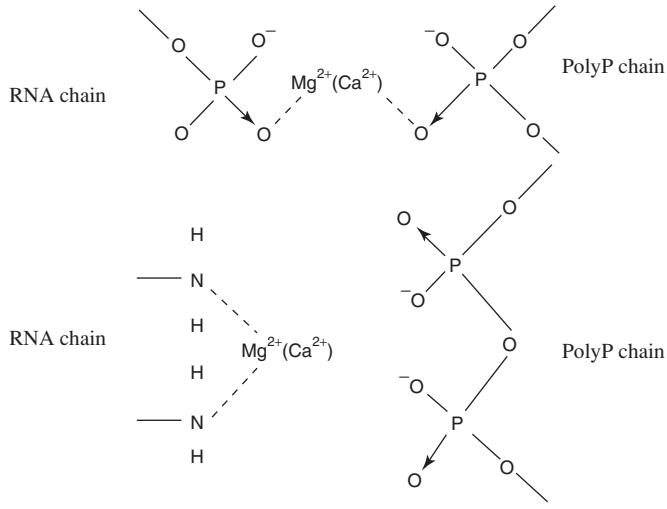

Figure 4.3 Possible modes of linkage of the chains of PolyP and RNA through divalent metal ions (Ebel *et al.,* 1963).

$PolyP_{60}$ was found in DNA preparations from filamentous fungal species of *Colleotrichum* (Rodriguez, 1993).

4.4 Binding of Polyphosphates with Proteins

Many years ago, Liss and Langen (1960a,b) showed that the most highly polymerized yeast PolyP fraction, extractable only with strong alkali (0.05 M) or when kept for a long period with diluted $CaCl_2$ solution, is apparently firmly bound to some cell components other than RNA. The removal of RNA by RNAase had no effect on the extraction rate of this PolyP fraction. It was considered that in this case PolyP was bound to a certain protein.

Later, numerous PolyP-binding proteins were detected in crude cell extracts from different organisms, including yeast and animals, using a filter-binding technique or affinity chromatography on PolyP–zirconia (Lorenz *et al.,* 1994a).

Some of the PolyP complexes with proteins are very important in cell regulatory processes. RNA polymerase isolated from the stationary-phase cells of *E. coli* was found to be closely bound with PolyP (Kusano and Ishihama, 1997). The ATP-dependent protease Lon formed a complex with PolyPs under degradation of ribosomal proteins at amino acids starvation (Kuroda *et al.,* 2001). PolyP is able to compete with DNA for the DNA binding sites at histones (Schröder *et al.,* 1999), while PolyPs can interact with non-histone proteins in the nucleus (Offenbacher and Kline, 1984).

PolyPs and PHB have been found to be associated with ion-conducting proteins such as the human erythrocyte Ca^{2+}–ATPase pump (Reush *et al.,* 1997) and the *Streptomyces lividans* potassium channel (Reusch, 1999b). Some enzymes of PolyP metabolism, such as polyphosphate glucokinase (Phillips *et al.,* 1999) and yeast high-molecular-weight exopolyphosphatase (Andreeva *et al.,* 2001, 2004), can contain tightly bound PolyP.

It is also possible that PolyPs in cells may be combined with other compounds, including polysaccharides, such as polyhexamines and chitin. PolyPs were shown to form complexes with polysaccharides of the cell wall of *N. crassa*, *in vitro* (Harold and Miller, 1961). The complex-forming reaction depended on both pH and the PolyP chain length.

The complexing ability of PolyP is one of the major properties of this negatively charged biopolymer, determining to a considerable extent its regulatory function in living cells. In our opinion, such metabolically active molecules as PolyPs do not exist in cells in the free form in large amounts. They are strongly compartmentalized and combined in the cell, either permanently or temporarily, via chelate bridges with other compounds. As described above, PolyPs can form complexes with such biologically active cations as Ca^{2+}, Mg^{2+}, K^{+}, etc., with polyhydroxybutyrate, and, which is of great importance, with nucleic acids and proteins. Probably, the interactions of PolyPs with other biopolymers are mediated in some cases by Ca^{2+}. However, this has only been established with certainty for PolyP–polyhydroxybutyrate–Ca^{2+} complexes. The ability of PolyPs to form complexes with different components of living cells allows them to perform many specific functions in such cells.

5

LOCALIZATION OF POLYPHOSPHATES IN CELLS OF PROKARYOTES AND EUKARYOTES

5.1 Prokaryotes

Prokaryotic cells have much simpler structures when compared with the simplest eukaryotes, such as yeast, fungi or algae. They have no nucleus enveloped by a membrane and no vacuoles, which are known to possess many PolyPs in eukaryotic cells. The compartmentalization of biochemical processes is not well developed in prokaryotic cells. However, PolyPs are found in all main compartments of the bacterial cell, i.e. cytoplasm, cell surface, periplasm and plasma membrane.

The presence of PolyPs in cellular inclusions, long known as *Babesh–Ernst bodies, metachromatic granules*, or *volutin granules*, was found long ago (Belozersky, 1945; Ebel, 1952d; Ebel and Muller, 1958; Ebel *el al.,* 1955, 1958a,b; Meissner and Diller, 1953; Drews, 1958a,b, 1959a,b, 1960a,b, 1962; Drews and Niklowitz, 1957; Guberniev *et al.,* 1961; Widra, 1959; Wilkinson and Duguid, 1960; Prokof'eva-Bel'govskaya and Kats, 1960; Hughes and Muhammed, 1962; Kulaev and Belozersky, 1962a,b; Verbina, 1964). PolyP granules were observed in cells of many prokaryotes (see the reviews of Kuhl, 1960, 1962, 1976; Harold, 1966; Kulaev and Vagabov, 1983; Wood and Clark, 1988), including PolyP-accumulating bacteria from activated sludge (Suresh *et al.,* 1985; Rees *et al.,* 1992; Serafim *et al.,* 2002).

The presence of PolyPs in these granules is indicated by the following indirect observations. First, volutin granules undergo metachromatic staining by basic dyes, 4′,6′-diamino-2-phenylindole (DAPI) fluorescence shifts, and other reactions specific for PolyPs (see

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Chapter 2), and they strongly absorb electrons, in exactly the same way as PolyPs (Drews, 1960a,b). Secondly, the accumulation of volutin granules almost always correlated well with the accumulation of specific PolyP fractions (Belousova and Popova, 1961). Bacterial mutants, which are unable to accumulate PolyPs, have no volutin granules in their cells (Harold and Harold, 1963, 1965). Thirdly, the utilization of PolyPs present in cells of certain bacteria by polyphosphate glucokinase is accompanied by the disappearance of volutin granules (Szymona and Szymona, 1961; Szymona, 1962). There is no doubt that PolyP-containing granules are actually present in cells and are not artifacts formed during the fixation and staining of cells by particular dyes. This may be concluded from the fact that they are readily visible without staining in living cells of microorganisms by phase-contrast microscopy.

It has already been pointed out that it is not only PolyPs but other anionic polymers, such as poly-β-hydroxybutyrate, which may form metachromatic granules in the cells of prokaryotes. However, recently a number of methods for differential staining of PolyPs and polyhydroxyalkanoate-containing granules in cells have been developed (see the review of Serafim *et al.,* 2002).

Various cytochemical methods were elaborated to detect volutin-like granules in different microorganisms (Keck and Stich, 1957; Ebel and Muller, 1958; Ebel *et al*., 1958a,b; Singh, 1959; Serafim *et al*., 2002). Cytological methods for detecting polyphosphate granules were boosted by the use of the electron microscope (Niklowitz and Drews, 1955; Ebel *et al*., 1958a,b; Drews, 1960a; Voelz *et al*., 1966; Jensen, 1968, 1969; Friedberg and Avigad, 1968; Jensen and Sicko, 1974; Jensen *et al*., 1977). The most comprehensive data on volutin granules in cyanobacteria were obtained by Jensen and co-workers (Jensen, 1968, 1969; Jensen and Sicko, 1974; Sicko-Goad *et al*., 1975; Sicko-Goad and Jensen, 1976; Jensen *et al*., 1977; Lawry and Jensen, 1979; Baxter and Jensen, 1980a,b). Using electron microscopy, Jensen and his colleagues investigated the accumulation of PolyP granules under various cultivation conditions in the cyanobacteria *Nostoc pumiforme* (Jensen, 1968), *Plectonema boryanum* (Jensen, 1969; Jensen and Sicko, 1974; Sicko-Goad and Jensen, 1976; Baxter and Jensen, 1980a,b; Jensen *et al*., 1982) and *Anacystis nidulans* (Lawry and Jensen, 1979). Under normal growth conditions, PolyP granules were found mainly near the DNA region and in a zone enriched with ribosomes. Under conditions of 'phosphate overplus', PolyP granules appeared in the polyhedral bodies involved in the dark reactions of photosynthesis in cyanobacteria (Stewart and Godd, 1975). In certain cells, PolyP granules formed close to thylakoids, which in these organisms perform phosphorylation reactions. Electron microscopy established that in cyanobacteria PolyP granules are localized in most cases in the region of nucleoid and sub-cellular structures participating in photosynthesis (Vaillancourt *el al*., 1978; Barlow *et al*., 1979). The data obtained, at least those on localization of polyphosphate granules in the vicinity of the bacterial nucleoid, correlated well with the previous findings using the same method on heterotrophic prokaryotes (Drews, 1958a, 1960a, Voelz *et al*, 1966; Friedberg and and Avigad, 1968; Kulaev and Vagabov, 1983).

Volutin granules were isolated from cells of *Agrobacterium tumefaciens* (Seufferheld *et al*., 2003) by gradient centrifugation. The volutin granule fraction contained 20 % of the total amount of PP_i and short-chain PolyPs and more than 35 % of total amount of P_i and long-chain PolyPs. The total extract of *Agrobacterium tumefaciens* contained ~ 315 nmol (mg protein)$^{-1}$ of PolyP with a chain length less than 50 residues, and 217 nmol (mg protein)$^{-1}$ of PolyP of about 700–800 P_i residues. X-ray microanalysis showed that the

granules contained magnesium and potassium, but if *Agrobacterium tumefaciens* was cultivated in the presence of $CaCl_2$, no magnesium and potassium was detected, but a dramatic increase in calcium content was revealed (Seufferheld *et al*., 2003). Transmission electron microscopy revealed that each granule was surrounded by a membrane (Seufferheld *et al*., 2003), similar to the poly-β-hydroxybutyric granules of some *Bacillus* (Williamson and Wilkinson, 1958). This supported the earlier data that under certain cultivation conditions, e.g. when grown on a medium containing oleic acid, the PolyP granules of mycobacteria were surrounded by lipid layers (Schaefer and Lewis, 1965).

Voelz *et al*. (1966) described a detailed investigation into the formation of PolyP granules in *Myxococcus xanthus* under various growth conditions. It was found that the PolyP granules in this organism are closely associated with glycogen inclusions, and are either distributed throughout the cytoplasm or localized within the nucleoids. A similar localization of PolyP granules was found in *Micrococcus lysodeikticus* (Friedberg and Avigad, 1968).

Some part of the PolyP may be located in the periplasmic region or in the cell capsule, i.e. outside the cytoplasmic membrane of bacterial cells. Ostrovsky *et al*. (1980), for example, believed that a marked increase in the intensity of the PolyP signals was observed when cells of *Mycobacterium smegmatis* were treated with EDTA, which points to localization of a certain amount of mobile PolyP in the periplasmic region. In the bacterial parasite *Bdellovibrio bacteriovorus,* most of the PolyP occur in the form of acid-insoluble highly polymerized fractions predominantly localized outward (Bobyk *et al*., 1980; Egorova *et al*., 1981). Similar observations were made with the oligotrophic bacteria *Tuberoidobacter* and *Renobacter* (Nikitin *et al*.,1979).

PolyP is a component of the cell capsule, which is loosely attached to the surface membrane of the *Neisseria* species. This capsular PolyP represents about a half of the PolyP content in *Neisseria* cells (Tinsley *et al*., 1993).

Bacterial membranes possess PolyPs as complexes with Ca^{2+} and poly-β-hydroxybutyrate (Reusch and Sadoff, 1988). The finding of these unusual structures is one of the most remarkable recent discoveries in PolyP biochemistry (see the reviews of Reusch, 1992; Reusch, 1999a; Reusch, 2000).

As a whole, it might be affirmed that PolyP is localized in prokaryotic cells in many cell compartments. For example, in the *Helicobacter pylori* bacteria colonizing gastric antrum, PolyP was found in at least three different locations: the cytoplasm, the flagellar pole and in association with the cell membrane (Bode *et al*., 1993). In PolyP-accumulating microorganisms of activated sludge, PolyP was observed by electron microscopy in the periplasm, cytoplasm and on the cell surface (Bond and Rees, 1999). One example of the cytochemical picture of PolyP localization in the bacterial cell is shown in Figure 5.1.

5.2 Eukaryotes

The basic difference between eukaryotes and prokaryotes is a much better developed compartmentalization of biochemical processes in eukaryotes, wherein some of the processes take place in specialized cell organelles. However, eukaryotic cells possess PolyP pools in all cell compartments studied in this respect.

The study of PolyP content in different cell compartments of eukaryotic cells is still a difficult task. Quantitative estimation of the PolyP content in the compartments of eukaryotic

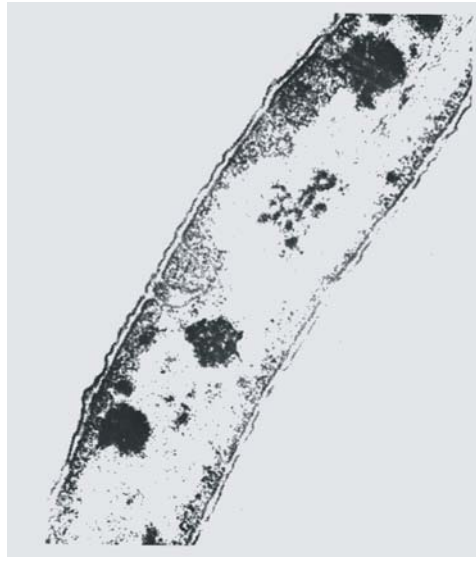

Figure 5.1 PolyP granules in *Myxococcus xanthus* (magnification 110 000×).

microorganisms may depend on the methods of extraction and assay. At cell fractionation, labile PolyP may degrade. The results obtained by ^{31}P NMR spectroscopy also have to be interpreted with a certain caution, considering the presence of 'NMR-invisible' PolyP in some compartments (see Chapter 2). The content of PolyP varies depending on the cultivation conditions. Nevertheless, using a combination of cytochemistry, chemical extraction, NMR spectroscopy and cell sub-fractionation methods, reliable data on PolyP localization in eukaryotic cells have been obtained.

Intracellular localization of PolyP in eukaryotes has been most extensively studied in yeast and fungi. Since the earliest works of Wiame (1946, 1947a,b, 1948, 1949, 1958), it has been known that PolyPs, or at least some part of them, are present in yeast cells as volutin granules. Such granules, containing about 14 % of the total PolyP content in yeast cells, four basic proteins and metal ions, were isolated by Jacobson *et al*. (1982). The chain length of the PolyP in isolated granules was estimated to be $>$ 3000 phosphate residues, but other methods give values of no more than 100–200 residues for the whole yeast cell (Vagabov *et al*., 1998; Ogawa *et al*., 2000a). Volutin granules were found in the cytosol and vacuoles by cytochemical method (Vořišek *et al*., 1982). The content of PolyP in the cytosol depends on culture age and cultivation conditions. The cytosol fraction may contain 10 % (Okorokov *et al*., 1980) to 70 % (Trilisenko *et al*., 2002) of the PolyP cell pool in cells of *S. cerevisiae*. In yeast, its amount in the cytosol increases about twofold under the so-called 'phosphate overplus', when cells are transferred from the medium without phosphate to a medium with phosphate (Trilisenko *et al*., 2002).

The first attempts to determine the intracellular localization of PolyPs in eukaryotes were made long ago. The earliest investigations aimed at establishing the localization of

the total intracellular PolyP were made by Harold and Miller (1961) on the mycelium of *Neurospora crassa*. They determined the distribution of PolyPs and some other compounds in various cell structures of this organism after disrupting of the mycelia in a Nossal's apparatus in 0.05 M tris(hydroxymethyl)amino methane (TRIS), pH 7.0, containing 0.25 M sucrose, and separation of cell structures by differential centrifugation (Table 5.1). It should be noted that, under mechanical disruption of the cells, a rapid degradation of PolyP may occur, and there is a possibility of secondary sorption and desorption of PolyP during sub-fractionation. Harold and Miller reported the occurrence of both of these processes. For example, acid-soluble PolyP degraded to P_i in the course of this work. Furthermore, Harold (1962a) showed that the presence of a large amount of acid-insoluble PolyP in the cell wall material from *N. crassa* could be due to secondary sorption by polysaccharides, such as chitin, which form a part of the cell wall of this organism.

Much more promising were the investigations which had no recourse to mechanical disruption of the cell. Weimberg and Orton (1965) treated the cells of *Saccharomyces mellis* with an enzyme preparation from the snail *Helix pomatia*. This treatment causes lysis of the fungal polysaccharide cell wall, resulting in the formation of spheroplasts devoid of cell walls. It was shown that approximately one fourth to one third of the total cellular PolyP was removed from the cells and degraded to P_i during the spheroplasts formation (Weimberg and Orton, 1965; Weimberg, 1970). It was concluded that these PolyPs were localized in the immediate vicinity of the external cytoplasmic membrane (Weimberg and Orton, 1965).

Souzu (1967a,b) showed that yeast cells during freezing and thawing underwent disturbance of the cytoplasmic membrane, accompanied by rapid hydrolysis of cellular PolyP to P_i. This was interpreted as evidence of localization of a significant portion of PolyP in yeast in the region of the cytoplasmic membrane. The localization of a portion of PolyP in yeast cells on the cell surfaces was proposed by Van Steveninck and Booij (1964), obtaining some evidence for PolyP participation in glucose transport.

Later, PolyP was revealed outside the plasma membrane of the yeast *Kluyveromyces marxianus* by fluorescence of 4′6-diamidino-2-phenylindole (Tijssen *et al*., 1982), by lead staining (Vořišek *et al*., 1982) and X-ray microanalysis (Tijssen and Van Steveninck, 1985), by a decrease in the ^{31}P NMR signal under UO_2^{2+} binding (Tijssen and Van Steveninck, 1984), by osmotic shock treatment (Tijssen *et al*., 1983), and by 9-aminoacrydine binding (Vagabov *et al*., 1990a). The cell envelope of yeast can contain about 20 % (and even more) of the total PolyP content of yeast cells (Vagabov, 1988; Ivanov *et al*., 1996).

Different cellular localization of various PolyP fractions on the basis of examination of their functions and metabolism was first proposed for *Chlorella* (Miyach, 1961; Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Kanai *et al*., 1963; Miyachi *et al*., 1964). In particular, it was shown that this alga possesses PolyP fraction A, which is a constituent of volutin granules and closely involved in nuclear metabolism, and another PolyP fraction C, which is sited adjacent to the chloroplasts and involved in photosynthetic processes. Fractions B and D appeared to be localized in other cell structures.

The investigation of intracellular localization of PolyP was carried out for the fungi *Neurospora crassa* and *Endomyces magnusii* (Kulaev *et al*., 1966a, 1967a,b, 1970a,b; Krasheninnikov *et al*., 1967, 1968; Kulaev and Afanas'eva, 1969, 1970; Skryabin *et al*., 1973). While mechanical disruption of cells was shown to be unsuitable for obtaining subcellular fractions, protoplast isolation was used in these studies. Although the total amount of phosphorus present in the intact cells and in the protoplasts was the same, nevertheless the

Table 5.1 Distribution of PolyPs and other compounds between fractions of sub-cellular structures in 24 h mycelia of *Neurospora crassa* enriched in phosphorus (per 1.5 g of fresh weight) (Harold and Miller, 1961).

	Acid-insoluble PolyP		Phospholipids		Nucleic Acids		Protein	
Fraction	P (μg)	% of initial value	P (μg)	% of initial value	P (μg)	% of initial value	P (μg)	% of initial value
Whole homogenate	740	100	480	100	15 500	100	82	100
Cell walls	465	63	190	40	1100	7	17.5	21
Mitochondria	145	20	275	57	1200	8	20.2	25
Ribosomes plus supernatant	68	9	48	10	11 000	71	34.0	42
Total	—	92	—	107	—	86	—	88

Table 5.2 Contents of PolyPs and other phosphorus compounds in the cells, protoplasts, and nuclei of *Neurospora crassa* (expressed as mg of P per g of dry mycelium) (Kulaev *et al*., 1966a; Krasheninnikov *et al*., 1967, 1968).

Phosphorus compound	Whole cells	Protoplasts	Nuclei
Total PolyP	5.6	3.0	0.2
Acid-soluble PolyP(I)	1.8	1.8	0.0
Salt-soluble PolyP(II)	1.0	1.2	0.2
Alkali-soluble PolyP(IV)	2.0	0.0	0.0
Hot-$HClO_4$-extractible PolyP(V)	0.8	0.0	0.0
Orthophosphate (P_i)	1.1	3.0	0.0
P_i plus total PolyP	6.7	6.0	0.2
Nucleotides	1.2	1.0	0.1
Nucleic acids	6.7	6.9	0.6
Phospholipids	2.1	2.1	0.4
Sugar phosphates	0.4	0.5	0.2
Total phosphorus	17.3	17.2	1.5

amount of different PolyP fractions differed considerably in *N. crassa* (Table 5.2). When the protoplasts were produced, the PolyPs of alkali and hot perchloric acid extracts disappeared completely, while the acid-soluble and salt-soluble fractions remain unchanged. The disappearance of some parts of the PolyPs was accompanied by a corresponding increase in the amount of P_i. This leads to the conclusion that the cell wall removal results in hydrolysis of the above-mentioned highly polymerized PolyP to P_i. It is possible that these PolyP fractions are in some way bound to the cytoplasmic membrane of *N. crassa* and the formation of protoplasts stimulates their hydrolysis. It is probable that these fractions are far more sensitive to the integrity of cell structure than the acid-soluble and salt-soluble fractions. If the cells of *N. crassa* were incubated with the snail enzyme for a longer time, substantial amounts of P_i and acid-soluble PolyPs were also lost. The salt-soluble fraction remained in the same amount as in the whole cells. The different behaviour of the PolyP fraction during protoplast formation from the cells of *N. crassa* indicated their different localization. It is likely that the fractions isolated by the 'Langen and Liss method' (Langen and Liss, 1958a,b) from *N. crassa* cells differ from each other not only in their molecular mass, but also in their intracellular localization and state in the cell. Thus, the most highly polymerized fractions are apparently located at the periphery of the cells, and removal of the cell walls results in their rapid hydrolysis. The less polymerized, acid-soluble PolyPs are evidently located within the cell, probably in the free state. In contrast to these fractions, the salt-soluble PolyPs in *N. crassa* are located in such a way that the protoplast formation has no effect on their amounts.

The removal of 30–35 % PolyPs from yeast cells during the lysis of cell walls by the snail enzyme was observed in *Saccharomyces carlbergensis* (Vagabov *et al*., 1973) – these were alkali-soluble fractions (Table 5.3). A comparative investigation of the amounts of various PolyP fractions was carried out in *E. magnusii* spheroplasts (Table 5.4.) and some sub-cellular fractions (Table 5.5). All of these data confirm the idea of PolyP localization in different compartments of cells of the lower eukaryotes.

Table 5.3 PolyP contents in cells and protoplasts of *Saccharomyces carlsbergensis* (expressed as mg of P per g of wet biomass) (Vagabov *et al*., 1973).

Phosphorus compound	Whole cells	Protoplasts
Total PolyP	1.786	1.050
Acid-soluble PolyP(I)	0.706	0.728
Salt-soluble PolyP(II)	0.516	0.299
Alkali-soluble PolyP(III) (pH 8–10)	0.208	0.023
Alkali-soluble PolyP(IV) (pH 12)	0.356	0

Table 5.4 PolyP contents in cells and protoplasts of *Endomyces magnusii* enriched in phosphorus (expressed as mg of P per g of dry biomass) (Kulaev *et al*., 1967).

Phosphorus compound	Whole cells	Protoplasts
Total PolyP	20.6	14.3
Acid-soluble PolyP(I)	11.2	12.1
Salt-soluble PolyP(II)	3.4	1.4
Alkali-soluble PolyP(III) plus PolyP(IV)	3.3	0.8
Hot $HClO_4$ extract, PolyP(V)	2.5	0.0

Table 5.5 Amounts of inorganic polyphosphates and other phosphorus compounds present in cells of *Endomyces magnusii*, and in the protoplasts, mitochondria and nuclei obtained from them (expressed as mg of P per g dry weight of cells) (Kulaev *et al*., 1967a,b; Afanas'eva *et al*., 1968; Skryabin *et al*., 1973).

Phosphorus compound	Whole cells	Protoplasts	Mitochondria	Nuclei
Total PolyP	2.0	1.5	0.0	0.4
Acid-soluble PolyP(I)	0.2	0.7	0.0	—
Salt-soluble PolyP(II)	0.7	0.4	0.0	0.4
Alkali-soluble PolyP(III) plus PolyP(IV)	0.9	0.4	0.0	0.0
Hot $HClO_4$ extract, PolyP(V)	0.2	0.0	0.0	0.0
Orthophosphate P_i	3.2	1.1	0.3	0.1
Total P_i and PolyP	5.2	2.6	0.3	0.5
Nucleotides	1.0	0.6	0.3	—
Nucleic acids	6.2	5.4	1.1	—
Phospholipids	1.2	1.2	0.9	0.2
Sugar phosphates	0.9	0.5	Trace	—
Total phosphorus	14.4	10.3	2.6	2.3

A part of the volutin granules was found in the cells of fungi and algae in vacuoles. The presence of PolyP granules in vacuoles was confirmed by cytochemistry and X-ray dispersion microanalysis in algae (Atkinson *et al*., 1974; Peverly *et al*., 1978; Adamec *et al*., 1979; Vořišek and Zachleder, 1984), and yeast (Vořišek *et al*., 1982). Indge (Indge, 1968a,b,c) was the first to indicate the presence of PolyPs in isolated yeast vacuoles. Since the work of Matile and his associates (Matile, 1978; Urech *et al*., 1978; Dürr *et al*., 1979; Wiemken *et al*., 1979), which examined isolated vacuoles and used the method of differential extraction of cell pools, an opinion has been formed in the literature that nearly all of the PolyPs of yeast cells are located in these organelles. This opinion was supported by the investigation of a vacuole-defective yeast mutant, where no 'NMR-visible' PolyPs were found (Shirahama *et al*., 1996). However, it should be noted that the PolyP content in vacuoles strongly depends on the cultivation conditions. Data on the quantity of PolyPs in yeast vacuoles are the most numerous, but are still quite contradictory, since the authors used different strains and cultivation conditions. When *Saccharomyces cerevisiae* are grown on a poor mineral medium with arginine as the only nitrogen source and the culture growth rate is low, vacuoles may contain the major part of the yeast-cell PolyP pool (Wiemken *et al*., 1979). The amount of PolyPs in yeast vacuoles sharply increases when this microorganism accumulates metal cations. *S. carlsbergensis* vacuoles accumulated seven times more PolyP than the cytosol under incubation with phosphate, glucose and K^+, and ten times more PolyP with Mn^{2+} (Lichko *et al*., 1982). Under other growth conditions, the vacuolar PolyP pool in *S. cerevisiae* was significantly lower. The vacuoles of *S. cerevisiae* growing on the 'Reader medium' contained nearly 15 % of the total amount of PolyPs in the cell (Trilisenko *et al*., 2002). The vacuoles of *Candida utilis* contained no more than 30 % of the total amount of PolyPs in the cell depending on the rate of culture growth and the nitrogen source in the medium (Nunez and Callieri, 1989). The vacuoles contained PolyPs of short chain lengths. These were determined to be $\sim$ 5 and 15–25 phosphate residues (Wiemken *et al*., 1979). Later, this evaluation was confirmed by Trilisenko and co-workers (Trilisenko *et al*., 2002). From an NMR spectroscopic study of the two PolyP fractions obtained from isolated vacuoles of *S. cerevisiae*, it was shown that these organelles contain two fractions of PolyPs: 5 ± 5 and 20 ± 2 phosphate residues. PolyPs were also found in the vacuoles of *Neurospora crassa* (Cramer *et al*., 1980; Cramer and Davis, 1984) and *Dunaliella salina* (Pick and Weiss, 1991). With regard to the role of vacuoles as the main compartment of reserve compounds in eukaryotic microorganisms, it can be expected that other fungi, yeast and algae have a vacuolar pool of PolyPs.

The vesicles of endoplasmic reticulum of yeast cells contain PolyPs and also a specific system of their biosynthesis related to glycoproteins (Shabalin *et al*., 1979, 1985).

The cells of protozoa (Docampo and Moreno, 2001; Ruiz *et al*., 2001a; Rodriguez *et al*., 2002a,b) and the alga *Chlamydomonas reinhardtii* (Ruiz *et al*., 2001b) possess specific PolyP and Ca^{2+} storage organelles–acidocalcisomes–which are similar to vacuoles in some properties. These organelles possess PolyPs with chain lengths of 3, 50 and 700–800 phosphate residues. The latter high polymeric PolyPs were observed in small amounts. PolyPs were also found in the lysosomes of human fibroblasts (Pisoni and Lindley, 1992).

PolyP complexes with poly-β-hydroxybutyrate, similar to those in bacteria, were identified in eukaryotic membranes, including animal cells (Reush, 1989, 1999a, 2000).

PolyPs with short chain lengths of 14 phosphate residues were found in yeast mitochondria by using ^{31}P NMR spectroscopy. This PolyP makes up to 10 % of the total content

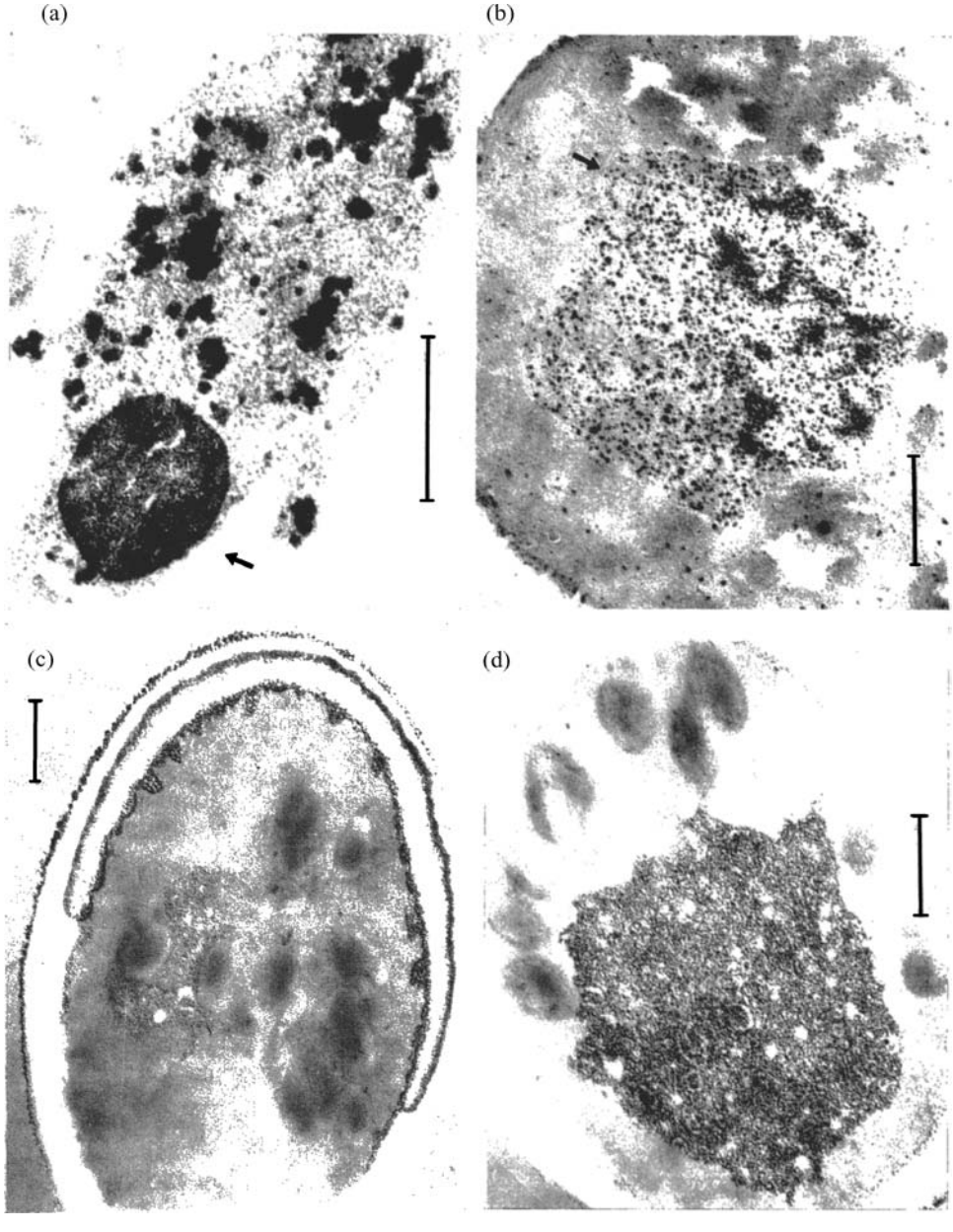

Figure 5.2 PolyPs in different compartments of the cells of *Saccahromyces cerevisiae* (lead staining) (Vořišek *et al*., 1982): (a) PolyP in vacuoles (the arrow indicates a metachromatic granule); (b) PolyP in a cell nucleus (the arrow indicates the nuclear membrane); (c) PolyP in internal cell wall layers at 'phosphate overplus'; (d) PolyP in vacuoles after 'phosphate overplus': the scale bars are equal to 0.5 μm.

of cellular PolyP detected by NMR spectroscopy (Beauvoit *et al*., 1989). PolyP was also found in a pure mitochondrial fraction of *Saccharomyces cerevisiae* by chemical extraction (Pestov *et al*., 2003). This acid-soluble PolyP, with an average chain length of about 25 phosphate residues, comprised ~ 7 % of the whole PolyP content of the cell. PolyP was also observed in mitochondria of mammalian cells (Kornberg *et al*., 1999).

We have no positive evidence of the presence of PolyPs in chloroplasts. In chloroplasts of *Acetabularia mediaterranea* (Rubtsov *et al*., 1977) and in cotton plants (Valikhanov and Sagdulaev, 1979), PolyPs were not found.

The presence of specific fractions of high-molecular-weight PolyPs in the nuclei of different organisms has been demonstrated by many researchers (Penniall and Griffin, 1964; Goodman *et al*., 1968; Sauer *et al*., 1956; Bashirelashi and Dallam, 1970; Kulaev *et al*., 1970b; Skryabin *et al*., 1973; Mansurova *et al*., 1975a; Offenbacher and Kline, 1984; Pilatus *et al*., 1989; Kumble and Kornberg, 1995, 1996).

PolyPs were found in isolated nuclei from *N. crassa* (Kulaev *et al*., 1970b). These were extracted with saturated salt solution (see Table 5.2). The presence of PolyPs in this fraction was proved exclusively by chromatography. Among the products of partial hydrolysis of this fraction by using the method of Thilo and Wicker (1957), cyclotriphosphate was found. The phosphorus contained in the salt-soluble nuclear fraction amounted to 15 % of the total PolyP phosphorus of the salt-soluble fraction of whole cells. Further purification of the nuclear fraction by centrifugation under a sucrose gradient or by other methods did not result in the removal of PolyPs from the nuclei. Similar results were obtained for *E. magnusii* (Skryabin *et al*., 1973). The PolyP content of the nuclei represented over 50 % of the total salt-soluble PolyP of the whole cells. No other PolyP fractions were found in the nuclei of this organism. PolyP represented ~ 13–15 % of the total phosphorus of isolated nuclei.

PolyPs with an average chain length of 100 residues were observed in the nuclei of *Physarium polycephalum* (Pilatus *et al*., 1989). Nuclear preparations of mammalian cells were found to be relatively enriched in PolyPs (Kumble and Kornberg, 1995; Kornberg, 1999).

Amounts of PolyP in the nuclei might be low; however, these compounds are conserved there during evolution, occurring in the nuclei of both the lower eukaryotes and mammalians.

As a whole, PolyPs in cells of eukaryotes are characterized by plural localization, depending on the cell age or environmental conditions. For example, cytochemical data on the localization of PolyPs in the cells of *Saccharomyces cerevisiae* are presented in Figure 5.2, according to Vořišek *et al*. (1982). Further studies of this problem may provide new data on the functions of these biopolymers.

6

ENZYMES OF POLYPHOSPHATE BIOSYNTHESIS AND DEGRADATION

6.1 Enzymes of Polyphosphate Biosynthesis

6.1.1 Polyphosphate Kinase (Polyphosphate:ADP Phosphotransferase, EC 2.7.4.1)

The reaction of reverse transfer of energy-rich phosphate residues from ATP to PolyPs and from PolyPs to ADP, thus linking energy-rich pools, was discovered by Kornberg and co-workers (Kornberg *et al*., 1956; Kornberg, 1957 a,b):

$$\text{PolyP}_n + \text{ATP} \rightleftharpoons \text{PolyP}_{n+1} + \text{ADP} \tag{6.1}$$

The enzyme was partly purified from *Propionibacterium shermanii* (Robinson *et al*., 1987), and was shown to be a monomeric enzyme with a molecular mass of $\sim$ 83 kDa. It was demonstrated that short-chain PolyPs of 6–80 residues serve as primers for the synthesis of long-chain PolyPs using ATP by a strictly processive mechanism. The largest PolyPs synthesized was PolyP_{750}.

The polyphosphate kinase (ppk1) purified from *Escherichia coli* was a membrane-bound homotetramer with a sub-unit molecular mass of 80 kDa (Ahn and Kornberg, 1990; Akiyama *et al*., 1992). The crystal structure of this enzyme has been determined (Zhu *et al*., 2003). This enzyme is responsible for the processive synthesis of long PolyP_{750} chains *in vivo* and needs Mg^{2+} for its activity (Ahn and Kornberg, 1990). The enzyme was shown to be multifunctional. It catalyses the reverse reaction of ATP synthesis from PolyPs (Kornberg, 1957a; Murata *et al*., 1988; Ahn and Kornberg, 1990; Kuroda and Kornberg, 1997) and

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can use PolyP as a donor instead of ATP, thereby converting GDP and other nucleoside diphosphates to nucleotide triphosphates (Kuroda and Kornberg, 1997). This reaction was observed in crude membrane fractions of *E. coli* and *Pseudomonas aeruginosa*, as well as in purified enzyme preparations obtained from *E. coli*. Membrane fractions obtained from *E. coli* mutants lacking the *ppk1* gene have no such activity. The substrate specificity order was ADP > GDP > UDP, CDP; the activity with ADP was twice as high as that with GDP. It was confirmed that polyphosphate kinase efficiently catalysed UTP regeneration in the cyclic system of *N*-acetyl lactosamine synthesis (Noguchi and Shiba, 1998). This activity of pure polyphosphate kinase was used to develop a method of oligosaccharide synthesis (Noguchi and Shiba, 1998). Although the transfer of a phosphate from PolyP to GDP by polyphosphate kinase to produce GTP was the predominant reaction, the enzyme also transferred a pyrophosphate group to GDP to form a linear guanosine 5′-tetraphosphate (Kim *et al*., 1998). The enzyme is also capable of authophosphorylation using ATP as a phosphate donor (Tzeng and Kornberg, 2000). The diverse functions of ppk1 in *E. coli* are realized by means of different sub-unit organization. Radiation target analysis revealed that the principal functional unit of ppk1 is a dimer, but the synthesis of linear guanosine tetraphosphate and authophosphorylation require trimeric and tetrameric states, respectively (Tzeng and Kornberg, 2000). The polyphosphate kinase of *Vibrio cholerae* is also a homotetramer (Ogawa *et al*., 2000b). It resembles the ppk1 of *E. coli* in size and multiple activities: processive PolyP synthesis from ATP, nucleoside diphosphate kinase activity with ADP and GDP as acceptors and PolyP as a donor, ppGpp synthesis from GDP, and autophosphorylation. The most notable differences are in the kinetic parameters for ATP: K_m is 0.2 and 2 mM for the ppk1 of *V. cholerae* and *E. coli,* respectively (Ogawa *et al*., 2000b).

Polyphosphate kinase was purified from *Acinetobacter* sp. (Trelstad *et al*., 1999). It was a 79 kDa monomer. In contrast to the *E. coli* enzyme, the polyphosphate kinase purified from this bacterium seems to work only in the forward direction, i.e. it produces but does not degrade PolyPs.

The *ppk1* genes of *E. coli* (Akiyama *et al*., 1992), *Klebsiella aerogenes* (Kato *et al*., 1993b), *Neisseria meningitidis* (Tinsley and Gotschlich, 1995), *Pseudomonas aeruginosa* (Ishige *et al*., 1998), *Acinetobacter* sp. (Geissdorfer *et al*., 1998), *V. cholerae* (Ogawa *et al*., 2000b), *Rhodocyclus tenuis* (McMahon *et al*., 2002) and many other bacteria (see http://www.expasy.org) have been cloned, sequenced and characterized. The deduced amino acid sequences of these enzymes show an extensive homology in different bacterial species (Figure 6.1) (Tzeng and Kornberg, 1998). Some conserved amino acid residues are important for enzymatic activity. Replacement of conserved His-441 and His-460 by either glutamine or alanine by site-specific mutagenesis rendered an enzymatically inactive protein in *E. coli* (Kumble *et al*., 1996).

High conservatism of the *ppk1* gene structure reveals polyphosphate kinase in microorganisms, microbial associates and activated sludges. Fragments of putative *ppk* genes were retrieved from a pure culture of *Rhodocyclus tenuis* and from microorganisms of activated sludge using PCR primers (McMahon *et al*., 2002). Four novel *ppk* homologs were found in the sludge, and two of them (types I and II) shared a high degree of amino acid similarity with *R. tenuis* ppk (86 and 87 %, respectively). Dot-blot analysis of total RNA extracted from the sludge demonstrated the presence of the Type I *ppk* mRNA, indicating that this gene is expressed during the process of phosphate removal. Inverse PCR was used to obtain a full Type I sequence from sludge DNA, and a full-length *ppk* was cloned, overexpressed, and purified to near homogeneity. The purified polyphosphate kinase has a specific activity

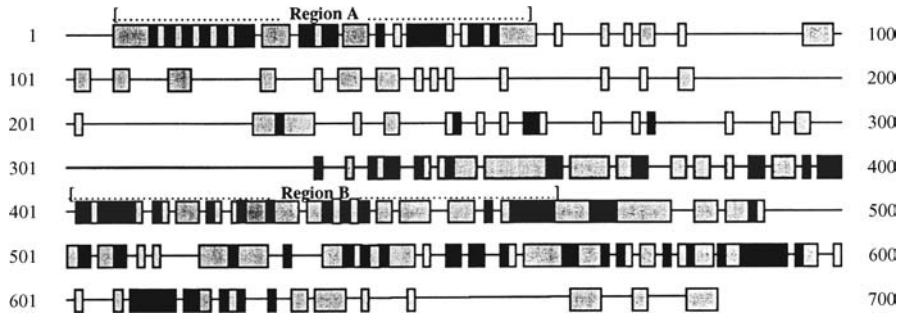

Figure 6.1 Identity of polyphosphate kinase (ppk1) among 12 bacteria. Based on length of the *E. coli* enzyme (687 amino acids), 100 % identity is represented by black and over 60 % identity by grey (Tzeng and Kornberg, 1998; Kornberg, 1999).

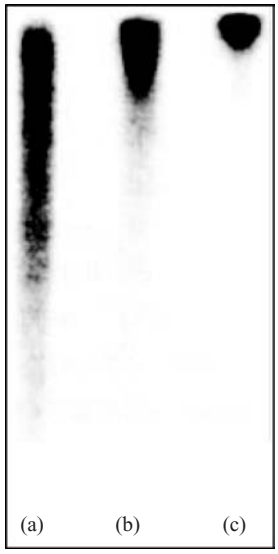

Figure 6.2 Cahin lengths of PolyPs synthesized by polyphosphate kinases: (a) ppk2 of *P. aeruginosa;* (b) ppk1 of *P. aeruginosa;* (c) ppk1 of *E. coli*. After 45 min at 37 °C, the products were separated by PAGE (20 % gel containing 7 M urea) (Zhang *et al*., 2002).

comparable with that of other polyphosphate kinases, requires Mg^{2+}, and does not appear to operate in reverse (McMahon *et al*., 2002).

In many bacteria, polyphosphate kinase is the main enzyme of PolyP metabolism. This was confirmed by a sharp decrease of PolyP content in *ppk1* mutants of *E. coli* (Crooke *et al*., 1994; Rao and Kornberg, 1996; Rao *et al*., 1998), *N. meningitidis* (Tinsley and Gotschlich, 1995), and *V. cholerae* (Ogawa *et al*., 2000b).

In a null mutant of *P. aeruginosa* lacking *ppk1*, another polyphosphate kinase activity distinguished from ppk1 was revealed (Ishige *et al*., 2002; Zhang *et al*., 2002). The enzyme has been purified 1300-fold to homogeneity from lysates of *P. aeruginosa*. As compared with ppk1, ppk2 produces PolyPs with a lower chain length (Figure 6.2) and has a preference for Mn^{2+} over Mg^{2+}. The ppk2 of *P. aeruginosa* differs from ppk1 in two other features. First, ppk2 utilizes PolyP to make GTP at a rate 75-fold higher than the synthesis of PolyP

from GTP. For ppk1, the activity of PolyP synthesis is 4-fold higher than the activity of PolyP utilization. Secondly, ppk2 uses GTP and ATP equally well in PolyP synthesis, but ppk1 is strictly specific for ATP in the PolyP synthesis. PolyPs of 15–700 phosphate residues can serve as a substrate, but PolyPs of 30–50 residues are optimal for GTP synthesis by ppk2. GDP is more preferable than ADP among nucleoside diphosphate acceptors. Thus, in *P. aeruginosa* the ppk2 function is first of all utilization of PolyP.

The gene encoding ppk2 (*ppk2*) was identified from the amino acid sequence of the purified protein. It encodes a protein of 357 amino acids with a molecular mass of 40.8 kDa. Both of the polyphosphate kinases ppk1 and ppk2 may be involved in regulation of the level of ribonucleoside triphosphates and deoxyribonucleoside triphosphates that modulate cell division and survival in the stationary phase (Ishige *et al*., 2002).

Sequences homologous to ppk2 were found in two other proteins in *P. aeruginosa*, in two Archaea, and in 32 other bacteria including several pathogenic species (Ishige *et al*., 2002; Zhang *et al*., 2002). Table 6.1 shows the distribution of *ppk1* and *ppk2* gene homologues in microorganisms (Zhang *et al*., 2002). These are lacking in most currently sequenced genomes of Eukarya and Archaea (Kornberg *et al*., 1999).

Polyphosphate kinase activity was observed in the yeast vacuolar membrane, but the activity when consuming PolyP and forming ATP was higher than in PolyP synthesis (Shabalin *et al*., 1977). The enzyme has not been purified, and the significance of PolyP synthesis using ATP in certain membrane fractions in yeast cells needs further investigations. The level of this activity observed in yeast was insufficient to explain the synthesis of a large amount of PolyP in this microorganism.

The polyphosphate kinase from the yeast cell homogenate purified by Felter and Stahl (Felter and Stahl, 1973) was shown to be actually diadenosine-5′,5‴-P^1, P^4 tetraphosphate a,b-phosphorylase (AP_4 phosphorylase). The enzyme acting in concert with one or more yeast polyphosphatases provided the production of ^{32}P-labelled ATP in the presence of ^{32}P-labelled PolyP and ADP (Booth and Guidotti, 1995):

$$[^{32}P]\,\mathrm{PolyP}_n \xrightarrow[\text{exopolyphosphatase}]{} [^{32}P]\,\mathrm{PolyP}_{n-1} + {}^{32}P_i \tag{6.2}$$

$$\text{diadenosine-5′, 5‴-P}^1\text{, P}^4\text{ tetraphosphate} \xrightarrow[\mathrm{AP_4\ phosphorylase}]{} 2\,\mathrm{ADP} \tag{6.3}$$

$$[^{32}P_i] + \mathrm{ADP} \longrightarrow [^{32}P]\,\mathrm{ADP} + P_i \tag{6.4}$$

$$[^{32}P]\,\mathrm{ADP} + \mathrm{ADP} \xrightarrow[\text{adenylate kinase}]{} [^{32}P]\,\mathrm{ATP} + \mathrm{AMP} \tag{6.5}$$

The resulting reaction was probably observed by Felter and Stahl (1973):

$$[^{32}P]\,\mathrm{PolyP}_n + \mathrm{ADP} \longrightarrow [^{32}P]\,\mathrm{PolyP}_{n-1} + [^{32}P]\,\mathrm{ATP} \tag{6.6}$$

It was reported that the archaeon *Sulfolobus acidocaldarius* possessed a glycogen-bound polyphosphate kinase, which was active only as a native complex with glycogen (Skorko *et al*., 1989). This result is doubted by Cardona *et al*. (2001) who repeated the purification

Table 6.1 Distribution of the *ppk1* and *ppk2* gene homologs among microorganisms[a] (Zhang *et al*., 2002).

ppk1 and ***ppk2***
Agrobacterium tumefaciens
Brucella melitensis
Burkholderia fungorum (2)
Campylobacter jejuni
Caulobacter crescentus
Chlorobium tepidum
Chloroflexus aurantiacus
Deinococcus radiodurans
Magnetospirillum magnetotacticum (2)
Mesorhizobium loti (2)
Methanosarcina acetivorans (2)[b]
Methanosarcina mazei (2)[b]
Mycobacterium tuberculosis
Myxococcus xanthus
Nostoc punctiforme (2)
Nostoc sp. PCC7120 (2)
P. aeruginosa (3)
Prochlorococcus marinus
Pseudomonas fluorescens (2)
Ralstonia metallidurans (5)
Ralstonia solanacearum
Rhodobacter sphaeroides (2)
Rhodopseudomonas palustris
Rhodospirillum rubrum
Sinorhizobium meliloti (3)
Streptomyces coelicolor
Synechococcus sp. WH 8102
Synechocystis sp. PCC6803
Thermosynechococcus elongatus
Vibrio cholerae
Xanthomonas axonopodis
Xanthomonas campestris

ppk2 only
Corynebacterium glutamicum (2)
Magnetococcus MC-1 (2)
Plectonema boryanum

ppk1 only
Genome complete
Bacillus anthracis
Bacillus halodurans
Clostridium acetobutylicum
Escherichia coli
Helicobacter pylori
Mycobacterium leprae
Neisseria meningitidis
Salmonella typhi
Salmonella typhimurium
Xylella fastidiosa
Yersinia pestis

Genome incomplete
Acidithiobacillus ferrooxidans
Acinetobacter baumannii
Acinetobacter calcoaceticus
Acinetobacter sp. ADP1
Aphanizomenon baltica
Aphanizomenon sp. TR183
Arthrobacter sp. KM
Bordeiella pertussis
Burkholderia cepacia
Campylobacter coli
Cytophaga hutchinsonii
Dictyostelium discoideum[c]
Geobacter sulfurreducens
Haloferax volcanii[b]
Klebsiella aerogenes
Leuconostoc mesenteroides
Microbulbifer degradans
Mycobacterium marinum
Mycobacterium ulcerans
Neisseria gonorrhoea
Nitrosomonas europaea
Nodularia spumigena
Oenococcus oeni
Porphyromonas gingivalis
Propionibacterium shermanii
Rhodcyclus tenuis
Salmonella dublin
Serratia marcescens
Shigella flexneri
Streptomyces griseus
Streptomyces lividans
Thermobifida fusca

Neither ***ppk1*** or ***ppk2***
Aeropyrum pernix[b]
Aquifex aeolicus
Archaeoglobus fulgidus[b]
Bacillus subtilis
Borrelia burgdorferi
Buchnera aphidicola str. Sg
Buchnera sp. APS
Chlamydia muridarum
Chlamydia trachomatis
Chlamydophila pneumoniae
Clostridium perfringens
Fusobacterium nucleatum
Haemophilus influenzae
Halobacterium sp. NRC-1[b]
Lactococcus lactis
Listeria innocua
Listeria monocytogenes
Methanococcus jannaschi[b]
Methanopyrus kandleri[b]
Methanothermobacter thermautotrophicus[b]
Mycoplasma genitalium
Mycoplasma pneumoniae
Mycoplasma pulmonis
Pasteurella multocida
Pyobaculum aerophilum[b]
Pyrococcus abyssi[b]
Pyrococcus furiosus[b]
Pyrococcus horikoshii[b]
Rickettsia conorii
Rickettsia prowazekii
Saccharomyces cerevisiae[c]
Schizosaccharomyces pombe[c]
Staphylococcus aureus
Streptococcus pneumoniae
Streptococcus pyogenes
Sulfolobus solfataricus[b]
Sulfolobus tokodaii[b]
Thermoanaerobacter tengcongensis
Thermoplasma acidophilum[b]
Thermoplasma volcanium[b]
Thermotoga maritima
Treponema pallidum
Ureaplasma urealyticum

[a] The numbers in parentheses indicate the number of homologs of *ppk2* genes in the organism.
[b] Archae.
[c] Eukaryotes.

procedure for a glycogen-bound protein of 57 kDa developed by Skorko *et al*. (1989). No polyphosphate kinase activity was found in the purified protein, when using recently developed enzymatic methods of PolyP analysis. Furthermore, no polyphosphate kinase activity was found associated with any of the proteins bound to the glycogen–protein complex. The gene corresponding to the 57-kDa protein was cloned and functionally characterized. The predicted product of the gene did not show similarity to any described ppk but to glycogen synthases instead. In agreement with these results, the protein showed only glycogen synthase activity (Cardona *et al*., 2001). It should be noted that PolyP identification in an earlier paper (Skorko *et al*., 1989) is based on electrophoresis in polyacrylamide gel, authoradigraphy, and subsequent acid hydrolysis or alkali phosphatase hydrolysis of radioactive spots. Such an assay could not exclude the possibility that the product obtained is not PolyP but phosphorylated protein(s).

In conclusion, it should be mentioned that PolyP synthesis using ATP or GTP has been reliably demonstrated in eubacteria only. In many bacteria, polyphosphate kinase is the main enzyme of PolyP synthesis. The existence of enzymes responsible for PolyP sythesis using ATP in eucaryotes and archaea is still in question.

6.1.2 3-Phospho-D-Glyceroyl-Phosphate:Polyphosphate Phosphotransferase (EC 2.7.4.17)

This enzyme, which is also called 1,3-diphosphoglycerate-polyphosphate phosphotransferase (Kulaev and Bobyk, 1971; Kulaev *et al*., 1971), catalyses the following reaction:

$$\text{3-phospho-D-glyceroyl-1-phosphate} + \text{PolyP}_n \longrightarrow \text{3-phosphoglycerate} + \text{PolyP}_{n+1} \tag{6.7}$$

This activity was found first in the *Neurospora crassa* mutant deficient in adenine, where the concentrations of ATP and other adenyl nucleotides were sharply reduced (Kulaev and Bobyk, 1971).

The incubation mixture, which afforded the maximum rate of incorporation of ^{32}P-orthophosphate into inorganic PolyP, contained glycilglycine buffer (pH 7.4), $MgCl_2$ (6 μM), $PolyP_{75}$ (0.015 μM), fructose-1,6-diphosphate (5.2 μM), 3-phosphoglyceraldehyde dehydrogenase (14.4 μg), NAD (8 μM), $Na_2H\ ^{32}PO_4$ (8 μM) and a cell-free extract of *N. crassa*. This enzyme system resulted in the incorporation of ^{32}P into high-molecular-weight PolyP only. The radioactive product obtained was undialysable and almost completely (80 %) hydrolysed to orthophosphate by treatment with 1 N HCl for 10 min at 100 °C. Tricyclophosphate was obtained among the products of incomplete hydrolysis by Thilo and Wiecker's method (Thilo and Wiecker, 1957). In order to prove that PolyP synthesis by this system is a result of glycolytic phosphorylation, the effects thereon of glycolytic and oxidative phosphorylation inhibitors were examined. It was found that iodoacetic acid (12 mM) and a mixture of sodium arsenate (50 mM) and sodium fluoride (2 mM) inhibited PolyP biosynthesis in this system by 96 and 95 %, respectively. Inhibitors of oxidative phosphorylation, 2,4-DNP (0.014 mM) and sodium azide (0.03 mM) had no effect on the incorporation of ^{32}P-orthophosphate into the PolyP, but an increase in the concentration to 1 mM retarded the process by 25%. These results therefore confirm the hypothesis that in such an enzyme system PolyP biosynthesis is associated with glycolytic phosphorylation reactions.

Table 6.2 Specific activities of 1,3-diphosphoglycerate–polyphosphate phosphotransferase in some microorganisms (Kulaev *et al*., 1971).

Microorganism	1,3-Diphosphoglycerate–polyphosphate phosphotransferase activity (mE per mg of protein)
Neurospora crassa (wild strain)	0.53
Neurospora crassa (adenine-deficient mutant)	1.45
Penicillium chrysogenum	0.28
Propionibacterium schermanii	0.04
Micrococcus lysodeikticus	0.06
Escherichia coli	0.14
Actinomyces aureofaciens	0.05

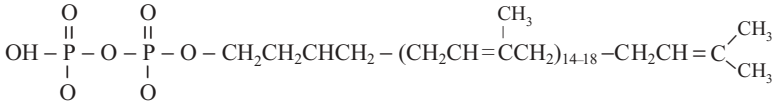

Figure 6.3 The structure of yeast dolichyl diphosphate.

Furthermore, this activity was found in a cell-free extract of the wild strain of *N. crassa* and in other microorganisms including bacteria, but was much lower than in an adenine-defficient *N. crassa* strain (Table 6.2). This pathway of PolyP synthesis probably occurs during glycolytic phosphorylation under a low ATP content in the cell and might actually be involved in the biosynthesis of some, presumably low-molecular-weight, PolyP fractions. Some authors, however, believe that PolyP biosynthesis in the lower eucaryotes to be apparently provided by 1,3-diphosphoglucerate:PolyP phosphotransferase (Schuddemat *et al*., 1989a). This enzyme has not been purified and therefore needs further investigation.

6.1.3 Dolichyl-Diphosphate:Polyphosphate Phosphotransferase (EC 2.7.4.20)

This enzyme's activity was found in the membrane fraction of yeast cells (Shabalin *et al*., 1979, 1985; Naumov *et al*., 1985; Kulaev *et al*., 1987; Shabalin and Kulaev, 1989), where PolyP synthesis using β-phosphate groups of dolichyl diphosphate (Figure 6.3) took place:

$$\text{dolichyl diphosphate} + \text{PolyP}_n \longrightarrow \text{dolichyl phosphate} + \text{PolyP}_{n+1} \qquad (6.8)$$

The enzyme was solubilized from the membrane fraction using Triton X-100 (Shabalin and Kulaev, 1989). The specific activity of the solubilized preparation was 20 times higher than that in protoplast lysate. The dolichyl-diphosphate:PolyP phosphotransferase activities of the membrane preparation and solubilized fraction were etal-dependent and exhibited the maximum activity in the presence of Mg^{2+} or Ca^{2+}. The same membrane fraction posessed

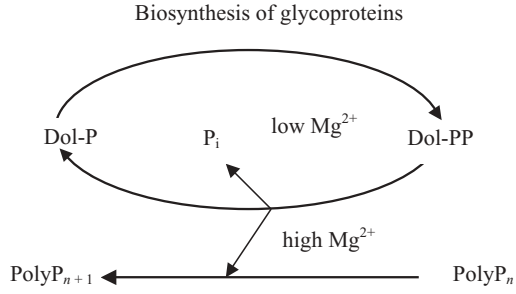

Figure 6.4 Two possible pathways for dolichyl diphosphate dephosphorylation in yeast cells: Dol-PP, dolichyl diphosphate; Dol-P, dolichyl phosphate.

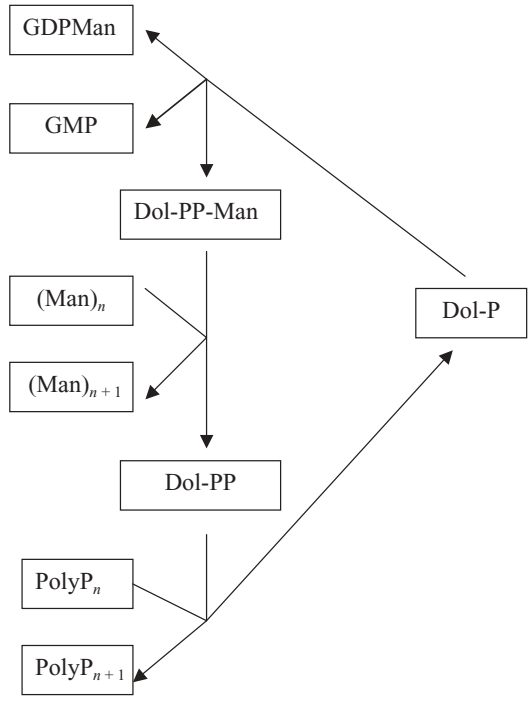

Figure 6.5 The tentative coupling of PolyP and mannan biosynthesis in yeast: Dol-PP, dolichyl diphosphate; Dol-P, dolichyl phosphate; GDPMan, GDP mannose; Dol-PP-Man, dolichyl diphosphate mannose; $(Man)_n$, mannan, where n is the number of mannose residues.

dolichyl-diphosphate:phosphohydrolase activity, which was, however, inhibited by divalent metal cations. Thus, depending on the divalent metal cations, two pathways of dolichyl diphosphate dephosphorylation may occur in some yeast cell membranes (Figure 6.4). The coupling of mannan and PolyP biosynthesis was proposed (Figure 6.5). However, no genetic data on this enzyme are available.

It is probable that a similar pathway of PolyP synthesis which is related to the biosynthesis of some biopolymers may exist in bacteria. Undecaprenyl diphosphate, which could be involved in this pathway, is found in bacteria.

6.2 Enzymes of Polyphosphate Degradation

6.2.1 Polyphosphate-Glucose Phosphotransferase (EC 2.7.1.63)

This enzyme catalyses the phosphorylation of glucose using polyP or ATP as the phosphoryl donor:

$$\text{PolyP}_n + \text{D-glucose} \longrightarrow \text{PolyP}_{n-1} + \text{D-glucose 6-phosphate} \qquad (6.9)$$

The enzyme activity was first observed in *Mycobacterium phlei* (Szymona, 1957) and then in numerous bacteria (Szymona *et al*., 1962; Szymona and Ostrowsky, 1964), including other Mycobacteria (Szymona and Szymona, 1978), *Corynebacterium diphtheriae* (Szymona and Szymona, 1961) and *Nocardia minima* (Szymona and Szymona, 1979). The screening for polyphosphate glucokinase activities in a variety of different organisms showed its presence in the phylogenetically ancient bacteria belonging to the *Actinomycetales* (Szymona *et al*., 1967, 1969, 1977; Kulaev and Vagabov, 1983). This activity was observed in *Microlunatus phosphorus,* a bacteria from activated sludge accumulating high levels of PolyPs (Kuroda and Ohtake, 2000).

The enzyme activity was not found in eucaryotes. The discovery of this enzyme was of the greatest significance for our understanding of the role of PolyPs: it provided the first evidence of the possible function of PolyPs as a phosphate and energy donor without the nucleoside phosphate system.

$PolyP_3$ and $PolyP_4$ were reported to be the end products formed from long-chain PolyPs in glucose phosphorylation (Szymona and Widomski, 1974; Kowalczyk and Phillips, 1993). PolyP glucokinase utilized polyP via a quasi-processive or non-processive mechanism (Pepin and Wood, 1986, 1987; Hsieh *et al*., 1996a,b). The enzyme from *Mycobacterium tuberculosis* utilizes a wide range of PolyP sizes by a non-processive mechanism (Hsieh *et al*., 1996a). The enzyme from *Propionibacterium shermanii,* on the other hand, shows a transition from a strictly processive mode with very long PolyPs to a strictly non-processive mode with short PolyPs below 100 residues. Intermediate sizes of PolyPs ($\sim$ 100–200 residues) are utilized by a quasi-processive mechanism, which is evidenced by a noticeable broadening of the range of polyP sizes with the reaction time.

There have been numerous reports on the occurrence of various isoenzymes of polyphosphate glucokinase in different microorganisms and on differences in the molecular weights of the enzyme in the same organism (Szymona *et al*., 1977; Kowalska *et al*., 1979; Pastuszak and Szymona, 1980). Szymona *et al*. (1977) found that the molecular mass of native enzyme from *M. tuberculosis* was 118 kDa, while Pastuszak and Szymona (1980) found a larger form of the enzyme. The enzyme from *M. phlei* was found to be a protein of 113 kDa (Szymona and Ostrowski, 1964) or 275–280 kDa (Girbal *et al*., 1989). The native enzyme from *P. shermanii* was reported to have a molecular mass of 31 kDa (Clark,

1990). The purified enzymes from *P. shermanii* (Phillips *et al*., 1993) and *M. tuberculosis* (Hsieh *et al*., 1993b) also showed multiple proteins by HPLC gel filtration, native PAGE and isoelectric focusing (lEF)–PAGE, although a single band was observed by SDS–PAGE. To explain the existence of multiple forms of glucokinase, it is assumed that the enzyme may contain residual amounts of strongly bound PolyPs of various chain lengths. As detailed by Phillips *et al*., (1999), the enzymes from *P. shermanii, M. tuberculosis* and *Propionibacterium arabinosum* were all found to be homodimers of 30 kDa sub units.

The common feature of PolyP glucokinases from different sources was that the extracts containing a polyphosphate glucokinase activity also contained an ATP-dependent activity. Stable co-purification of these activities suggested that both of them can be catalysed by a single enzyme (Szymona *et al*., 1977; Pepin and Wood, 1986). To answer the question of bifunctionality, Phillips and co-workers carried out extensive purification of the enzymes from *P. shermanii* (Phillips *et al*., 1993), *M. tuberculosis* (Hsieh *et al*., 1993a) and *P. arabinosum* (Phillips *et al*., 1999). A detailed characterization of enzyme preparations unequivocally revealed that a single enzyme from these sources catalyses both polyP- and ATP-dependent glucokinase activities (Hsieh *et al*., 1993a; Phillips *et al*., 1993; Kowalczyk *et al*., Phillips *et al*., 1999).

The most convincing evidence was cloning the gene from *M. tuberculosis* (Hsieh *et al*., 1996a). It was shown that the recombinant protein, expressed and purified from *E. coli*, contained both activities. The ability to utilize both inorganic (PolyP) and organic (ATP) phosphoryl donors in glucose phosphorylation suggested that, due to fundamental differences in the structures of the two phosphate donors, the residues involved in their binding may also be different. Horn *et al*. (1991), Phillips *et al*. (1993a), and Hsieh *et al*. (1993a) provided evidence of separate binding sites for the substrates.

Despite the lack of sequence similarities between eukaryotic hexokinases and prokaryotic glucokinases in the putative adenosine site, Hsieh (1996) found some structure similarities between the adenosine site in polyphosphate glucokinase and the proposed adenosine site in yeast hexokinase. Comparison of the kinetic features of PolyP- and ATP-dependent reactions for the enzymes from different sources supports the hypothesis that glucokinase in the earliest organisms may have predominantly been dependent on PolyP rather than ATP (Phillips *et al*., 1999). There is a progressive decrease in the efficiency of PolyP utilization by glucokinases, from older to newer organisms. The polyphosphate glucokinase from *Microlunatus phosphovorus* was closely related to the polyphosphate/ATP–glucokinase of *Mycobacterium tuberculosis,* but it could not phosphorylate glucose with ATP (Tanaka *et al*., 2003).

An enzyme responsible for the PolyP- and ATP-dependent mannokinase activities was purified to homogeneity from a cell extract of the bacterium *Arthrobacter* sp. (Mukai *et al*., 2003). The enzyme concerned was a monomer with a molecular mass of 30 kDa. This enzyme phosphorylated glucose and mannose with a high affinity for glucose, utilizing PolyP as well as ATP. The catalytic sites for PolyP-dependent phosphorylation and ATP-dependent phosphorylation of the enzyme were found to be shared, and the PolyP-utilizing mechanism of the enzyme was shown to be non-processive (Mukai *et al*., 2003). The deduced amino acid sequence of the polypeptide exhibited homology to the amino acid sequences of the PolyP/ATP–glucokinase of *M. tuberculosis* (level of homology, 45 %), ATP-dependent glucokinases of *Corynebacterium glutamicum* (45 %), *Renibacterium salmoninarum* (45 %) and *Bacillus subtilis* (35 %) (Mukai *et al*., 2003).

All of these observations suggest a hypothesis that PolyP was a precursor of ATP in bioenergetic processes at the earliest stage of evolution (Kulaev, 1971, 1974). There might

have been a gradual transition from PolyP to ATP as the phosphoryl donor in glucose phosphorylation (Phillips *et al*., 1999).

6.2.2 NAD Kinase (ATP:NAD 2′-Phosphotransferase, EC 2.7.1.23)

This enzyme catalyses the following reaction:

$$\mathrm{ATP} + \mathrm{NAD} \longrightarrow \mathrm{ADP} + \mathrm{NADP} \tag{6.10}$$

The above reaction was known many years ago (Kornberg, 1950; Wang and Kaplan, 1954) and found both in procaryotes and eucaryotes. In *Brevibacterium ammoniagenes* (Murata *et al*., 1979), *Micrococcus luteus* and *Corynebacterium ammoniagenes* (Fillipovich *et al*., 2000) phosphorylation of NAD using PolyP as a phosphate donor was revealed:

$$\mathrm{PolyP}_n + \mathrm{NAD} \longrightarrow \mathrm{PolyP}_{n-1} + \mathrm{NADP} \tag{6.11}$$

It has been established that in some bacteria one enzyme displays both activities (Kawai *et al*., 2000). An enzyme with both PolyP- and ATP-dependent NAD kinase activities was isolated from *Micrococcus flavus*. This enzyme is a dimer consisting of 34 kDa subunits. A gene *Rv1695* has been found in *Mycobacterium tuberculosis* and proposed to also be a PolyP-dependent NAD kinase. By cloning and expression in *E. coli*, *Rv1695* was shown to encode PolyP/ATP–NAD kinase and was named as *ppnk*. The *ppnk* product, a recombinant PolyP/ATP–NAD kinase (Ppnk), was purified and characterized. This enzyme was a tetramer consisting of 35 kDa sub-units when expressed in *E. coli*. PolyP/ATP–NAD kinases of *M. flavus* and Ppnk of *M. tuberculosis* H37Rv phosphorylated NAD, using PolyP and nucleoside triphosphates as the phosphoryl donors (Kawai *et al*., 2000).

NAD kinase was purified to homogeneity from *E. coli*. The enzyme was a hexamer consisting of 30 kDa sub-units and utilized ATP or other nucleoside triphosphates as phosphoryl donors for the phosphorylation of NAD. This enzyme could not use PolyP. The deduced amino acid sequence exhibited a homology with that of *M. tuberculosis* PolyP/ATP–NAD kinase (Kawai *et al*., 2001). Therefore, NAD kinases show the same features as PolyP/ATP glucose kinases. The enzymes are active or not active with PolyP, depending on the microorganism under study. The evolutionally older *Mycobacteria* possess both activities, in contrast to the evolutionally younger *E. coli*. This fact, together with data on the distribution of polyphosphate glucokinase in bacteria, confirms the idea of the greater role of PolyPs in cell energetics at the earliest stages of evolution (Kulaev, 1971, 1974).

6.2.3 Exopolyphosphatase (Polyphosphate Phosphohydrolase, EC 3.6.1.11)

One of the most important enzymes involved in PolyP metabolism is exopolyphosphatase, the enzyme that splits P_i from the end of the PolyP chain:

$$\mathrm{PolyP}_n + \mathrm{H_2O} \longrightarrow \mathrm{PolyP}_{n-1} + \mathrm{P_i} \tag{6.12}$$

The first data on this enzyme were obtained by Kitasato (1928), Ingelman and Malmgren (1947, 1948, 1949) Krishnan (1952), Malmgren (1949, 1952) and Grossman and Lang (1962).

Much data on the properties of exopolyphosphatases were obtained from different sources, and have been reviewed (Kulaev, 1979; Kulaev and Vagabov 1983). The greatest difficulty in the investigation of exopolyphosphatases was their low stabilities at purification. The first purified preparation of an exopolyphosphatase with high activity and stability was obtained from the *S. cerevisiae* cell envelope (Andreeva *et al*., 1990).

In recent years, the overview of the exopolyphosphatases classification and function has been renewed a great deal. A significant diversity of exopolyphosphatases in microorganisms and sufficient differences in their structure and properties in procaryotes and eucaryotes have come to light.

The most important enzymes exhibiting exopolyphosphatase activity in bacteria are the exopolyphosphatase encoded by the *ppx* gene (Akiyama *et al*., 1993) and the guanosine pentaphosphate phosphohydrolase encoded by the *gppA* gene (Keasling *et al*., 1993).The enzymes encoded by the *ppx* and *gppA* genes demonstrate a great sequence similarity, i.e. 39 % identity over an overlapping region of 492 residues (Reizer *et al*., 1993). These proteins are of about the same length (513 amino acid residues for ppx and 494 for pppGpp phosphohydrolase). Both enzymes possess one hydrophobic region. The *ppx* and *gppA* possess five conserved boxes, which suggest that the two phosphatases belong to the sugar kinase/actin/heat-shock protein hsp70 superfamily (Reizer *et al*., 1993).

The exopolyphosphatase encoded by the *ppx* of *E. coli* is a dimer with a sub-unit molecular mass of about 58 kDa (Akiyama *et al*., 1993). Its affinity to high-molecular-weight PolyP was nearly 100-fold higher than that of yeast polyphosphatases ($K_m = 9$ nM $PolyP_{500}$ as a polymer). This enzyme exhibits a high requirement for K^+ (21-fold stimulation by 175 mM of K^+) (Akiyama *et al*., 1993). It is 'low-active' with short-chain PolyPs.

The exopolyphosphatase ppx of *E. coli* is a highly processive enzyme demonstrating the ability to recognize PolyPs of long chain lengths. Multiple PolyP binding sites were identified in distant portions of the enzyme and shown to be responsible for the enzyme polymer length recognition (Bolesch and Keasling, 2000a). In addition, two independently folded domains were identified. The genes for the *N*- and *C*-terminal domains were generated by using PCR and overexpressed in *E. coli*. The purified domain proteins were immobilized and used for the study of PolyP binding constants. The purified *N*- and *C*-terminal domains lacked exopolyphosphatase activity. However, the activity could be recovered in cases where the polypeptides were combined (Bolesch and Keasling, 2000a). The *N*-terminal domain contained a quasi-processive polyphosphatase active site belonging to the sugar kinase/actin/heat-shock protein hsp70 superfamily. The *C*-terminal domain contained a single polyphosphate-binding site and was responsible for nearly all affinity for PolyP. This domain was also found to confer a highly processive mode of action (Bolesch and Keasling, 2000a).

The exopolyphosphatase encoded by *ppx* from *A. johnsonii* is a monomeric protein of 55 kDa (Bonting *et al*., 1993b). The K_m value for a polyphosphate with an average chain length of 64 phosphate residues is 5.9 μM. The activity is maximal in the presence of 2.5 mM Mg^{2+} and 0.1 mM K^+. No activity is observed in the absence of cations or in the presence of Mg^{2+} or K^+alone. The enzyme of *A. johnsonii* was active with $PolyP_3$ and $PolyP_4$ in the presence of 300 mM NH_4 and 10 mM Mg^{2+}, while no activity with $PolyP_3$ was observed in the presence of 0.1 mM K^+ and 2 mM Mg^{2+}.

The purified exopolyphosphatases from *E. coli* and *A. johnsonii* have low specific activities (1 μmol P_i per min per mg protein for the enzyme from *A. johnsonii* and 22 μmol P_i per min per mg of protein for the enzyme from *E. coli*) in comparison with the yeast enzymes (200–400 μmol P_i per min per mg of protein and more). They are low-active with $PolyP_3$ and short-chain PolyPs and require K^+ for the maximal activity. These properties represent the most appreciable difference between the majority of yeast and bacterial exopolyphosphatases. The low activity of bacterial exopolyphosphatases is probably explained by the fact that polyphosphate kinase in procaryotes is able both to synthesize and to hydrolyse PolyP (Kornberg *et al*., 1999). The gene *ppx* encoding the major *E. coli* exopolyphosphatase has been cloned and sequenced (Akiyama *et al*., 1993). The *ppx* genes were cloned and sequenced from *Pseudomonas aeruginosa* (Miyake *et al*., 1999) and*Vibrio cholerae* (Ogawa *et al*., 2000b). It should be noted that in *E. coli* (Akiyama *et al*., 1993) and *Vibrio cholerae* (Ogawa *et al*., 2000b) *ppk1* and *ppx* are in one operon, which suggests a co-regulation of their transcription activities, while in *Pseudomonas aeruginosa the ppx* is located in the opposite direction from the *ppk* gene and they do not constitute an operon (Miyake *et al*., 1999).

Another enzyme encoded by the *gppA* gene and possessing exopolyphosphatase activity was purified from *E. coli* (Keasling *et al*., 1993). This enzyme is a dimer with a monomer molecular mass of 50 kDa; K_m is 0.5 nM for $PolyP_{500}$. It has a preference for long-chain polyPs, but one of its substrates is guanosine pentaphosphate (pppGpp), an important second messenger in bacteria.

One cannot exclude, however, that some bacteria possess other enzymes, which can split PolyPs. The acid phosphatase of *E. coli* was demonstrated to split long-chain PolyPs with a high specific activity (190 μmol P_i per min per mg of protein). The enzyme is active without divalent cations, but has the optimal pH of 2.5 (Dassa and Boquet, 1981). Two exopolyphosphatases have been detected in a cell-free extract of *Microlunatus phosphovorus* (Lichko *et al*., 2002a),a bacterium isolated from activated sludge. One of them has a molecular mass of 93 kDa, pH optimum of 4.5, does not require K^+ for its activity and is stimulated by divalent cations. The other exopolyphosphatase has a molecular mass of 55 kDa, pH optimum of 7.5, and displays its optimal activity in the presence of K^+ and divalent cations. The content of the former exopolyphosphatase increased during the growth, while that of the latter varied only slightly (Lichko *et al*., 2002a). Exopolyphosphatase activity was found in some methanotrophs (Trotsenko and Shishkina, 1990).

There are little data on exopolyphosphatase activity in Archae. In *Halobacterium salinarium*, it was very low (Andreeva *et al*., 2000). In a crude extract of *Sulfolobus solfactaricus* (Cardona *et al*., 2002), the specific activity was 0.6 nmol P_i per min per mg of protein. This is much less than that found in bacteria. In this termophilic archae, a functionally active gene of exopolyphosphatase was found, cloned and overexpressed. This gene encoded a protein of 417 amino acid residues (47.9 kDa). Purified recombinant exopolyphosphatase degraded long-chain PolyPs (700–800 residues) and needed Mn^{2+} for its activity. The deduced amino acid sequence of *S. solfactaricus ppx* showed the highest (25–45 %) similarity to the sequences of bacterial *ppx* and possessed all of their conserved motifs. While *in vitro* the enzyme splits pppGpp, the authors believe that Archae do not seem to possess the genes responsible for the pppGpp synthesis and that the role of exopolyphosphatase *in vivo* is only PolyP hydrolysis (Cardona *et al*., 2002).

As for eucaryotes, exopolyphosphatases of the yeast *Saccharomyces cerevisiae* have been most extensively studied. Exopolyphosphatase activity in a cell homogenate of *S. cerevisiae* is high (0.10–0.13 μmol P_i per min per mg of protein) (Andreeva *et al*., 1994; Wurst and Kornberg, 1994) when compared with bacteria (0.02–0.04 μmol P_i per min per mg of protein) (Akiyama *et al*., 1993; Bonting *et al*., 1993b). Exopolyphosphatases from the cell envelope (Andreeva *et al*., 1990; Andreeva and Okorokov, 1993), cytosol (Andreeva *et al*., 1996, 1998a, 2003), vacuolar sap (Andreeva *et al*., 1998b) and mitochondrial matrix (Lichko *et al*., 2000) of *S. cerevisiae* were purified and characterized.

Two polyphosphatases have been purified from an homogenate of *S. cerevisiae* (Wurst and Kornberg, 1994; Lorenz *et al*., 1994b). These enzymes have neutral pH optima, similar kinetic properties and substrate specificity, and require divalent cations, preferably Mg^{2+} or Co^{2+}, for the maximal activity. Their activities on tripolyphosphate is nearly 1.5-fold higher than those on long-chain PolyPs. These enzymes are monomeric proteins, one of 45 kDa (Wurst and Kornberg, 1994) and the other of 28 kDa (Lorenz *et al*., 1994b). The properties of these enzymes are similar to those of cytosolic and cell-envelope exopolyphosphatases (Andreeva and Okorokov, 1993; Andreeva *et al*., 1996).

Exopolyphosphatase activities were found and characterized in the purified preparations of yeast cell nuclei (Lichko *et al*., 1996, 2002b) and mitochondrial membranes (Lichko *et al*., 1998). The presence of specific exopolyphosphatases in these sub-cellular fractions was confirmed by their insensitivity to inhibitors of other phosphohydrolases occurring in the cell compartments and differences in pH optima.

All studied exopolyphosphatases of *S. cerevisiae* exhibited several common features (Andreeva *et al*., 1990, 1998 a,b, 2001; Andreeva and Okorokov, 1993; Lichko *et al*., 1996, 1998, 2000, 2002b, 2003a,b). They hydrolysed PolyPs of various chain lengths with release of P_i, and failed to hydrolyse *p*-nitrophenylphosphate (the substrate of phosphatases with a broad spectrum of action), ATP and other nucleoside triphosphates, and PP_i. The enzyme–substrate affinity for all exopolyphosphatases under study was considerably higher with PolyPs of greater chain lengths (Table 6.3). The sensitivity to a number of inhibitors was also similar. They were insensitive to molybdate, a commonly used phosphohydrolase inhibitor, and fluoride, the inhibitor of pyrophosphatases. They were not inhibited with azide, oligomycin, orthovanadate, *N*,*N*′-dicyclohexylcarbodiimide, diethylstilbesterol and nitrate – the known inhibitors of ATPases of different types. SH reagents such as *N*-ethylmaleimide and iodacetamide had little or no effect on exopolyphosphatase activities, showing that the

Table 6.3 The Michaelis constants (μmol) of purified exopolyphosphatases of different cell compartments of *S. cerevisiae* (Andreeva and Okorokov, 1993; Andreeva *et al*., 1998a,b, 2001, 2004; Lichko *et al*., 1996, 1998, 2000).

	Cell compartment				
		Cytosol			
Substrate	Cell envelope, 40 kDa enzyme	40 kDa enzyme	High-molecular-weight enzyme	Vacuolar sap	Mitochondrial matrix
$PolyP_{15}$	15	11	75	93	18
$PolyP_{208}$	0.9	1.2	3.5	2.4	0.25

enzyme active centers contained no SH groups necessary for the activity. Insensitivity of all exopolyphosphatases types to orthovanadate suggests their inability to form a phosphorylated intermediate during the PolyP hydrolysis reaction.

It should be noted that, in spite of the solubilities of most yeast exopolyphosphatases, the detergent 'Triton X-100' was the best stabilizer of these enzymes during purification and storage (Andreeva *et al*., 1990; Andreeva *et al*., 1998a,b).

The common inhibitor for all exopolyphosphatases was heparin, which suppressed the activities of both sub-cellular preparations and purified enzymes as a competitive inhibitor (Table 6.4.) (Andreeva *et al*., 1994).

Exopolyphosphatases of *S. cerevisiae*, except for that from the mitochondrial membrane, were stimulated by divalent metal cations (Table 6.5). The degree of stimulation was different and depended on the cation and its concentration.

Monovalent metal cations including K^+ had little or no effect on the yeast exopolyphosphatases when compared with bacterial enzymes, which exhibited a strong dependence on K^+ for their activities (Lichko *et al*., 2003a).

Exopolyphosphatases of *S. cerevisiae* were optimal at neutral pH, although the profiles of pH dependence had their own peculiarities for each enzyme. While the cell envelope and cytosolic exopolyphosphatases were able to hydrolyse substrates at acid and alkaline pH,

Table 6.4 Effect of some reagents on exopolyphosphatases (exopolyPase) of various cell compartments of *S. cerevisiae* (Andreeva and Okorokov, 1993; Andreeva *et al*., 1998a,b, 2001, 2004; Lichko *et al*., 1996, 1998, 2000).

	Activity (%)			
Enzyme	Without effector[a]	Heparin 20 μg ml^{-1}	EDTA, 1 mM	Antibodies[b], 5 μl ml^{-1}
40 kDa exopolyPase of cell envelope[c]	100 (220)	6	145	10
40 kDa exopolyPase of cytosol[c]	100 (320)	8	143	20
High-molecular-weight exopolyphosphatase of cytosol[c]	100 (135)	5	0	135
ExopolyPase of mitochondrial matrix[c]	100 (2)	50	100	80
ExopolyPase of vacular sap[c]	100 (60)	10	0	100
ExopolyPase of mitochondrial membrane[d]	100 (0.085)	0	100	100
ExopolyPase of nucleus[d]	100 (0.055)	10	70	100

[a] The values of specific activities (U mg protein^{-1}) corresponding to 100 % are given in brackets. $PolyP_{15}$ and 2.5 mM Mg^{2+} were used.
[b] Antibodies were obtained against purified cell-envelope exopolyphosphatase.
[c] Purified enzymes.
[d] The exopolyphosphatase activity of isolated sub-cellular fractions is shown.

Table 6.5 Effect of divalent cations on exopolyphosphatase activities of cell compartments of *S. cerevisiae* (Andreeva and Okorokov, 1993; Andreeva *et al*., 1998a,b, 2001, 2004; Lichko *et al*., 1996, 1998, 2000).

	Degree of stimulation[a]		
Source of enzyme	2.5 mM Mg^{2+}	0.1 mM Co^{2+}	0.1 mM Zn^{2+}
Cell envelope[b]	10	14	7.5
Cytosol, 40 kDa enzyme[b]	39	66	30
Mitochondrial matrix[b]	4	4	4
Cytosol, high-molecular-weight enzyme[b]	2	6	2
Vacuolar sap[b]	2	6	2
Mitochondrial membrane[c]	0.7	0.7	0.6
Nucleus[c]	1.8	3.2	1.7

[a] Activity was measured with $PolyP_{15}$ used as the substrate and the values (expressed as '*n*-fold') were related to the activity observed in the absence of exogenous cations.
[b] Purified enzymes.
[c] The exopolyphosphatase activities of isolated sub-cellular fractions is shown.

though to a lesser extent, the exopolyphosphatases of vacuoles, nuclei and mitochondria had sharper pH profiles.

Other properties of exopolyphosphatases from separate cell compartments of *S. cerevisiae* differed essentially from each other. At least 45–60 % of the total exopolyphosphatase activity of the yeast cell is localized in the cytosol (Andreeva *et al*., 1994, 2001). Cytosol and the cell envelope possess very similar forms of the enzyme (Andreeva *et al*., 1990, 1996). This is a monomeric protein of 40 kDa. Antibodies obtained against the purified cell envelope exopolyphosphatase inhibited only the enzyme from the cell envelope and cytosol, but were inefficient towards other exopolyphosphatases of the yeast cell (Table 6.5). Based on substrate specificity, 40 kDa exopolyphosphatases of the cytosol and cell envelope should be termed tripolyphosphatases, since their activity with $PolyP_3$ is 1.5-fold higher than with the long-chain PolyPs. These can hydrolyse adenosine- and guanosine-tetraphosphates. The specific activity with PolyPs of different degrees of polymerization, with the exception of $PolyP_3$, did not depend on the PolyP chain length (Table 6.6). This exopolyphosphatase was essentially inactive without divalent metal cations (Table 6.5). A complex of PolyP and Mg^{2+} in the ratio of 1:1 was a substrate of the reaction with $PolyP_3$ (Andreeva *et al*., 1998a; Kulakovskaya *et al*., 1999) and, probably, with high-molecular-weight PolyPs. An unusual property of this enzyme, which is lacking in other exopolyphosphatases, is stimulation with EDTA: 1 mM of this complexon in the presence of 2.5 mM Mg^{2+} increased the enzyme activity 1.5-fold (Table 6.4.). This effect apparently resulted from the presence in the enzyme molecule of a regulatory centre for binding divalent metal cations. Other yeast exopolyphosphatases are inhibited by EDTA, in accordance with its ability to bind divalent cations, or are not affected by this complexon. The cytosol of *S. cerevisiae* posesses an additional high-molecular-weight exopolyphosphatase (Andreeva *et al*., 2001, 2004), whose properties will be discussed below.

The exopolyphosphatase purified from the mitochondrial matrix of *S. cerevisiae* has the same molecular mass of $\sim$ 40 kDa, substrate specificity (Table 6.6), and the requirement

Table 6.6 Substrate specificity of exopolyphosphatases of *S. cerevisiae* (Andreeva and Okorokov, 1993; Andreeva *et al*., 1998a,b, 2001, 2004; Lichko *et al*., 1996, 1998, 2000).

	Activity (%)[a]			
Compartment	$PolyP_3$	$PolyP_9$	$PolyP_{15}$	$PolyP_{208}$
Cell envelope[b]	180	120	110	100
Cytosol, 40 kDa enzyme[b]	160	120	110	100
Mitochondrial matrix[b]	210	140	110	100
Cytosol, high-molecular-weight enzyme[b]	12	44	90	100
Vacuolar sap[b]	7	21	86	100
Mitochondrial membrane[c]	36	41	77	100

[a] Specific activities were the same as those shown in Table 6.4.
[b] Purified enzyme preparations.
[c] Exopolyphosphatase activity in isolated sub-cellular fractions.

for divalent cations (Table 6.5). This enzyme, however, has a higher affinity to long-chain PolyPs (Table 6.3), is less sensitive to antibodies against cell-envelope exopolyphosphatase, and is not activated by EDTA (Table 6.4).

Quite a different form of exopolyphosphatase was purified from the vacuolar sap of *S. cerevisie* (Andreeva *et al*., 1998b). Its molecular mass determined by gel filtration was ~ 245 kDa. This exopolyphosphatase hydrolysed $PolyP_3$ only slightly, and its specific activity increased with the increase in PolyP chain length (Table 6.6). It was unable to hydrolyse adenosine- and guanosine-tetraphosphates and was insensitive to antibodies inhibiting the low-molecular-mass exopolyPase of the cytosol (Table 6.4). This enzyme was stimulated by divalent metal cations to a much lesser extent than 40 kDa exopolyphosphatase (Table 6.5) and was inhibited by EDTA (Table 6.4). The inhibitory effect of EDTA is explained by the binding of Co^{2+}, which is the best activator of the vacuolar exopolyphosphatase at 0.1 mM.

The exopolyphosphatase of a membrane fraction of a these organelles resembles the soluble form in the properties studied (Andreeva *et al*., 1993).

A specific exopolyphosphatase was tightly bound to the mitochondrial membranes of *S. cerevisiae* (Lichko *et al*., 1998). This was the first known example of membrane-bound exopolyphosphatases. It was characterized by its higher activity with PolyPs of greater chain lengths (Table 6.6). Under gel filtration of a solubilized preparation of mitochondrial membranes, this activity was shown to be associated with proteins of 76 and 140 kDa. A special feature of this exopolyphosphatase was its inhibition by divalent metal cations (Table 6.5).

The exopolyphospahatase found in the nuclei of *S. cerevisiae* was stimulated 2–3-fold by divalent metal cations (Table 6.5) and was insensitive to EDTA and antibodies against cell-envelope exopoylphosphatase (Table 6.4).

Under phosphate overplus, i.e. a transfer of yeast cells from P_i-limited to complete media, a new exopolyphosphatase was observed in the cytosol of *S. cerevisiae*, demonstrating a sufficient difference from the 40 kDa exopolyphosphatase (Andreeva *et al*., 2001, 2004). It appears as a complex of ~ 830 kDa, probably comprising PolyPs and other proteins. The

activity of high-molecular-weight exopolyphosphatase under phosphate overplus gradually increased up to 10-fold in the logarithmic phase and then remained stable, while the activity of the 40 kDa exopolyphosphatase decreased 1.3-fold in the same growth phase and dropped almost 10-fold in the stationary growth phase. The high-molecular-weight exopolyphosphatase of cytosol was more active with PolyPs of longer chain lengths. Its activity with $PolyP_3$ was only 13 % of that with $PolyP_{208}$ (Andreeva *et al*., 2001, 2004). The antibodies against 40 kDa exopolyphosphatase had no effect on the high-molecular-weight ones. The stimulation of activity by divalent cations was less in the case of high-molecular-weight exopolyphosphatase when compared with the 40 kDa form (Table 6.4). High-molecular-weight exopolyphosphatase of the cytosol also differed from exopolyphosphatases of the cell envelope (Andreeva and Okorokov, 1993), nuclei (Lichko *et al*., 1996) and mitochondria (Lichko *et al*., 1998), but was similar to the vacuolar exopolyphosphatase of the same yeast (Andreeva *et al*., 1998b). It should be noted that, despite the simultaneousness of PolyP accumulation and increase in high-molecular-weight exopolyphosphatase activity, no direct interrelation has been found between these procecces. Cycloxemide blocked the increase in high-molecular-weight exopolyphosphatase activity but had no effect on PolyP accumulation.

The only known gene encoding the yeast exopolyphosphatase is the *PPX1* gene, which was cloned and sequenced (Wurst *et al*., 1995). The *PPX1* gene belongs to the PPase C family and has no sufficient similarity to the bacterial *ppx* gene (see http://www.expasy.org). Exopolyphosphatase PPX1, a protein of 396 amino acids with a molecular mass of $\sim$ 45 kDa, was purified from a homogenate of *S. cerevisiae* (Wurst *et al*., 1995). A *PPX1*-deficient strain was obtained using the gene elimination method (Wurst *et al*., 1995). Surprisingly, this had an exopolyphosphatase activity of $\sim$ 50 % of the parent strain level. Thus, the existence of other genes, encoding exopolyphosphatases in the yeast genome, was proposed (Wurst *et al*., 1995).

Considerable changes in exopolyphosphatase spectrum were observed on *PPX1* elimination (Figure 6.6). In the *PPX1*-deficient strain, 40 kDa exopolyphospatases were not observed in the cytosol, cell envelope and mitochondrial matrix (Lichko *et al*., 2002b, 2003a). Although PPX1 was absent in the cytosol of the mutant, exopolyphosphatase activity in this compartment decreased only twofold. This was explained by a concurrent fivefold increase in the activity of high-molecular-weight exopolyphosphatase in this compartment, whose properties were the same as those of the high-molecular-weight exopolyphosphatase which appeared in the cytosol under phosphate overplus. No exopolyphosphatase activity was found in a cell-envelope fraction of the PPX1 null mutant.

Inactivation of *PPX1* did not result in any considerable changes in the content and properties of vacuolar, nuclear and membrane-bound mitochondrial exopolyphosphatases when compared with the parent strain of *S. cerevisiae* (Lichko *et al*., 2002b, 2003b). This allows us to conclude that the 40 kDa exopolyphosphases of the cytosol, cell envelope and the mitochondrial matrix of *S. cerevisiae* are encoded by the same *PPX1* gene, and the cytosolic high-molecular-weight enzyme and those of vacuoles, nuclei and mitochondrial membranes are encoded by other genes (Lichko *et al*., 2002b, 2003a,b).

Distinction of the soluble mitochondrial exopolyphosphatase from those localized in the yeast cytosol and cell envelope could be explained by post-translational modification of this enzyme.

Under two different statuses of the yeast cell, P_i overplus and *PPX1* disruption, a drastic increase in high-molecular-weight exopolyphosphatase activity and disappearance

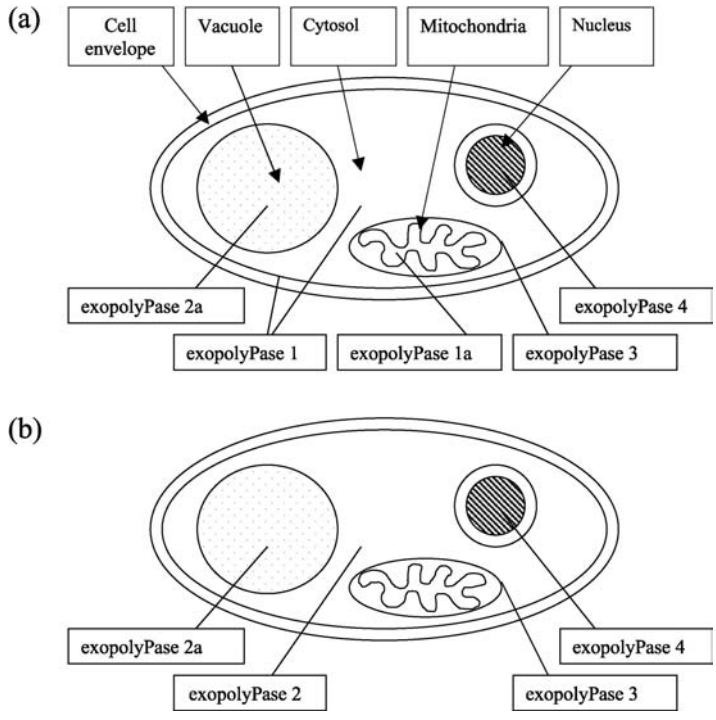

Figure 6.6 Effect of *PPX1* inactivation on the exopolyphosphatase spcetrum of *S. cerevisiae*: (a) the parent strain, and (b) the *PPX1*-deficient strain: exopolyPase 1, 40 kDa enzyme of cell envelope and cytosol; exopolyPase 1a, enzyme of mitochondrial matrix; exopolyPase 2, high-molecular-weight enzyme of cytosol; exopolyPase 2a, enzyme of vacuolar sap; exopolyPase 3, enzyme of mitochondrial membrane; exopolyPase 4, enzyme of nucleus.

of the 40 kDa exopolyphosphatase were observed in the yeast cytosol. This suggests an existence of a joint mechanism of regulation of both enzymes. These are likely to be involved in a co-regulation of the levels of some PolyP fractions and nucleoside polyphosphates such as adenosine-tetraphosphate. A search of such mechanisms is important to elucidate the involvement of exopolyphosphtases in regulation of yeast cell metabolism.

The comparison of exopolyphosphatases from different cell compartments of the yeast *S. cerevisiae* suggests that they are a typical example of 'compartment-specific' enzymes. The latter differ from each other in their physico-chemical properties, substrate specificity, response to changing cultivation conditions, and presumably, in the functions and ways of regulation. The compartment-specificity of exopolyphosphatases should be taken into account in the study of PolyP metabolism in the eukaryotic cell.

The cloning, overexpression, purification and characterization of the exopolyphosphatase (LmPPX) from Protozoa *Leishmania major* have been reported (Rodrigues *et al.*, 2002a). The gene sequence shows a similarity with *PPX1*. The product of this gene (LmPPX) has 388 amino acids and a molecular mass of 48 kDa. Heterologous expression of LmPPX in *Escherichia coli* produced a functional enzyme that was similar to the

yeast exopolyphosphatase with respect to its Mg^{2+} requirement, optimal pH and sensitivity to cations, amino acids and heparin (Rodrigues *et al*., 2002). In contrast to the yeast enzyme and other known exopolyphosphatases, it hydrolysed $PolyP_3$ with a higher rate and affinity. This processive enzyme did not hydrolyse pyrophosphate, ATP or *p*-nitrophenylphosphate. Immunofluorescence microscopy using affinity-purified antibodies against the recombinant enzyme indicated its acidocalcisomal and cytosolic localization (Rodrigues *et al*., 2002).

Exopolyphosphatases purified from *Neurospora crassa* (Umnov *et al*., 1974) and *Endomyces magnusii* (Afanas'eva and Kulaev, 1973) are close to the yeast cytosol exopolyphosphatase through its molecular mass and divalent cations requirements. The fact that it actually did not hydrolyse $PolyP_3$ may be due to its low affinity to this substrate, which was used in a 10-fold lower concentration than K_m for the yeast enzyme (Umnov *et al*., 1974). Two other exopolyphosphatase activities were observed in the 'slime' variant of *N. crassa* which cannot synthesize cell walls (Trilisenko *et al*., 1985a,b). One of these was K^+- and Mg^{2+}-dependent, hydrolysing high-molecular-weight polyPs, while the other was K^+- and Mg^{2+}-independent, hydrolysing low-molecular-weight polyPs. The study of a number of exopolyphosphatases from the lower eucaryotes is important to clarify the PolyP functions in each individual compartment of these microorganisms.

As regards animals, the first exopolyphosphatases were purified from the marine sponge *Tethya lyncurum* (Lorenz *et al*., 1995). Two exopolyphosphatases were identified in this simple metazoa. Exopolyphosphatase I had a molecular mass of 45 kDa, a pH optimum of 5.0, and did not required divalent cations for its activity, while exopolyphosphatase II had a molecular mass of 70 kDa, a pH optimum of 7.5, and displayed optimal activity in the presence of Mg^{2+} (Lorenz *et al*., 1995).

Exopolyphosphatase activity is also present in human osteoblasts (Leyhausen *et al*., 1998). The specific activity of the enzyme in osteoblasts was much higher than those in other mammalian cells and tissues tested (Schröder *et al*., 2000) (Table 6.7.). More than 50 % of the exopolyphosphatase activity in osteoblast cells was 'membrane-bound'. Exopolyphosphatase activity has also been found extracellularly, e.g. in synovial fluid (Schöder *et al*., 1999), as well as in human blood plasma and serum (Schröder *et al*., 1999, 2000) (Table 6.7).

Table 6.7 Exopolyphosphatase activities with $PolyP_{35}$ as the substrate in different cells, tissues and extracellular fluids from mammals (Schröder *et al*., 2000).

Cell/tissue	Exopolyphosphatase activity (nmol of P_i per h per mg of protein)
Rat liver	48
Rat brain	54
Human plasma	5.5
Human serum	5.4
Human osteoblasts	210
Human HL-60 cells	25
Human peripheral blood mononuclear cells	15

It was demonstrated that the intestinal isoform of alkaline phosphatase from calf was able to degrade PolyPs with a wide range of chain lengths, in addition to PP_i (Lorenz and Schröder, 2001). The enzyme splits P_i from PolyP in a processive manner. The pH optimum is in the alkaline range. Divalent cations are not required for catalytic activity but instead inhibit PolyP degradation. The rate of hydrolysis of short-chain PolyPs is comparable with that of the standard alkaline phosphatase substrate, i.e. *p*-nitrophenyl phosphate. The specific activity of the enzyme decreases with increasing chain length of the polymer, both in the alkaline and neutral pH ranges. The K_m of the enzyme also decreases with increasing chain length. The mammalian tissue non-specific isoform of alkaline phosphatase was not able to hydrolyse PolyP under the conditions applied, while the placental-type alkaline phosphatase displayed PolyP-degrading activity (Lorenz and Schröder, 2001).

Therefore, the exopolyphosphatases are quite different both in various organisms and in different cell compartments of the same organism. This suggests their possible different functions in cells.

6.2.4 Adenosine–Tetraphosphate Phosphohydrolase (EC 3.6.1.14)

$$\text{adenosine–5′-tetraphosphate} + H_2O \longrightarrow \text{ATP} + \text{phosphate} \qquad (6.13)$$

In yeast, adenosine–tetraphosphate phosphohydrolase and guanosine–tetraphosphate phosphohydrolase activities are an inherent property of exopolyphosphatase PPX1. It was demonstrated both with the cytosol preparations, where this enzyme is localized (Kulakovskaya *et al*., 1997), and with a purified PPX1 preparation (Guranowski *et al*., 1998). Exopolyphosphatase PPX1 of the cytosol of *S. cerevisiae* is able to hydrolyse adenosine–5′-tetraphosphate and guanosine–5′-tetraphosphate about twice more actively than $PolyP_{15}$, with an apparent K_m value of 80–100 μM (Kulakovskaya *et al*., 1997). Thus, in yeast PPX1 may link the metabolism of PolyP and some nucleoside polyphosphates.

The enzyme splitting both adenosine–tetraphosphate and guanosine–tetraphosphate was purified to homogeneity from yellow lupin seeds (Guranowski *et al*., 1997). The polypeptide of $\sim$ 25 kDa catalysed the hydrolysis of nucleoside–5′-tetraphosphate to nucleoside triphosphate and P_i, and hydrolysed $PolyP_3$, but neither pyrophosphate nor $PolyP_4$. The divalent carions Mg^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+} were required for the reaction.

The enzyme with adenosine–tetraphoshatase activity was obtained earlier from rabbit muscle (Small and Cooper, 1966). This enzyme had an effect on inosine tetraphosphate and tripolyphosphate but showed little or no activity with other nucleotides or PolyPs.

6.2.5 Triphosphatase (Tripolyphosphatase, EC 3.6.1.25)

Only earlier data (Meyerhof *et al*., 1953; Kornberg, 1957b) are available for trimetaphosphate hydrolase (EC 3.6.1.2) catalysing the following reaction:

$$\text{cyclotriphosphate} + H_2O \longrightarrow \text{PolyP}_3 \qquad (6.14)$$

Many enzymes, however, were demonstrated to catalyse the reaction:

$$\text{PolyP}_3 + \text{H}_2\text{O} \longrightarrow \text{Pyrophosphate} + \text{P}_\text{i} \tag{6.15}$$

First, there are inorganic pyrophosphatases (EC 3.6.1.1) which can hydrolyse tri- and tetraphosphate (Baykov *et al*., 1999). The ability of some pyrophosphatases to split these substrates depends on pH and divalent cations (Baykov *et al*., 1999). The most effective hydrolysis of low-molecular-weight PolyPs was observed for inorganic pyrophosphatase isolated from the archaeon *Metanotrix soehgenii*. This hydrolyses PolyP_3 and PolyP_4 for 44 and 8 %, respectively, of the PP_i hydrolysis rate (Jetten *et al*., 1992) and may therefore be involved in their metabolism.

Secondly, adenosylmethionine synthetase, in addition to synthetase reaction, catalyses tripolyphosphatase reactions stimulated by adenosylmethionine. Both of the enzymatic activities of the enzyme, which has been purified to homogeneity from *E. coli*, require a divalent metal ion and are markedly stimulated by certain monovalent cations (Markham *et al*., 1980). Tripolyphosphatase activity is also associated with *S*-adenosylmethionine synthetase isozymes from rat liver (Shimizu *et al*., 1986).

Thirdly, some RNA triphosphatases possess a weak tripolyphosphatase activity, and PolyP_3 is a potential competitive inhibitor (Yu *et al*., 1997; Gong and Shuman, 2002).

Specific tripolyphosphatase was purified from *Thermobacterium thermoautotrophicum* (Van Alebeek *et al*., 1994). The enzyme of 22 kDa hydrolyses tripolyphosphates five times more actively than PolyP_{15}.

Tripolyphosphatase was purified from *Neurospora crassa*. This had a molecular mass of 50 kDa, and its activity strongly depended on divalent metal cations (Kulaev *et al*., 1972a,c; Umnov *et al*., 1974; Egorov and Kulaev, 1976).

In *S. cerevisiae*, tripolyphosphatase activity is an inherent property of exopolyphosphatases of the cell envelope, cytosol and mitochondrial matrix (Andreeva and Okorokov, 1993; Andreeva *et al*., 1996; Lichko *et al*., 2000), which are encoded by the *PPX1* gene. In *Leishmania major,* exopolyphosphatase is also responsible for PolyP_3 degradation (Rodrigues *et al*., 2002a).

The putative ability of some tripolyphosphatases from microorganisms to split long-chain PolyPs under suitable conditions needs further investigation.

6.2.6 Endopolyphosphatase (Polyphosphate Depolymerase, EC 3.6.1.10)

Endopolyphosphatase splits long-chain PolyP molecules into shorter ones. The product contains four to five phosphate residues.

$$\text{PolyP}_n + \text{H}_2\text{O} \longrightarrow \text{Oligopolyphosphates} \tag{6.16}$$

Malmgren (1952) and Mattenheimer (1956) were the first to observe this reaction in yeast and fungi. Endopolyphosphatase activity was investigated in the fungi *Aspergillus niger* (Malmgren, 1952) and *Neurospora crassa* (Kritsky *et al*., 1972; Kulaev *et al*., 1972a–c).

This enzyme was purified from the yeast (Kumble and Kornberg, 1996). It is a dimer of 35 kDa sub-units, and its activity requires divalent metal cations. Mn^{2+} is more active

than Mg^{2+}, with an optimum concentration of about 2.5 mM. The enzyme hydrolyses PolyPs to shorter chain lengths and even to tripolyphosphate. These authors suggest the endopolyphosphatase to be localized in vacuoles (Kumble and Kornberg, 1996). The activity has been partially purified from rat and bovine brain, where its abundance is about 10 times higher than in other tissues but much less than in yeast (Kumble and Kornberg, 1996). Endopolyphosphatase has escaped detection in procaryotes (Kumble and Kornberg, 1996). The presence of this enzyme in eucaryotes is supposed to be associated with the redistribution of PolyP pools in different compartments and PolyP transport between the compartments.

6.2.7 PolyP:AMP Phosphotransferase

The most well-known databases (http://www.expasy.org, and http://www.chem.qmul.ac.uk/iubmb/enzyme) have no mention of this enzyme.

The reaction was found first in *Corynebacterium xerosis* (Dirheimer and Ebel, 1965):

$$\text{PolyP}_n + \text{AMP} \rightleftharpoons \text{PolyP}_{n-1} + \text{ADP} \tag{6.17}$$

PolyP:AMP phosphotransferase was partly purified from *A. jonsonii* strain 210A (Bonting *et al*., 1991). This had a molecular mass of 55 kDa. The kinetic studies showed apparent K_m values of 0.26 mM for AMP and 0.8 μM for PolyP_{35}. The highest activity was found with PolyPs of 18 to 44 phosphate residues. The PolyPs were degraded completely by a processive mechanism. No activity was revealed with pyrophosphate, PolyP_3 and PolyP_4.

Some authors believed that this enzyme, in concert with adenylate kinase, is responsible for utilization of the greater part of PolyP in *A. johnsonii* (Kortstee and van Aeen, 1999; Kortstee *et al*., 2000):

$$\text{PolyP}_n + \text{AMP} \rightleftharpoons \text{PolyP}_{n-1} + \text{ADP} \tag{6.18}$$

$$2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \tag{6.19}$$

The resulting reaction is as follows:

$$\text{PolyP}_n + \text{ADP} \rightleftharpoons \text{PolyP}_{n-1} + \text{ATP} \tag{6.20}$$

This pathway retains the phosphoanhydride-bound energy of PolyP. The enzymes from *A. johnsonii* were used to create an ATP-regenerating system (Resnick and Zehnder, 2000).

The significance of PolyP:AMP phosphotransferase in different bacteria is still in question. Therefore, in *E. coli* the ADP formation from PolyP by chain shortening was explained by a joint action of polyphosphate kinase and adenylate kinase, which formed a complex with the PolyP (Ishige and Noguchi, 2000):

$$\text{PolyP}_n + \text{ADP} \rightleftharpoons \text{PolyP}_{n-1} + \text{ATP} \tag{6.21}$$

$$\text{AMP} + \text{ATP} \rightleftharpoons \text{ADP} \tag{6.22}$$

This conclusion was grounded on the observation that over-expression of polyphosphate kinase in *E. coli* caused a sharp increase of the PolyP:AMP phosphotransferase activity (Ishige and Noguchi, 2000). Moreover, *in vitro* PolyP:AMP phosphotransferase activity

Table 6.8 The occurrence of PolyP-dependent enzymes in prokaryotes and eukaryotes.

Enzyme	Procaryotes	Eucaryotes
PolyP kinase	+	−
1,3-Diphosphoglycerate–PolyP phosphotransferase	+	+
Dolichyl-PP:PolyP phosphotransferase	−	+
PolyP-glucose phosphotransferase	+	−
Exopolyphosphatase	+	+
Endopolyphosphatase	−	+
PolyP-dependent NAD kinase	+	−
PolyP:AMP phosphotransferase	+	−

required equimolar concentrations of polyphosphate kinase and adenylate kinase (Ischige and Noguchi, 2000).

In *Pseudomonas aeruginosa*, massive PolyP:AMP phosphotransferase and PolyP:ADP phosphotransferase activities were found (Ishige and Noguchi, 2001). Partially purified PolyP:ADP phosphotransferase was independent of polyphosphate kinase encoded by the *ppk1* gene and could act as a PolyP-dependent nucleoside diphosphate kinase, which preferred GDP as a phosphate aceptor (Ishige and Noguchi, 2001). It has been demonstrated that PolyP:AMP phosphotransferase activity in this bacterium originated mainly from the combined action of PolyP:ADP phosphotransferase and adenylate kinase (Ishige and Noguchi, 2001). The PolyP:ADP phosphotransferase activity is probably due to a new polyphosphate

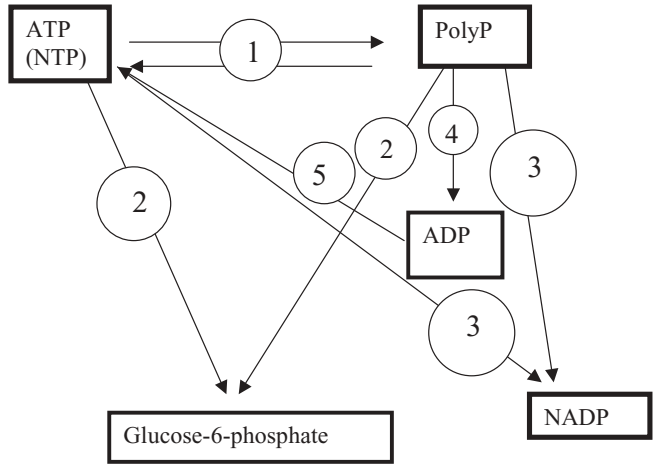

Figure 6.7 Enzymes coupling metabolism of PolyPs and nucleoside phosphates in bacteria: (1) polyphosphate kinases; (2) glucokinases; (3) NAD kinases; (4) PolyP:AMP phosphotransferase; (5) adenylate kinase.

kinase, ppk2, discovered in *Pseudomonas aeruginosa* in compliance with its substrate specificity and predominant reaction pathway (Ishige *et al*., 2002, Zhang *et al*., 2002).

Thus, in *E. coli* and *P. aeruginosa* the PolyP:AMP phosphotransferase activity is a result of the joint action of adenylate kinase and polyphosphate kinases. The occurrence of PolyP:AMP phosphotransferase in the above bacteria has, however, no genetic confirmation. No such activity was observed in eucaryotes, in line with the absence of polyphosphate kinase.

The analysis of the properties of PolyP-dependent enzymes has offered some interesting observations. The sets of PolyP-dependent enzymes in procaryotes and eucaryotes show a significant difference. Some enzymes are found in only procaryotes or in eucaryotes (Table 6.8). Even the exopolyphosphatases of procaryotes and eucaryotes belong to different protein families. Tight interrelations between PolyPs and the enzymes metabolizing nucleoside compounds are most demonstrated in procaryotes (Figure 6.7), whereas in eucaryotes these interrelations are weaker. Nevertheless, some reactions linking PolyPs and nucleoside–polyphosphate pools are also observed (Booth and Guidotti, 1995; Kulakovskaya *et al*., 1997; Guranowski *et al*., 1998).

It should be noted that many PolyP-dependent enzymes (polyphosphate kinase, exopolyphosphatases, PolyP glucokinase and NAD kinase) are multifunctional and can catalyse reactions both with PolyPs and nucleotide triphosphates. Some PolyP-dependent enzymes, especially exopolyphosphatases, provide excellent examples of cell-compartment specific enzymes. Cell-compartment specificity is a characteristic feature of eucaryotic ATPases (Nelson, 1992) and pyrophosphatases (Baltscheffsky and Baltscheffsky, 1992; Davies *et al*., 1997; Baykov *et al*., 1999). This means that the same reaction may be performed in cell compartments by specific enzymes, which differ in their properties, encoding genes and functions. All of the above properties of PolyP-dependent enzymes suggest their important role in the regulation of living cell functions as a whole.

7

THE FUNCTIONS OF POLYPHOSPHATES AND POLYPHOSPHATE-DEPENDENT ENZYMES

7.1 Phosphate Reserve

Many authors have adhered to the view that PolyPs are primarily a reserve of phosphate, on which the cells of microorganisms are able to draw on at any time, but especially during the periods of phosphorus starvation (Mudd *et al*., 1958; Harold, 1966; Kulaev and Vagabov, 1983; Wood and Clark, 1988; Kornberg, 1995). This function of PolyPs is confirmed by a strong dependence of PolyP content in the cells of microorganisms on the phosphate content in the medium (see Chapter 8).

In the opinion of Harold (1966), PolyPs, being polymers, constitute highly convenient compounds for the storage of large amounts of P_i in the cell, since the accumulation of polymeric phosphate molecules has little effect on osmotic pressure within the cells and, on the other hand, serves to maintain a constant level of important metabolites such as free P_i and ATP. Since phosphorus is an element of vital importance, which organisms are absolutely uncapable to live without, in the course of evolution microorganisms have developed the ability to store surplus phosphate in the form of PolyPs. It has been shown more than once that many microorganisms, both prokaryotes and eukaryotes, may occasionally accumulate sufficient amounts of PolyPs, thus enabling them to grow on a phosphorus-free medium (Langen and Liss, 1958a,b; Liss and Langen, 1962; Harold, 1966; Kulaev and Vagabov, 1983).

PolyPs are the principal regulators of the intracellular level of P_i in microorganisms. In all cases of rapid P_i uptake by cells, when the pathways of its utilization are limited, PolyPs are accumulated and the intracellular concentration of P_i remains low (Harold, 1966;

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Kaltwasser, 1962; Lichko *et al*., 1982; Kulaev and Vagabov, 1983). The same effect is observed when free P_i accumulates in a cell as a result of degradation processes, especially the degradation of nucleic acids (Harold, 1962b). Phosphate overplus (hypercompensation effect) is observed for both prokaryotes and eukaryotes. Phosphorus starvation results in de-repression of phosphatases localized on cell surfaces and of phosphate uptake systems (Harold, 1966; Nesmeyanova *et al*., 1974a,b, 1975a). Both processes, the cleavage of organic phosphorus compounds and P_i uptake from the medium, may increase the P_i level when phosphorus-starved cells are placed in a medium containing this element. In order to maintain a sensibly constant level of P_i, it is converted into PolyP (Ehrenberg, 1960; Harold, 1962b, 1966).

The importance of maintaining a constant low level of P_i in cells is relevant on account of several considerations. First, P_i concentration is, in turn, a powerful controlling factor of biochemical processes. Secondly, accumulation of any significant amounts of P_i in cells would result in a considerable change of its osmotic pressure and pH. It is also possible that free P_i in high concentrations is toxic for cells. One example of such toxicity was observed during P_i accumulation by the halophilic archae *Halobacterium salinarium* (Smirnov *et al*., 2002a,b). This archaeon is able to take up about 90 % of P_i from culture medium but unable to synthesize PolyPs in large amounts. As a result, massive P_i uptake leads to an accumulation of magnesium phosphate in cells, a change in cell morphology, and the death of some part of the population. In fact, it is likely that the large amounts of PolyPs, which are accumulated in cells of microorganisms under certain culture conditions, are a detoxification product of P_i entering the cells. Some features of the PolyP function as a P_i reserve in prokaryotes and eukaryotes will be described below.

7.1.1 In Prokaryotes

Many bacteria are able to accumulate PolyPs if the P_i content in the medium is high. In *Acinetobacter johnsonii*, these polymers make up to 30 % of dry biomass (Deinema *et al*., 1985). Large amounts of PolyPs are characteristic of the bacteria from wastewaters with a high phosphate content. *A. johnsonii* (Deinema *et al*., 1985; Kortstee *et al*., 1994, 2000), *Microlunatus phosphovorus* (Nakamura *et al*., 1995), *Microthrix parvicella* (Erhardt *et al*., 1997) and *Rhodocyclus* sp. (Keasling *et al*., 2000), isolated from activated sludge, are examples of such bacteria. The biotechnology of 'enhanced biological phosphate removal' (EBPR) has become a field of rapid development. This is based on the ability of bacterial communities of activated sludge to remove P_i from waste and to accumulate PolyPs in sludge biomass. Many reviews describe the biochemical and biotechnological aspects of this process (Kortstee *et al*., 1994, 2000; Van Loosdrecht *et al*., 1997; Ohtake *et al*., 1999; Keasling *et al*., 2000; Mino, 2000; McGrath and Quinn, 2003). We will return to this topic in Chapter 9.

In *E. coli*, the level of PolyP drops drastically under phosphate starvation, and the subsequent addition of orthophosphate to the medium restores the initial phosphate level (Nesmeyanova *et al*., 1973, 1974a,b; Nesmeyanova, 2000). Some genetic manipulations increased the ability of *E. coli* to accumulate PolyP (Kato *et al*., 1993a; Hardoyo *et al*., 1994; Ohtake *et al*., 1994; Sharfstein *et al*., 1996). High levels of accumulation were achieved

by genetic regulation and increase in the dosage of *E. coli* genes encoding polyphosphate kinase 1, acetate kinase, and phosphate-inducible transport systems (PSTS, PSTC, PSTA, and PSTB) and by genetic inactivation of *ppx* encoding exopolyphosphatase. The best recombinant strains of *E. coli* eliminated approximately two- and threefold more P_i from the medium than the control strain (Hardoyo *et al*., 1994). These strains accumulated in the cells approximately 10-fold more P_i than the control strain. The phosphorus content of these recombinant strains reached a maximum of 16 % of dry biomass. About 65 % of cellular phosphorus was stored as PolyP (Ohtake *et al*., 1994). These data suggest that the systems providing PolyP accumulation in bacteria include many genes in addition to those encoding the major bacterial PolyP metabolizing enzymes, i.e. polyphosphate kinase and exopolyphosphatase.

In some culture conditions, extracellular PolyP was identified as a good source of phosphate (Curless *et al*., 1996). Using a typical medium in a high-cell-density fermentation of *E. coli*, 40 % higher cell density was obtained when using PolyP instead of P_i as a phosphate source (Curless *et al*., 1996). It is probable that the expression of specific porins allows PolyP transfer from the culture medium into the cells. The outer membrane porin PhoE of *E. coli* (Bauer *et al*., 1989) and the OprO porin of *Pseudomonas aeruginosa* (Siehnel *et al*., 1992; Hancock *et al*., 1992), induced by phosphate starvation, are examples of proteins which prefere PP_i and PolyP rather than P_i.

7.1.2 In Eukaryotes

The accumulation of phosphate reserves as PolyPs and their use at phosphate starvation also occur in eukaryotic microorganisms. The yeast *Saccharomyces cerevisiae* (Liss and Langen, 1962; Kulaev and Vagabov, 1983) and *Neurospora crassa* (Kulaev and Afanasieva, 1969, 1970) are characterized by the phenomenon of phosphate overplus. These accumulate higher contents of PolyPs after phosphate starvation, followed by transfer to a phosphate-containing medium. Such processes touch upon all different PolyP fractions of eukaryotic microbial cells (Kulaev and Afanasieva, 1969, 1970; Kulaev and Vagabov, 1983; Vagabov *et al*., 2000).

The increase in PolyP level in yeast may be due to phosphate uptake stimulation. Cells of *Candida humicola* demonstrated a 4.5-fold increase in phosphate uptake from the medium and accumulated 10-fold more PolyP during growth at pH 5.5, when compared with growth at pH 7.5 (McGrath and Quinn, 2000). Further details on PolyP accumulation and utilization in eukaryotes are given in Chapter 8.

Whereas mainly cytosolic PolyP performs the function of phosphorus reservation in bacteria, in eukaryotic microorganisms phosphorus is also reserved as PolyP in other cell compartments. Under yeast growth on a medium without phosphate, the PolyP content drops by more than an order in the cytosol, vacuoles and cell walls (Kulaev and Vagabov, 1983; Kulaev *et al*., 1999). PolyP granules of the cytosol quickly disappear after the yeast has been placed in a phosphate-deficient medium. In a P_i-deficient medium, a sharp decrease of the PolyP level, both in whole cells and in vacuoles, was noted, and after 7 h of starvation the PolyP level in vacuoles decreased by 85 %, which indicates an active utilization of the entire PolyP pool for the needs of the cell under these growth conditions (Kulaev *et al*., 1999; Trilisenko *et al*., 2002).

Vacuoles also contain an important phosphorus reserve in yeast and fungi (Indge, 1968a,b,c; Urech *et al*., 1978; Cramer and Daves, 1984). Under phosphate overplus, the content of PolyP in vacuoles of *Saccharomyces carlsbergensis* grew dramatically (Lichko *et al*., 1982). Some mutants of *S. cerevisiae* having no vacuoles possess low levels of PolyP and are unable to grow on a medium without P_i (Shirahama *et al*., 1996).

The cells of protozoa (Docampo and Moreno, 2001) and alga *Chlamydomonas reinhardtii* (Ruiz *et al*., 2001b) possess specific PolyP and Ca^{2+} storage organelles, i.e. acidocalcisomes, which are similar to vacuoles in some properties, especially in the presence of proton-pumping pyrophosphatase. These organelles act as phosphate storage systems for the above lower eukaryotes.

In eukaryotes, the function of PolyP as a phosphate reserve is probably related to the action of different forms of exopolyphosphatases and endopolyphosphatase.

It is possible, however, that in some cases utilization of PolyP does not involve hydrolysis to P_i, but rather phosphate transfer without loss of the energies of the phosphoric anhydride bonds to other compounds. It seems unlikely that the energy stored in PolyPs would be dissipated without being utilized for energy-requiring processes.

7.2 Energy Source

The phosphoanhydride bonds of PolyPs have free energies of hydrolysis similar to that of ATP and occupy an intermediate position in the free energy scale of phosphorylated compounds. Thermodynamically, the standard free energy of hydrolysis of the anhydride linkage yields about 38 kJ per phosphate bond at pH 5. It can therefore act as both a donor and an acceptor of phosphate groups. Belozersky was the first to suggest that PolyP in very primitive organisms could perform the functions of energy-rich compounds as an evolutionary percursor of ATP (Belozersky, 1958).

7.2.1 Polyphosphates in Bioenergetics of Prokaryotes

In many prokaryotes, PolyP is a direct phosphorus donor for biochemical reactions due to the action of enzymes such as polyphosphate–glucose phosphotransferase and NAD kinase. Polyphosphate kinases and PolyP:AMP phosphotransferase link nucleoside–polyphosphate and inorganic PolyP. Polyphosphate kinases 1 and 2 can use PolyPs for the synthesis of different nucleoside triphosphates.

A specific way of using PolyP as an energy source was found in the PolyP-accumulating bacterium *Acinetobacter johnsonii*. When high-P_i-grown cells of strictly aerobic *A. johnsonii* 210A are incubated anaerobically, their PolyP is degraded and P_i is excreted (Van Veen *et al*., 1994; Kortstee *et al*., 2000). The energy of PolyP is mobilized by two systems. The polyphosphate:AMP phosphotransferase/adenylate kinase system is responsible for the direct formation of ATP from PolyP, while a constitutive, bidirectional, low-affinity P_i transport system mediates the uptake and efflux of $MeHPO_4$. The uptake is driven by the proton motive force, while the electrogenic excretion of $MeHPO_4$ in conjunction with a proton generates this force (Van Veen *et al*., 1994). Exopolyphosphatase may enhance the

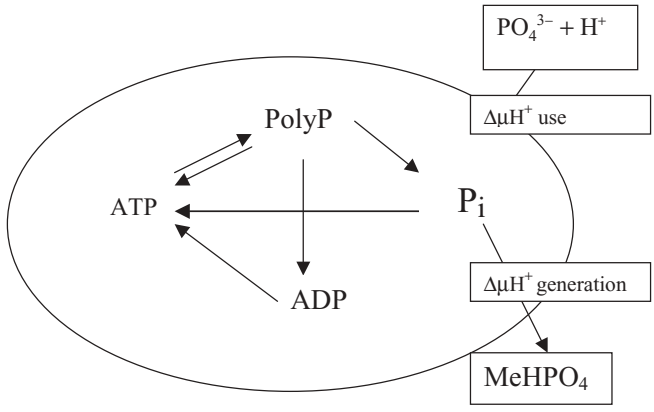

Figure 7.1 The interrelations between ATP and PolyP in bacteria ($\Delta\mu H^+$ is the electrochemical H^+ potential).

latter energy recycling mechanism by providing the efflux process with a continuous supply of P_i (Kortstee *et al*., 2000). The known interrelations between ATP and PolyP metabolism in bacteria are shown in Figure 7.1.

7.2.2 Polyphosphate in Bioenergetics of Eukaryotes

In eukaryotes, little direct evidence of the interrelation between the AMP–ADP–ATP system and PolyPs has been found. Polyphosphate kinase genes are absent in the known eukaryotic genomes (Kornberg *et al*., 1999; Zhang *et al*., 2002). NAD kinases and glucokinase of eukaryotes have lost the ability to use PolyP as a phosphodonor. It seems that the role of PolyP in bioenergetics is diminished in eukaryotic cells. However, some data suggest preservation of the PolyP function as an energy reserve in eukaryotic microorganisms.

First, the synthesis of ATP from PolyP has been observed in isolated vacuoles of yeast (Schabalin *et al*., 1977). However, the significance of this process needs further investigation.

Secondly, the induction of high-molecular-weight PolyP synthesis in yeast cells took place in parallel with the exit of K^+ ions from the cells under accumulation of divalent cations in the presence of glucose (Okorokov *et al*., 1983a,b). This accumulation (Figure 7.2) was not affected by antymicine A but completely prevented by ionophores, stimulating K^+/H^+ exchange and disturbance of the K^+ gradient on the plasma membrane. This suggests a possibility of PolyP participation in retention of the energy of transmembrane K^+ gradient. PolyP accumulation in the yeast cell under phosphate overplus conditions was inhibited by 50 % by the protonophoric uncoupler FCCP (Trilisenko *et al*., 2003). This suggests involvement of the energy of proton motive force in PolyP synthesis. At the same time, it was observed that PolyP hydrolysis in *S. cerevisiae* was induced by the protonophoric uncoupler CCCP (Beauvoit *et al*., 1991).

Thirdly, observations of the PolyP dynamics during the growth of *S. cerevisiae* give additional indirect evidence of PolyP participation in the processes of energy conservation in

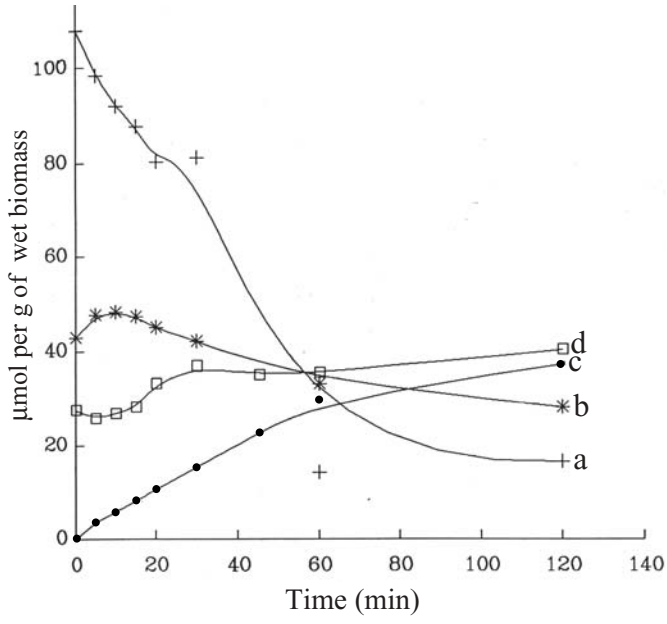

Figure 7.2 The content of (a) K^+, (b) high-molecular-weight PolyP, (c) Zn^{2+}, and (d) low-molecular-weight PolyP during Zn^{2+} uptake by the yeast *Saccharomyces carlbergensis*. The incubation medium contained 100 mM glucose and 3 mM $ZnSO_4$ (Okorokov *et al*., 1983b).

cells. Both the total content and distribution of PolyP by fractions in the yeast *Saccharomyces cerevisiae* depend on the growth phase (Vagabov *et al*., 1998) (for details, see Chapter 8). Before glucose was consumed from the medium, the biomass and total cellular PolyP content had increased in parallel. After glucose depletion, the content of PolyP in the cells fell sharply and then increased again. A significant decline of the content of intracellular PolyP while P_i was present in the growth medium at high concentrations may imply that in this growth phase PolyP is an energy rather than a phosphate source (Vagabov *et al*., 1998, 2000). The active synthesis of PolyPs, accompanied by a dramatic decrease in their chain lengths in the logarithmic phase of *S. cerevisiae* growth in a carbon- and phosphorus-sufficient medium, also suggests that the energy derived from PolyP hydrolysis is necessary to maintain the high rate of yeast growth (Vagabov *et al*., 1998, 2000). It was reported that PolyP participates in the repair of yeast cells after radiation damage as an alternative energy supply and phosphate source (Holahan *et al*., 1988).

Furthermore, in an adenine-deficient mutant of *N. crassa*, where the concentrations of ATP and other adenyl nucleotides are sharply reduced, PolyP is alternatively synthesized during glycolytic phosphorylation by 1,3-diphosphoglicerate:PolyP phosphotransferase (Kulaev and Bobyk, 1971). Thus, under certain conditions PolyP can replace ATP as an energy reserve in eukaryotes.

PolyPs of 12–25 P_i residues were found in the mitochondria of *S. cerevisiae* (Beauvoit *et al*., 1989). The amounts increase sharply under phosphate overplus (Pestov *et al*., 2003). The function of PolyPs in mitochondria needs further investigation. PolyPs also occur

in these organelles under glucose repression. Therefore, their role as an alternative energy reserve seems to be similar to that of pyrophosphate (Mansurova *et al*., 1973a,b; Mansurova, 1989).

It should be noted that the relation of PolyP and transmembrane gradients in yeast has been confirmed more conclusively than that of PolyP and ATP pools.

7.3 Cations Sequestration and Storage

7.3.1 In Prokaryotes

Complexes of PolyP with common cations (Mg^{2+}, Ca^{2+} and K^{+}) have been found in many prokaryotes. One more important function of the PolyP is involvement in the detoxication of heavy metal cations. PolyP sequesters Ni^{2+} in *Staphylococcus aureus* (Gonzales and Jensen, 1998). The cells of *Anacystis nidulans* with high intracellular PolyP levels showed a greater tolerance to Cd^{2+} than those with small PolyP reserves (Keyhani *et al*., 1996). The Cd^{2+} tolerance of *E. coli* also depends on PolyP metabolism (Keasling and Hupf, 1996). The PolyP produced in a recombinant *E. coli* strain with *mer* operon encoding mercury transport systems was capable of chalating and reducing the cytotoxity of Hg^{2+} (Pan-Hou *et al*., 2002). However, degradation of PolyP was observed during growth in the presence of heavy metals (Keyhani *et al*., 1996; Keasling and Hupf, 1996; Keasling, 1997; Keasling *et al*., 2000). The PolyP metabolic pathways in *E. coli* were genetically manipulated to test the effect of PolyP on tolerance to cadmium (Keasling and Hupf, 1996; Keasling *et al*., 2000). A strain mutant in the genes for polyphosphate kinase (*ppk1*) and polyphosphatase (*ppx*) produced no PolyP, whereas the same strain carrying multiple copies of *ppk* on a high-copy plasmid produced significant amounts of PolyP. The cell-doubling time of both strains increased with increasing Cd^{2+} concentration. In contrast, the mutant strain carrying multiple copies of *ppk* and *ppx* produced one tenth of the PolyP found in the strain carrying multiple copies of *ppk* only and showed no significant increase in cell-doubling time over the same Cd^{2+} concentration range. Therefore, not only the large amount of intracellular PolyP but also the ability to synthesize and degrade PolyP is important for tolerance to heavy metals (Keasling and Hupf, 1996; Keasling *et al*., 2000). The following mechanism of PolyP participation in the detoxication of heavy metals has been proposed. PolyP sequesters heavy metals, on the one hand, and the entry of metal cations into the cells stimulates exopolyphosphatase activity, which releases P_i from PolyP, on the other hand. The $MeHPO_4^{-}$ ions are then transported out of the cells (Keasling, 1997; Keasling *et al*., 2000).

7.3.2 In Eukaryotes

In the lower eukaryotes, cation sequestration and storage are observed in vacuoles. Vacuoles of yeast accumulate amino acids (Wiemken and Dürr, 1974), K, Mg^{2+} and Mn^{2+} (Okorokov *et al*., 1980; Lichko *et al*., 1982) (Table 7.1), and Ca^{2+} (Ohsumi and Anraku, 1983; Dünn *et al*., 1994). PolyP, which is able to confine different cations in an osmotic inert form, was also found in these storage organelles (Indge, 1968a,b,c; Westenberg *et al*., 1989).

Table 7.1 The concentrations of K^+, Mg^{2+} and P_i in cytosol and vacuoles of *Saccharomyces carlsbergensis* (Okorokov *et al*., 1980; Lichko *et al*., 1982).

	Concentration (mM)	
Ion	Cytosol	Vacuoles
K^+	60	470
Mg^{2+}	5	73
P_i	1	110

Table 7.2 The accumulation of Mn^{2+} (μmol per g of wet biomass), P_i (μmol per g of wet biomass) and PolyP (μmol of P per g of wet biomass) in the cells of *S. carlsbergensis*. The cells were pre-incubated with KH_2PO_4 and glucose (Lichko *et al*., 1982).

Compartment	Compound	Cells before incubation with Mn^{2+}	Cells after 60 min incubation with Mn^{2+} and glucose
Cytoplasm	P_i	1.9	1.3
	PolyP	3.7	3.9
	Osmotically free Mn^{2+}	< 0.1	1.2
Vacuole	P_i	34.0	27.4
	PolyP	25.5	38.4
	Osmotically free Mn^{2+}	< 0.1	2.8
	Bound Mn^{2+}	< 0.1	9.5

Arginine accumulated in vacuoles was shown to form a complex with PolyP (Dürr *et al*., 1979; Cramer and Davis, 1984). In the vacuoles of *Neurospora crassa*, spermidine was found along with arginine, and almost half of the PolyP in these organelles was considered to form complexes with these positively charged compounds (Cramer and Davis, 1984), in spite of the existence of an independent regulation of vacuolar pools of basic compounds and PolyP under some culture conditions (Cramer *et al*., 1980).

The accumulation of Mn^{2+} in the vacuoles of *Saccharomyces carlbergensis* (Lichko *et al*., 1982) correlated well with the increase in PolyP content (Table 7.2). During the accumulation of Mn^{2+} by *S. carlsbergensis*, both of the PolyP and Mn^{2+} contents increased simultaneously. This accumulation took place even when the incubation medium contained no P_i and was accompanied by a simultaneous decrease of P_i content in the vacuoles. This complex-forming function of PolyP may be very important for the yeast cell, since under a short-term phosphate starvation in the presence of metal cations in the medium the vacuolar PolyP content slightly decreases (Table 7.3) (Lichko *et al*., 1982). A stable P_i content in the cytosol under the above conditions is maintained mainly due to a decrease in the vacuolar P_i pool but not in the vacuolar PolyP pool. It is probable that the ability of fungi to accumulate large amounts of heavy metals is connected with the PolyP pools, especially in vacuoles.

Table 7.3 The contents (μmol of P per g of wet biomass) of P_i and PolyP in vacuoles of *S. carlsbergensis* under phosphate starvation and phosphate overplus (Lichko *et al.*, 1982). The cells were grown for 5 h.

	Culture conditions		
Compound	Control[a]	P_i starvation[b]	Phosphate overplus[c]
P_i	13.7	17.1	16.3
PolyP	23.5	17.4	88.9

[a] Cells were transferred from a complete medium to a new complete medium.
[b] Cells were transferred from a medium free from potassium phosphate to a new phosphate-free medium.
[c] Cells were transferred from a medium free from potassium phosphate to a complete medium.

In protozoa and some algae, cation sequestration is one of the functions of acidocalcisome (Docampo and Moreno, 2001; Ruiz *et al.*, 2001a,b). This is an electron-dense acidic organelle, which contains pyrophosphate and PolyP bound with Ca^{2+} and other cations. Its membrane possesses a number of pumps and exchangers for the uptake and release of these elements.

It should be noted that the PolyPs of the cell envelope could also be the first barrier on the route of penetration of heavy metal cations into a cell, both in prokaryotes and eukaryotes.

7.4 Participation in Membrane Transport

PolyP is a participant of transmembrane ion transport processes, both in procaryptes and eukaryotes. It is widely accepted that ion channels are exclusively proteins, but recently the formation of ion-selective, voltage-activated channels by complexes of PolyP and poly-(*R*)-3-hydroxybutyrates (PHBs) has been demonstrated (Reusch and Sadoff, 1988; Reusch, 1992, 1999a, 2000). Each of these have unique molecular characteristics that facilitate ion selection, solvation and transport.

PHBs provide solvation of PolyP salts by encircling them. A relatively weak solvation ability of the carbonyl ester oxygens (when compared with the oxygens of water) and the absence of hydrogen-bond donors for solvation of anions means that PHBs will preferentially interact with salts composed of cations with high solvation energies and anions with diffused charges. As stated above, the critical factors in achieving this solvation are the flexible backbones of PHBs and the optimal distances between the carbonyl oxygens along the backbone. The result is a flexible structure of two discrete polymers bridged together by lanes of cations. Since PolyPs are fully charged at the physiological pH level, they will select divalent cations. The major physiological divalent cations are Mg^{2+} and Ca^{2+}. PolyPs do not distinguish between these two cations, but the irregular binding cavities formed by the phosphoryl oxygens of PolyPs with the ester carbonyl oxygens of PHBs strongly favour Ca^{2+} (Reusch, 1999a, 2000).

Complexes of the two polymers, isolated from bacterial plasma membranes or prepared from synthetic polymers, form voltage-dependent, Ca^{2+}-selective channels in planar lipid bilayers that are selective for divalent over monovalent cations, permeant for Ca^{2+}, Sr^{2+}

and Ba^{2+}, and blocked by transition metal cations in a concentration-dependent manner. Recently, both PolyPs and PHBs have been found to be components of ion-conducting proteins, namely, the human erythrocyte Ca^{2+}–ATPase pump (Reusch *et al*., 1997) and the *Streptomyces lividans* potassium channel (Reusch, 1999b). The contributions of PolyPs and PHBs to ion selection and/or transport in these proteins is yet unknown, but their presence gives rise to the hypothesis that these and other ion transporters are supramolecular structures, where proteins, PolyPs and PHBs co-operate to form well-regulated and specific cation transfer systems.

The ability of *E. coli PolyP*–PHB complexes to form calcium-selective channels in planar bilayers was investigated first of all (Reusch *et al*., 1995; Reush, 1999a, 2000). PolyP–PHB complexes were extracted from cell membranes into chloroform and then pre-mixed with the phospholipid solution before obtaining the bilayers. Single-channel currents were again observed with voltage steps of 60 mV or more. When the complexes are extracted from membranes or cells, the chloroform solutions contain protein and lipopolysaccharides in addition to PolyP–PHB. To remove these components and to evaluate their influence on channel activity, the complexes were further purified by size-exclusion column chromatography. This eliminated all detectable contaminants and in addition provided an estimate of the molecular weight of the complexes as 17000 ± 4000. Purified complexes were found to be more labile, although the single-channel activity they produced closely resembled that observed for the membrane complexes (Reusch *et al*., 1995).

To still further determine the composition of the channels, the PHB–Ca^{2+}–PolyP complexes were reconstituted. PHB was recovered from *E. coli* and carefully purified, and Ca^{2+}–PolyP was prepared from commercial sodium PolyP and calcium chloride. Single-channel currents similar to those described above were obtained by three different experimental procedures, as described by Reusch *et al*. (1995). The chain length of chemically synthesized PolyP was determined by acrylamide gel electrophoresis to be in the same range (55–65 residues) as in the *E. coli* complexes (Castuma *et al*., 1995).

The channels formed in planar bilayers by synthetic complexes were virtually identical to those formed by PolyP–PHB complexes extracted from *E. coli* (Reusch *et al*., 1995; Reusch, 1999a). The conductances of synthetic and *E. coli* channels were equivalent. The channels formed by PolyP–PHB complexes, *E. coli* or synthetic, show strong selectivity for divalent over monovalent cations (Reusch *et al*., 1995).

One of the characteristics of protein calcium channels is their sensitivity to a block by transition metal cations. Lanthanum is a particularly potent blocker. It is suggested that permeant and blocking ions compete for the common binding sites in the channels. The PolyP–PHB channel complexes are also blocked by transition metal cations in a concentration-dependent manner. A nearly complete block of single-channel currents was observed in the synthetic complexes at concentations > 0.1 mM La^{3+} (0.1 % of Ca^{2+}) (Das *et al*., 1997). Evidently, PHB–PolyP complexes are versatile ion carriers whose selectivities may be modulated by small adjustments of the local pH. The results may be relevant to the physiological function of PHB–PolyP channels in bacteria and the role of PHBs and PolyPs in the *Streptomyces lividans* potassium channel (Das and Reusch, 2001).

The mechanism of ion conduction by PolyP–PHB channel complexes can be rationalized in terms of the structures and properties of the component polymers (Reusch, 1999a). One of the notions of how the channel may operate in the cell membrane or planar bilayer is as follows. Ca^{2+}–PolyP surrounded by PHB forms a salt bridge extending from the cytoplasm

to the periplasm. A multi-lane channel is formed between the two polymers, where the outer wall is lined with solvation oxygens, and the inner wall is girdled by monovalent phosphoryl anions. At the outer interface, cations are drawn to the 'mouth' of the channel by PolyP and divalent cations are preferentially bound. Ca^{2+} occupies most of the binding sites within the channel and the strong bonds between Ca^{2+} and PolyP prevent ion movement, so that the channel is 'closed'. The PolyP 'wire' of negative charges across the bilayer acts as a sensor of membrane potential. PolyP reacts to membrane depolarization (or a voltage step of sufficient strength) by stretching or sliding within the PHB pore, thus dislodging the resident Ca^{2+} and initiating an ion flow. Ca^{2+} at the interface then preferentially permeate into binding cavities at the end of the channel by virtue of their well-suited coordination geometry and the relatively rapid rate, at which they undergo replacement of hydration water. Sr^{2+} and Ba^{2+} are also permeant, but they are not normally found in physiological systems. These cations have the same coordination geometry as Ca^{2+} and, evidently, the flexible PHB envelope can adjust to accommodate the larger ion size. Since the binding sites on PolyPs are identical and spaced at frequent intervals, there is no net potential energy consumption during cation movement within the channel. Segmental motions of the PHB backbone and librational movements of ester carbonyl oxygens carry Ca^{2+} from site to site in parallel single-file lanes, until the internal concentrations rise to an appropriate level or the membrane is again polarized. Transition metal cations, particularly trivalent cations such as La^{3+}, bind tightly to PolyPs at the interface but have difficulty with entering because of their unsuitable coordination preferences, and consequently they block the ion flow.

This organization implies that Ca^{2+} could be transported out of the cell by extending the PolyP chain on the cytoplasmic side of the membrane and transporting it through the PHB pore. As the appended phosphate units move into the PHB channel, Ca^{2+} is sequestered from the cytoplasm, and PolyP–Ca^{2+} is exported at the outer face of the membrane (Figure 7.3).

The *Streptomyces lividans* KcsA potassium channel, a homotetramer of 17.6 kDa sub-units, was found to contain PHB and PolyP (Reusch, 1999b). PHB was detected in both the tetramer and monomer species of KcsA by reaction to anti-PHB IgG on Western blots and estimated as 28 monomer units of PHB per KcsA tetramer by a chemical assay, which converts PHB into its unique degradation product, crotonic acid. PolyP was detected in KcsA tetramers, but not in monomers, by metachromatic reaction to *o*-toluidine blue stain on SDS-PAGE gels. A band of free PolyP was also visible, suggesting that PolyP is released when tetramers dissociate. The exopolyphosphatase of *S. cerevisiae* degraded free PolyP, but tetramer-associated PolyP was not affected, thus indicating it was inaccessible for the enzyme. PolyP in KcsA was estimated as 15 monomer units per tetramer by an enzymatic assay with polyphosphate kinase. It was suggested that PHB is covalently bound to the KcsA sub-units, while PolyP is held within the tetramers by ionic forces.

Complexes of PolyP and PHB were found in the membranes of the endoplasmic reticulum and mitochondria of animal cells (Reusch, 1989), which suggests their participation in the processes of transmembrane transfer. The most intriguing report was that the Ca^{2+}–ATPase purified from human erythrocytes contains PolyP and PHB and that the plasma membrane Ca^{2+}–ATPase may function as a polyphosphate kinase; it exhibits ATP–PolyP transferase and PolyP–ADP transferase activities (Reusch *et al*., 1997). These findings suggest a novel supramolecular structure for the functional Ca^{2+}–ATPase and a new mechanism of uphill Ca^{2+} extrusion coupled with ATP hydrolysis (Reusch *et al*., 1997).

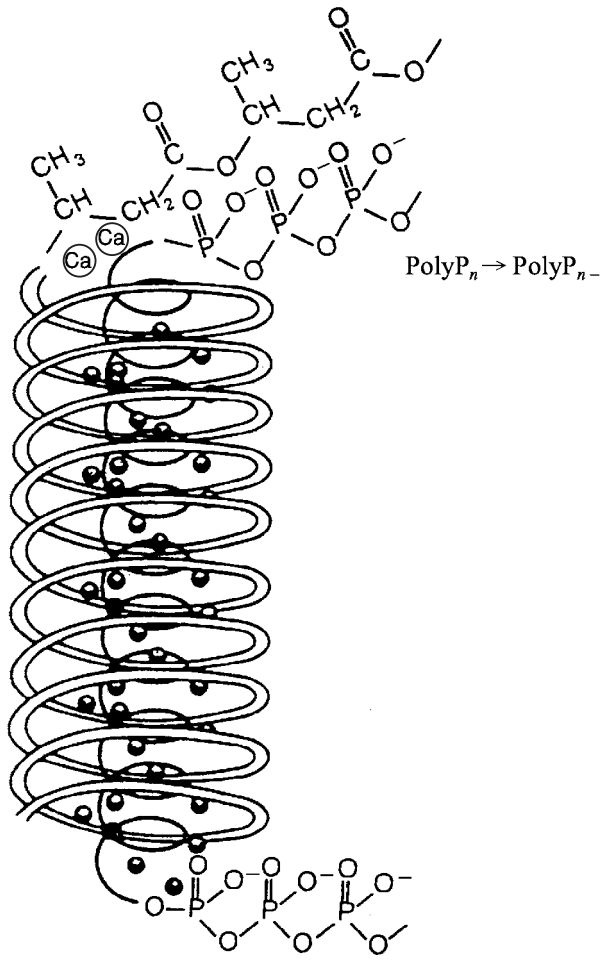

Figure 7.3 Model of a putative PolyP–PHB–Ca^{2+} pump, indicating a hypothetical mechanism for co-translocation of Ca^{2+} and PolyP across the membrane (Reusch, 1992).

The next important property of PolyP–PHB complexes is their effect on DNA transfer into bacterial cells. It was the striking correlation between PolyP–PHB concentrations and transformation efficiencies in *Azotobacter vinelandii, Bacillus subtilis* and *E. coli* that led Reusch and Sadoff (1988) to postulate that the complexes are involved in DNA transmembrane transport.

Nevertheless, it was found that, regardless of the method used to develop competence, the result is a conspicuous increase in the concentration of the PolyP–PHB complexes in the plasma membranes. When formation of the complexes is prevented by any means, transformation is inhibited (Reusch *et al.*, 1986; Huang and Reusch, 1995). DNA binding

requires divalent cations, and only certain cations are fit for this – Mg^{2+}, Ca^{2+}, Mn^{2+} and Sr^{2+}. All of these cations form strong ionic bonds with phosphate and can 'cross-bridge' the phosphate residues of DNA and PolyP. For DNA uptake to occur, cells must return to normal growth media. Examination of the thermotrophic fluorescence spectrum of the cells therein has revealed a rapid decrease in the intensity of the 56 °C fluorescence peak, indicating that the PolyP–PHB complexes are being removed from the membrane. Hence, a mechanism of DNA transmembrane transfer has been proposed. As PolyP is retrieved by cytoplasmic enzymes, it may draw the bound DNA molecule into and through the PHB channel. From this viewpoint, various procedures for competence development and DNA transformation are simply resourceful methods to change the direction of PolyP movement within the PHB pore from outward to inward. The cells are first placed into an environment that leads to a substantial increase in PolyP–PHB, with a sufficient number of divalent cations to bind DNA to PolyP. Then, they are transferred to a medium where they ordinarily sustain much lower levels of membrane complexes, thus inducing an inward flow of PolyP. In support of this hypothesis, a single-stranded donor DNA was found in complex with PHB, when DNA uptake in *E. coli* RR1 was interrupted in the first few minutes (Reusch *et al.*, 1986; Huang and Reusch, 1995; Reusch, 1999a).

Little is known of the ways of biosynthesis and insertion in the membranes of PolyP–PHB–Ca^{2+} complexes. In polyphosphate kinase 1 mutants of *E. coli*, the amounts of the complexes did not change (Castuma *et al.*, 1995). Therefore, the PolyP in the complexes is synthesized not by polyphosphate kinase 1 but by another enzyme. *E. coli* strain, which lacks the AcrA component of a major multi-drug resistance pump, had greatly reduced amounts of the complexes and was defective in its ability to maintain sub-micromolar levels of free Ca^{2+} in the cytoplasm (Jones *et al.*, 2003). This indicates that the AcrAB transporter may have a novel, hitherto undetected, physiological role, either directly in the membrane assembly of the PHB complexes or the transport of a component of the membrane, which is essential for assembly of the complexes into the membrane.

It should be noted that complexes of different proteins with PolyPs (Schröder *et al.*, 1999) or PHBs (Reusch *et al.*, 2002) were found in cells. The prokaryotic histone-like protein, *E. coli* H-NS, and eukaryotic calf thimus histone proteins, Hq, H2A, H2B, H3 and H4, were found to be post-translationally modified by conjugation with short-chain PHBs. The presence of these compounds in proteins with similar functions in such diverse organisms suggests that PHBs play a certain role in shaping the structure and/or in facilitating the function of these important proteins (Reusch *et al.*, 2002). It cannot be excluded that complexes of proteins with PolyP, PHB, and both polymers together, may be found in different cell compartments, not only in the membranes, and have any regulatory role, which needs further investigation.

7.5 Cell-Envelope Formation and Function

7.5.1 Polyphosphates in the Cell Envelopes of Prokaryotes

The cell envelopes of bacteria play an essential role in bacterial virulence, surface attachment and biofilm formation (O'Toole *et al.*, 2000). This cell compartment possesses PolyPs, and thereby its role in the above functions was intensively investigated. The conclusion was

made on the essential role of polyphosphate kinase and PolyP in bacterial pathogenesis (Kornberg, 1999; Kornberg *et al*., 1999).

PolyP was shown to be a component of the cell capsule of *Neisseria*. These capsular PolyPs were about a half of the cellular content of PolyP (Tinsley *et al*., 1993). The polyphosphate kinase deficient mutant of *Neisseria* had a reduced PolyP pool and a lower pathogenicity than the wild-type strain (Tinsley and Gotschlich, 1995).

The effects on the cell-envelope functions of mutations in the *ppk1* gene encoding the polyphosphate kinase 1 were studied (Rashid and Kornberg, 2000; Rashid *et al*., 2000a,b). The *ppk1* null mutants were prepared from *Pseudomonas aeruginosa, Vibrio cholerae, Salmonella enterica, E. coli* and *Klebsiella pneumoniae*, and the motility of these mutants was compared with that of the corresponding wild-type strains on swim plates (1 % tryptone, 0.5 NaCl, 0.3 % agar). The swim areas were decreased in *ppk* null mutants to 13–79 % of the corresponding areas of wild strains. When the mutants were transformed by PPK-expressing plasmids, the motility was completely restored. Electron microscopy revealed that the mutants possessed apparently intact flagella. Thus, the effect of the mutation on swimming motility was proposed to be due to altered functioning of the flagella (Rashid *et al*., 2000a). In a liquid culture, however, the *ppk* mutants were motile (Rashid *et al*., 2000a).

The *ppk* mutant of *P. aeruginosa* was also deficient in type-IV pili-mediated twitching and in swarming motility (Rashid and Kornberg, 2000). Some suggestions on the molecular mechanisms of PolyP– PPK action in motility were made (Rashid and Kornberg, 2000). These included the possible role of PolyP in the phosphorylation of Che-Y-like proteins or modulation of the Ca^{2+} level (Rashid and Kornberg, 2000).

The role of PolyP in the cell envelope of prokaryotes may be connected with their anionic properties, important for providing the negative charge of this compartment. In addition, PolyPs may affect the cell-envelope functions by gene activity regulation, as will be discussed below.

7.5.2 Polyphosphates in the Cell Envelopes of Eukaryotes

PolyPs are present in the cell envelopes of the lower eukaryotes, where their contents may vary depending on the cultivation conditions. PolyP was found at first in the cell envelope of *Neurospora crassa* (Krascheninnikov et al., 1967; Kulaev et al., 1970) and *Endomyces magnusii* (Kulaev *et al*., 1967; Kulaev and Afanasieva, 1970). This high-molecular-weight PolyP was located outside of the cell, adjacent to the outer side of the cytoplasmic membrane.

PolyP was revealed outside of the plasma membrane of the yeast *Kluyveromyces marxianus* by fluorescence of 4′6-diamidino-2-phenylindole (Tijssen *et al*., 1982), by osmotic shock treatment (Tijssen *et al*., 1983), by decrease of the ^{31}P NMR signal under UO_2^{2+} binding (Tijssen and van Steveninck, 1984), and by lead staining and X-ray microanalysis (Tijssen and Van Steveninck, 1985). When the cells of *K. marxianus* were subjected to osmotic shock, they released a limited amount of PolyP into the medium, which represented about 10 % of the total cellular content. The procedure of osmotic shock caused no substantial membrane damage, as was judged from limited K^+ and unimpaired cell viability. The released PolyP fraction differed from other cellular PolyPs by the higher chain length and the lower metabolic turnover rate (Tijssen *et al*., 1983).

The PolyP in the cell envelope is of great importance for maintenance of the negative charge on the cell surfaces of fungi (Vagabov *et al*., 1990a; Ivanov *et al*., 1996). The cell-envelope PolyPs can bind with a monovalent cation dye, 9-aminoacridine (9AA), in the presence of an inhibitor of translocation of the dye across the plasma membrane, namely thiamine (Theuvenet *et al*., 1983). From the results of measuring the absorption rate of 9AA, one can determine variations in the PolyP content in the cell envelope. Using various P_i concentrations in the medium, it is possible to initiate significant variations in the PolyP content in yeast and to observe their effects on 9AA absorption by the cell envelope. Phosphate starvation of cells resulted in a significant decline of their ability to absorb 9AA, while their subsequent growth on a phosphate-rich medium promoted an increase in absorption of the dye. Interestingly, in this case the pre-treatment of cells with UO^{2+} caused a decrease in 9AA sorption of almost 80 %. These results are evidence of an appreciable contribution of PolyP to the total negative charge of the cell envelope (Vagabov *et al*., 1990).

The PolyP content in cell envelopes affects the extent of cytoplasmic membrane damage induced by different ionic compounds, in particular, a cationic surfactant – cetyltrimethylammonium bromide (CTAB). It was observed that the higher the PolyP content of the cell envelope, then the more CTAB is concentrated there, thus resulting in an intensification of its damaging effect on the cell (Ivanov *et al*., 1996).

The putative pathway of coordination of mannan and PolyP biosynthesis by cell-wall formation has been proposed (Kulaev, 1994), which explains the presence of PolyP outside of the cytoplasmic membrane (Figure 7.4). Dolichyl–phosphates (Dol–Ps) act as transmembrane carriers of carbohydrate residues in glycoprotein biosynthesis. GDP–mannose at the

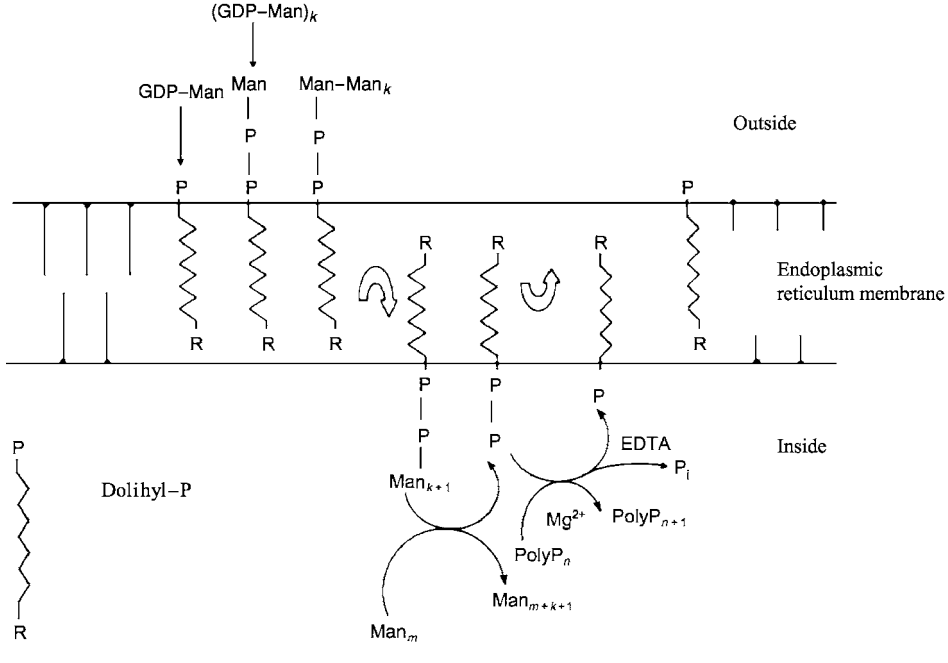

Figure 7.4 The putative pathway of coordination of mannan and PolyP biosynthesis in yeast.

cytoplasmic side of the endoplasmic reticulum interacts with the phosphate residue of Dol–P. The Dol–P–P–mannose is transported across the membrane so that the phosphomannose residue enters the lumen, where mannosyl transferase and Dol–P–P:PolyP phosphotransferase reactions occur. As a result, Dol–P is formed, which again crosses the membrane and could interact on its cytoplasmic side with a new molecule of GDP–mannose. The mannoproteins and PolyP are transported to the cell envelope by special vesicles.

One of the specific processes of cell–cell interactions in the lower eukaryotes is the symbiosis between fungi and plants. It was observed that mycorrhiza possesses a lot of phosphorus and PolyPs. For example, microsclerotia of the root-inhabiting fungus *Phialocephala fortinii* at an early stage of interaction with the roots of *Asparagus officinalis* was shown to accumulate PolyPs (Yu *et al*., 2001). PolyPs were found in vacuoles of fungal cells in *Eucalyptus pilularis*/*Pisolithus tinctorius* ectomycorrhizas (Ashford *et al*., 1999). The mycorrhization of corn plants by the fungi *Glumus mosseae* and *Glumus fasciculatum* was shown to stimulate phosphorus uptake and accumulation (Shnyreva and Kulaev, 1994).

It cannot be excluded that PolyPs, located on cell surfaces of the lower eukaryotes, may play a certain role in cell–cell interactions and especially in the interactions of fungi and plant cells during mycorrhiza development.

7.6 Regulation of Enzyme Activities

Being a polyanion, PolyP can interact with many proteins and enzymes, especially those rich in cationic amino acid residues. For example, in the presence of PolyP, cytochrome C forms stable protein aggregates as a result of binding of the polymer at a single site close to lysines 13, 86 and 87 on the protein surface (Concar *et al*., 1991).

It should be noted that the effect of PolyP on enzymatic activities might involve different mechanisms. First, there is a competition with the substrate for the binding site. It is probable that inhibition by PolyP of polygalacturonase activity, which is important for pathogenicity of the fungus *Botrytis cinerea* (Mellerharel *et al*., 1997) and restriction endonucleases of the fungus *Colleotrichum* (Rodriguez, 1993), is realized in such a way.

Secondly, there is an interaction of PolyP with polycationic activators. As for yeast trehalase, the inhibitory effect is probably due to the interactions with polyamines, which are activators of the enzyme (App and Holzer, 1985). PolyP inhibited trehalase from vegetative yeast cells and, to a lesser extent, that from the spores (Wolska-Mitaszko, 1997).

As for deaminase, the kinetic analysis suggests a partial mixed-type inhibition mechanism. Both the K_i value of the inhibitor and the breakdown rate of the enzyme–substrate–inhibitor complex are dependent on the chain length of the PolyP, thus suggesting that the breakdown rate of the enzyme–substrate–inhibitor complex is regulated by the binding of Polyphosphate to a specific inhibitory site (Yoshino and Murakami, 1988). More complicated interactions were observed between PolyP and two oxidases, i.e. spermidine oxidase of soybeen seedling and bovine serum amine oxidase. PolyP competitively inhibits the activities of both enzymes, but may serve as an regulator because the amino oxydases are also active with the polyamine–PolyP complexes (Di Paolo *et al*., 1995).

The complexing of cations important for enzyme activities may be the third way of PolyP action on enzyme activity. An example of such action is the mechanism leading to growth inhibition, morphological changes and lysis of *Bacillus cereus* when challenged

by a long-chain PolyP (Maier *et al*., 1999). At a concentration of 0.1 % or higher, PolyP had a bacteriocidal effect on logarithmic-phase cells. This activity was strictly dependent on active growth and cell division, since PolyP failed to induce lysis in cells treated with chloramphenicol and in stationary-phase cells, which were, however, bacteriostatically inhibited by PolyP. The 0.1 % PolyP inhibited spore germination and outgrowth, and a higher concentration (1.0 %) was even sporocidal. Addition of Mg^{2+} and Ca^{2+} could almost completely block and reverse the antimicrobial activity of PolyP. While DNA replication and chromosome segregation were undisturbed, electron microscopy revealed a complete lack of septum formation. It was proposed that PolyP might have an effect on the ubiquitous bacterial cell division protein FtsZ, whose GTPase activity is known to be strictly dependent on divalent metal ions. (Maier *et al*., 1999). The bacteriostatic effect of PolyP on *Staphylococcus aureus* was also observed (Jen and Shelef, 1986). The addition of PolyP did not significantly inhibit the growth of *Listeria monocytogenes* and *S. aureus* in milk, probably because of high concentrations of divalent metal cations in this growth medium (Rajkowski *et al*., 1994).

Some other effects of PolyP on the important proteins were found, the mechanisms of which are still unclear. PolyP had a stimulatory effect on the regeneration of GTP-bound from the GDP-bound form of human and yeast *ras* proteins. These authors suggested possible mechanisms of participation of such effects in the regulation of ras-dependent pathways (De Vendittis *et al*., 1986).

PolyPs, as well as nucleoside di-, tri- and tetraphosphates and phosphorylated sugars, caused a dose-dependent (1–5 mM range) delay in the appearance of the cytopathogenic effect of *Clostridium difficile* toxin B on human lung fibroblasts. With a longer phosphate chain, the delay was more pronounced. By analogy with the P site on diphtheria toxin, it was postulated that *C. difficile* toxin B contains a PolyP-binding site. This site is separate from the receptor-binding site but is involved in the interaction of toxin B with cell surfaces (Florin and Thelestam, 1984).

The effects of PolyPs on the enzymes of RNA metabolism may be a way of participation of such biopolymers in gene-activity modulation. RNA polymerase isolated from the stationary-phase cells of *E. coli* was found to be closely associated with PolyP (Kusano and Ishihama, 1997). The inhibitory effects of PolyPs on transcription were examined by using two forms of the holoenzyme, one containing σ^{70} (the major sigma-factor for transcription of the genes expressed during exponential cell growth) and the other containing σ^{38} (the sigma-factor operating in the stationary phase). At low salt concentrations, PolyPs inhibited the transcription by both forms of the RNA polymerase, with σ^{70} and with σ^{38}. At high-salt concentrations, the σ^{38}-containing enzyme is activated, while the σ^{70}-containing enzyme is unable to function. These results show that PolyPs may play a certain role in the promoter-selectivity control of RNA polymerase in *E. coli* growing under high osmolarity and during the stationary-growth phase.

The polyphosphate kinase was found to be an additional component of *E. coli* degradosome (Blum *et al*., 1997). This multi-enzyme complex, whose function is RNA processing and degradation, consists of four major proteins, i.e. endoribonuclease Rnase E, exoribonuclease PNPase, RNA helicase and enolase. The *ppk*-deleted mutant showed an increased stability of the *ompA* mRNA. Purified polyphosphate kinase was shown to bind RNA, while RNA binding was prevented by ATP (Blum *et al*., 1997). PolyPs were found to inhibit RNA degradation by the degradosome *in vitro*. This inhibition was overcome by ADP, required

for ATP regeneration when using PolyP. It was suggested that polyphosphate kinase in the degradosome maintained an appropriate micro-environment, removing inhibitory PolyPs and regenerating ATP (Blum *et al*., 1997).

In addition, PolyPs are most likely involved in the regulation of enzyme activities by participation in their phosphorylation. A protein phosphorylation process, using not ATP but high-polymer PolyPs, was revealed in the archae *Sulfolobus acidocaldarius* (Skorko, 1989). Tripolyphosphate was observed to be a phosphodonor of selective protein phosphorylation of rat liver microsomal membrane (Tsutsui, 1986).

7.7 Gene Activity Control, Development and Stress Response

7.7.1 In Prokaryotes

The involvement of PolyPs in the regulation of enzyme activities and expression of large groups of genes is the basis of their effects on survival under stress conditions and adaptation to the stationary-growth phase. The genes encoding the enzymes of PolyP metabolism in *E. coli* were proposed to form a phosphate regulon together with a number of other genes, the products of which are involved in phosphate metabolism and transport (Nesmeyanova *et al*., 1975 a,b). At present, the interrelation of PolyP metabolism and the activities of PHO and PHOB regulons is supplemented with new details. A number of works of A. Kornberg and co-workers show that polyphosphate kinase and PolyPs synthesized by this enzyme play the key role in the transition of bacteria from active growth to the stationary phase, as well as in their survival in the stationary phase and under stress. These are summarized in a number of publications (Kornberg, 1999; Rao and Kornberg, 1999; Kornberg *et al*., 1999).

It should be noted that in bacteria there is a tight interrelation between PolyP and a signal compound, guanosine 3,5-bispyrophosphate (ppGpp). PolyP accumulation requires the functional *PHOB* gene and higher levels of (p)ppGpp. The latter serves as an alarmon in prokaryotes, which distributes and coordinates different cellular processes according to the nutritional potential of the growth medium (Svitil *et al*., 1993; Nystrom, 1994, 2003; Faxen and Isaksson, 1994; Schreiber *et al*., 1995). This polyfunctional signalling compound is accumulated in bacteria in response to either amino acid or energy source starvation (Svitil *et al*., 1993; Nystrom, 1994). The major role in the control of its level in *E. coli* is played by the genes *spoT* (encoding guanosine 3′5′-bis(diphosphate) 3′-pyrophosphohydrolase and, probably, guanosine 3′5′-bis(diphosphate) synthetase, designated as PSII) and *relA* (encoding ppGpp synthetase I, PSI) (Gentry and Cashel, 1996). Activation of RelA results in a global change of cellular metabolism, including enhanced expression of the stationary-phase sigma factor RpoS. The product of the gene *gppA* participates in the hydrolysis of this compound (Keasling *et al*., 1993). When the intracellular level of ppGpp in *E. coli* was enhanced by expression of truncated *relA*, encoding the more catalytically active ppGpp synthetase, the rate of protein synthesis was inhibited to the level characteristic of amino acid starvation (Svitil *et al*., 1993). The stringent response genes *relA* and *spoT* are important for *Escherichia coli* biofilms-formation slow-growth conditions (Balzer and McLean, 2002). Inhibition of transcription of ribosomal RNA in *Escherichia coli* upon amino acid

starvation is thought to be due to the binding of ppGpp to RNA polymerase (Chatterji *et al*., 1998). The ppGpp directly inhibits rRNA promoter *in vitro* (Barker *et al*., 2001). In addition to the role of inhibition of ribosome synthesis, ppGpp participates in coordination of DNA replication and cell division (Schreiber *et al*., 1995). In ppGpp-deficient relA spoT mutants, the expression of *rpoS* is strongly reduced (Lange *et al*., 1995). PolyP and ppGpp are factors (Ishihama, 2000) coordinating in the activation of *rpoS*. A recent review (Venturi, 2003) analyses the main studies on rpoS transcriptional regulation in *E. coli* and *Pseudomonas*.

However, in some cases these compounds act independent of, or contrary to, rpoS. In *E. coli* and *S. typhimurium*, the regulatory protein leuO, which is potentially involved in the regulation of many genes, is expressed when bacteria are in the process of transition from the exponential to the stationary growth phase. *LeuO* expression is very sensitive to the cellular level of ppGpp but not dependent on the *rpoS* (Fang *et al*., 2000).

The second example is the biosynthesis of antibiotics. The ppGpp is a positive effector in the synthesis of antibiotics in *Streptomyces*. The disruption of the ppGpp synthetase *relA* gene of *Streptomyces coelicolor* (Chakraburty and Bibb, 1997) and *Streptomyces antibioticus* (Hoyt and Jones, 1999) gives phenotypes unable to produce antibiotics. The disruptants were unable to accumulate ppGpp to the level sufficient for initiation of morphological differentiation and antibiotics production.

The antibiotic producer *Streptomyces lividans* possesses a *ppk* gene, which was cloned (Chouayekh and Virolle, 2002). Its transcription was only detectable during the late stages of growth in a liquid minimal medium. A mutant strain interrupted for *ppk* was characterized by increased production of the antibiotic actinorhodin. This production was completely abolished by the addition of KH_2PO_4 to the medium. In the *ppk* mutant strain, this increased production correlated with enhanced transcription of *actII-ORF4* encoding a specific activator of the actinorhodin pathway. In that strain, the transcription of *redD* and *cdaR*, encoding specific activators of the undecylprodigiosin and calcium-dependent antibiotic biosynthetic pathways, respectively, was also increased, but to a lesser extent. The enhanced expression of these regulators did not seem to relate to increased relA-dependent ppGpp synthesis, as no obvious increase in *relA* expression was observed in the *ppk* mutant strain. These results suggested that the negative regulatory effect exerted by *ppk* on antibiotic biosynthesis was most probably caused by repression exerted by the endogenous P_i, resulting from the hydrolysis of PolyP synthesized by polyphosphate kinase, on the expression of specific activators of the antibiotic biosynthetic pathways (Chouayekh and Virolle, 2002). Earlier, the interaction of PolyP metabolism and antibiotic biosynthesis has been studied in *Streptomyces aureofaciens* (Hostalek *et al*., 1976; Kulaev *et al*., 1976) and in *Streptomyces levorini* (Zuzina *et al*., 1981) and the competition between PolyP accumulation and antibiotic biosynthesis was revealed (Figures 7.5 and 7.6). The low-productive strains contained a 10-fold higher PolyP level than the high-productive ones. The excess of P_i in the culture medium increased PolyP accumulation and decreased the synthesis of antibiotics (Zuzina *et al*., 1981).

There are other examples of the influence of PolyPs on the expression of some genes omitting *rpoS*. If the level of cellular PolyP in *E. coli* was reduced to a barely detectable concentration by overproduction of exopolyphosphatase (Shiba *et al*., 1997), the cells were more sensitive to UV or mitomycin C than the control cells. PolyP accumulation was observed after treatment with mitomycin C, whereas the PolyP level was below the detectable level

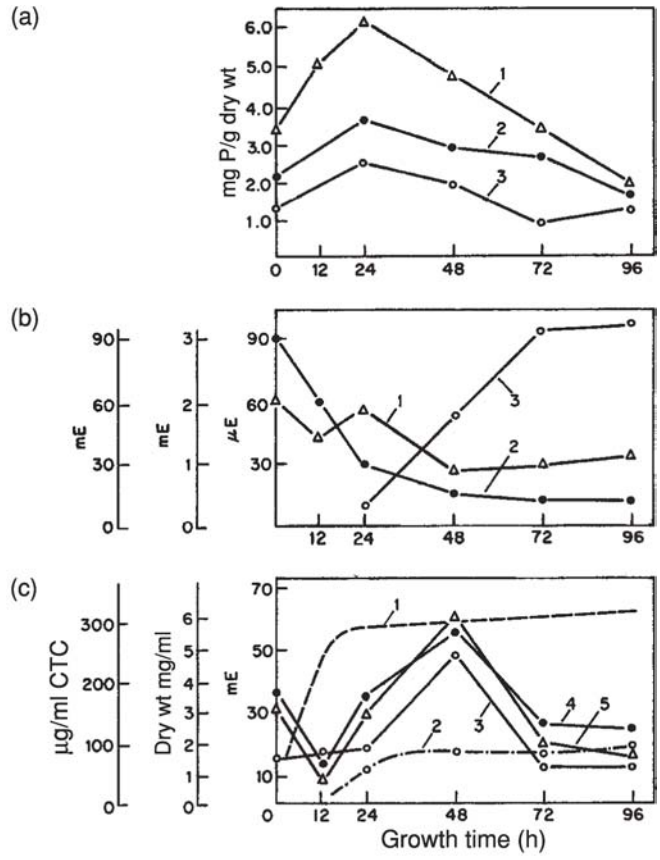

Figure 7.5 Changes in (a) PolyP content, (b and c) PolyP-metabolizing enzymes activities, and (c) biomass and production of chlortetracycline during growth of the low-producing strain of *Streptomyces aureofaciens* 2209 (Kulaev *et al*., 1976). (a) (1) total acid-insoluble PolyP; (2) PolyP extracted with hot perchloric acid; (3) salt-soluble PolyP: (b) (1) polyphosphate kinase (centre scale); (2) 1,3-diphosphoglycerate-polyphosphate phosphotransferase (right-hand scale); (3) PolyP glucokinase (left-hand scale): (c) (1) biomass; (2) chlortetracycline; (3) exopolyphosphatase with $PolyP_{290}$; (4) pyrophosphatase; (5) tripolyphosphatase.

in cells that overproduced exopolyphosphatase. When exopolyphosphatase-overproducing cells were transformed again by a multicopy plasmid that carried the polyphosphate kinase gene (*ppk*), the cells accumulated a great amount of PolyP and restored the UV and mitomycin C sensitivities to the level of the control cells. In addition, a strain containing multiple copies of *ppk* accumulated a large amount of PolyP. It is probable that PolyP is necessary to regulate the expression of SOS genes (Shiba *et al*., 1997, 2000; Tsutsumi *et al*., 2000).

The important role of polyphosphate kinase in the survival of *E. coli* under stress and starvation was established by the study of a mutant deficient in the *ppk1* gene and lacking the most part of PolyP (Rao and Kornberg, 1996; Rao *et al*., 1998). Mutant cells show no

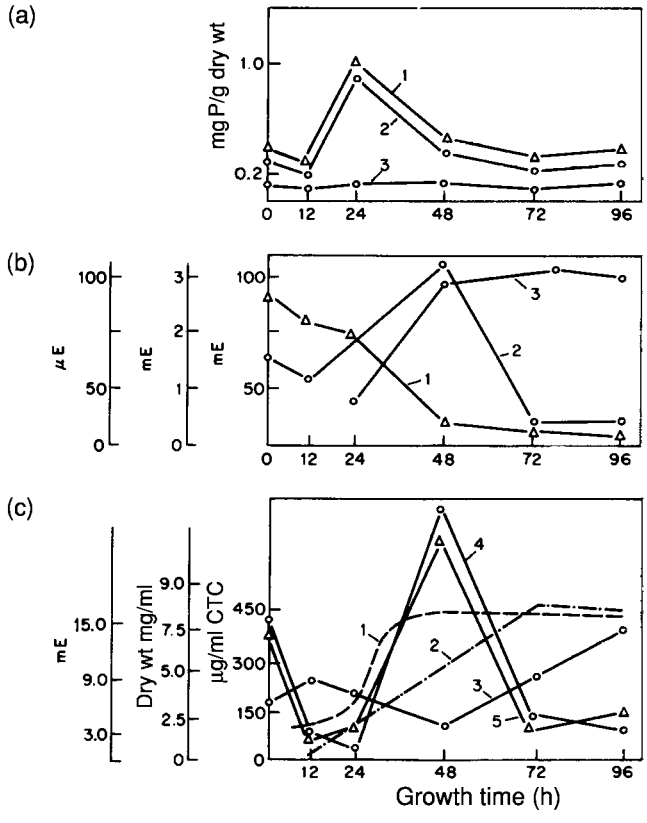

Figure 7.6 Changes in (a) PolyP content, (b and c) PolyP-metabolizing enzymes activities, and (c) biomass and production of chlortetracycline during growth of the high-producing strain of *Streptomyces aureofaciens* 8425 (Kulaev *et al.*, 1976). (a) (1) total acid-insoluble PolyP; (2) PolyP extracted with hot perchloric acid; (3) salt-soluble PolyP: (b) (1) polyphosphate kinase (centre scale); (2) 1,3-diphosphoglycerate-polyphosphate phosphotransferase (left-hand scale); (3) PolyP glucokinase (right-hand scale): (c) (1) biomass; (2) chlortetracycline; (3) exopolyphosphatase with $PolyP_{290}$; (4) pyrophosphatase; (5) tripolyphosphatase.

phenotypic changes during the exponential phase of growth. During the stationary phase, the mutants survive poorly and are less resistant to heat, H_2O_2 oxidants and osmotic challenge with 2.5 M NaCl. After a week in the stationary phase, the mutant is replaced by a small-colony variant with improved viability and stress resistance. In as much as the levels of polyphosphate kinase activity and PolyP remain low, some other genetic alteration can be inferred (Rao and Kornberg, 1996). The suppressive influence of the *rpoS* gene on the *ppk1* mutation was observed (Rao and Kornberg, 1996). The product of *rpoS* is the σ^{38} sub-unit of RNA polymerase responsible for the expression of nearly 50 genes involved in adjustments to the stationary-growth phase, high osmolarity and other stressful agents (Loewn *et al.*, 1998; Ishihama, 2000). When multiple-copy *rpoS* plasmids were introduced into the *ppk1* mutant, heat resistance was elevated to the wild-type level (Rao and Kornberg,

1996). Therefore, the interrelation of PolyP, polyphosphate kinase and the σ^{38} sub-unit of RNA polymerase was confirmed.

The interrelation of PolyP and induction of *rpoS* expression were studied when the PolyP level in *E. coli* was down-shifted by expression of the yeast PPX1 (Shiba *et al*., 1997). As a consequence, a 10-fold increase of H_2O_2 sensitivity was observed. The sensitivity increased 1000-fold in a mutant lacking HPI catalase. Thus, the catalase most dependent on PolyP was stationary-phase *rpoS*-dependent catalase HPII (Shiba *et al*., 1997). Induction of the expression of both catalase HPII and the stationary-phase σ factor was prevented in cells with low PolyP levels. The resistance was restored to the parent-strain level by complementation with plasmids expressing *ppk1*. The levels of ppGpp and pppGpp were not changed in mutants possessing yeast exopolyphosphatase PPX1 with enhanced PolyP hydrolysis. In view of the capacity of additional *rpoS* expression to suppress the sensitivity to H_2O_2, the PolyP action was attributed to the induction of *rpoS* (Shiba *et al*., 1997).

E. coli mutants lacking cytoplasmic superoxide dismutases (SODs) show an inability to survive in the stationary phase and a high sensitivity to redox-cycling reagents and H_2O_2 (Carlioz and Touati, 1986) similar to the *ppk1* mutants. *E. coli* mutants lacking SODs accumulate as much PolyP as the parent strain (Al-Maghrebi and Benov, 2001), when grown in the PolyP-accumulating conditions described by Rao *et al*. (1998). The increase of PolyP content makes the SODs mutants more resistant to H_2O_2, and the cells show the higher rate of H_2O_2 consumption (Al-Maghrebi and Benov, 2001). No direct protective effect of PolyP on oxidative DNA damage was observed. Indeed, *rpoS* dependent-HPII catalase was much higher in those cells with high levels of PolyP (Al-Maghrebi and Benov, 2001). Thus, the results of Shiba *et al*. (1997) were confirmed. The reason for the protective effect of PolyP is the induction of catalase and probably some DNA repair enzymes as members of the *rpoS* regulon (Shiba *et al*., 1997, 2000; Al-Maghrebi and Benov, 2001).

Many other bacteria show similar phenotypic defects, when the *ppk1* gene is knocked out. The *ppk1* mutants of *Neisseria gonorrhoeae* and *N. meningitidis* grew less vigorously than the wild-type cells and showed a striking increase in sensitivity to human serum (Tinsley and Gotschlich, 1995). The *Vibrio cholerae ppk* mutant was similar to that of *E. coli* in response to heat and oxidants and in a long-term survival on synthetic medium (Ogawa *et al*., 2000b). The *P. aeruginosa* PAO1 *ppk* mutant shows no defects in adaptive responses but is severely impaired in motility and surface attachment (Rashid and Kornberg, 2000; Rashid *et al*., 2000a,b). The *ppk* mutants of *Porfiromonas gingivalis* (Chen *et al*., 2002) failed to survive in the stationary phase, while those of *Shigella* and *Salmonella* (Kim *et al*., 2002) have defects in growth on minimal media. It appears that the *ppk* gene is essential for stationary-phase long-term survival of *P. gingivalis* (Chen *et al*., 2002), although this gene may be not the only enzyme responsible for PolyP production in this organism. Unlike the *ppk1* mutant of *E. coli*, the sensitivity of the *ppk1* mutant of *P. gingivalis* to heat and oxidants remains the same as in the parent strain.

Biofilms are sessile microbial communities, the formation of which is initiated by surface attachment of individual bacteria, followed by cell–cell interactions and development in a three-dimensional structure of the colonies (O'Toole *et al*., 2000). Biofilm formation is a multi-step development process over a period of several hours (Costerton *et al*., 1995). The initial surface interaction is mediated by flagella and pili functioning, then the exopolysaccharides stabilize the biofilm and, finally, intercellular communication occurs through signaling molecules (Watnic and Kolter, 1999).

The *ppk1* null mutation affects the adherence properties of bacteria. The *Salmonella ppk* mutant showed a 20–35 % decrease of adherence to abiotic surface (polystyrene) relative to the wild type. The mutant was only half as invasive in epithelium cells (Kim *et al*., 2002). The *P. aeruginosa ppk1* mutant was moderately defective at an early stage of attachment to polystyrene surface, but the biofilm maturation was greatly affected (Rashid *et al*., 2000b). The *ppk* mutant of *Porphyromonas gingivalis*, which seems to be important in the etiology of periodontitis, was attenuated in biofilm formation on poly(vinyl chloride) and glass, while the insertion of an intact *ppk* gene copy restored its biofilm formation (Chen *et al*., 2002).

In view of the fact that the motility and biofilm formation of pathogens are essential to invade and establish systemic infections in host cells, these data suggest a crucial and essential role of polyphosphate kinase or PolyP in bacterial pathogenesis.

The quorum-sensing mechanism allows bacteria to coordinate the expression of particular genes. In *Pseudomonas aeruginosa*, a complex quorum-sensing circuitry, associated with RpoS expression, is required for cell-density-dependent production of many secreted virulence factors, including LasB elastase. The overexpression of *relA* activated the expression of rpoS in *P. aeruginosa* and led to premature, cell-density-independent LasB elastase production. It was suggested that the stringent response can activate two quorum-sensing systems of *P. aeruginosa* independent of cell density (Van Delden *et al*., 2001).

In as much as the *ppk1* mutant of *P. aeruginosa* is defective in three types of motility, surface attachment and biofilm differentiation, Rashid *et al*. (2000b) determined the levels of the quorum-sensing molecules, AI-1 (*N*-3-(oxododecanoyl)-L-homoserine lactone) and AI-2 (*N*-butyryl-L-homoserine lactone). Their levels in the*ppk1* mutant were reduced to $\sim$ 50 % of those of the parent type. The complementation of the mutant with the *ppk1* gene doubled the parent strain level (Rashid *et al*., 2000b). The extracellular virulence factors, elastase and rhamnolipid, also decreased in the *ppk1* mutant and were restored when the mutant was complemented by *ppk*-containing plasmid (Rashid *et al*., 2000b). As a result, the lethality of the *P. aeruginosa ppk* mutant in the burn-mouse model decreased to $\sim$ 7 % of the lethality of the parent strain (Rashid *et al*., 2000b).

Thus, PolyPs and polyphosphate kinase affect the development processes in many bacteria.

One example of the adaptation of bacteria to an unfavourable environment is their response to amino acid starvation. In an environment rich in amino acids, cells do not produce enzymes of amino acid synthesis. However, in the case of a nutritional downshift in the environment, cells must use their own proteins as sources of amino acids for building enzymes required for amino acid biosynthesis pathways (Gottesman and Maurizi, 2001).

The mutant of *E. coli* lacking ppk1 exhibited an extended lag phase of growth when shifted from a rich to minimal medium. Supplementation of amino acids to the minimal medium abolished the extended growth lag of the mutant. Levels of the stringent response factor, ppGpp, increased in response to the nutritional downshift, but unlike in the wild type, the levels were sustained in the mutant. These results suggested that the mutant was impaired in the induction of amino acid biosynthetic enzymes. The rate of protein degradation increased in response to the nutritional downshift in the wild type, whereas it did not in the mutant. Thus, polyphosphate kinase ppk1 is required to stimulate protein degradation and for adaptation to amino acid starvation in *E. coli* (Kuroda *et al*., 1999).

Convincing evidence was obtained that protein degradation in *E. coli* during amino acid starvation depends on the ATP-dependent proteases Lon and Clp (Kuroda *et al*., 2001). Mutations in Lon and Clp proteases produced the same phenotype as *ppk1* mutation – the

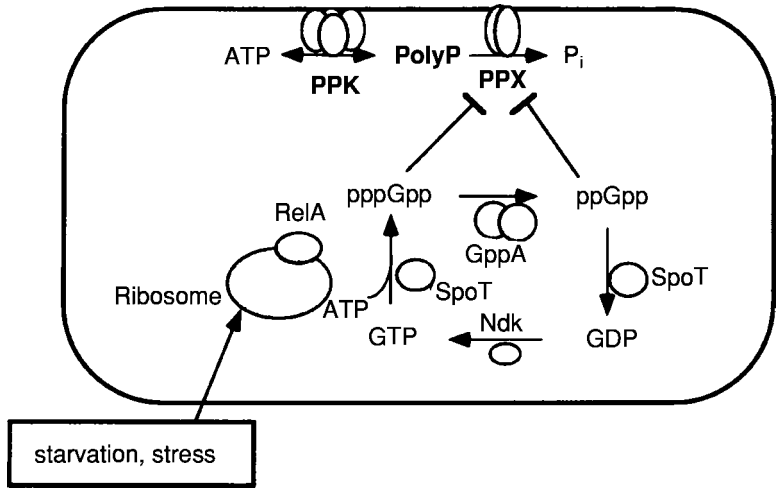

Figure 7.7 Simplified scheme for participation of PolyP and PolyP-metabolizing enzymes in the regulation of (p)ppGpp level in *E. coli:* Ndk, nucleoside diphosphate kinase; PPK, polyphosphate kinase; PPX, exopolyphosphatase (Rao and Kornberg, 1999).

cells failed to overcome a nutritional downshift. Moreover, the ATP-dependent protease Lon formed a complex with PolyP and degraded most of the ribosomal proteins, including S2, L9 and L13. PolyP may stimulate ribosomal protein degradation by the Lon protease, thereby supplying the amino acids needed to respond to starvation (Kuroda *et al.*, 2001). Earlier, it was shown that amino acid starvation in *E. coli* results in a significant accumulation of PolyP.

Intriguingly, ppGpp is also required for PolyP accumulation and for increasing degradation of otherwise stable proteins during starvation. The effects of PolyP and ppGpp are related. The PolyP level in *E. coli* is dependent on the activities of polyphosphate kinase and exopolyphosphatases. Exopolyphosphatase is inhibited by ppGpp and, consequently, when ppGpp builds up in the cells, a decrease of exopolyphosphatase activity results in PolyP accumulation (Figure 7.7). The ppGpp-dependent increase of protein degradation can be explained by the increase of PolyP content in the cells, because PolyPs have an ability to bind ribosomal proteins, so making them available for Lon protease. How do PolyPs promote protein degradation? Gottesman and Maurizi (2001) proposed that PolyPs may provide the proximity of substrate protein and protease, or the Lon protease may recognize a motif in the degradable protein, which becomes exposed once the protein interacts with the PolyP. The complexes of PolyPs with many proteins were observed in cells, and probably some of them are specific (Rao and Kornberg, 1999; Reush, 1999a,b; Schröder *et al.*, 1999).

The co-regulation of the synthesis of alginate, PolyP, GTP and ppGpp in *Pseudomonas aeruginosa* has been observed (Kim *et al.*, 1998). The mutant lacking the regulatory protein AlgR2, which positively regulated nucleoside diphosphate kinase (NDK), had a low level of these compounds. This was restored by overexpression of AlgR2 or NDK genes. It was proposed that the production of alginate and the high level of PolyP under starvation were an attempt by the cells to synthesize PolyP as an energy reserve for further use and to secrete alginate outside the cells (Chakrabarty, 1998). This may be a mechanism to guard against continued accumulation of metabolically active nucleotide triphosphates (Chakrabarty, 1998).

Evidence for the participation of (p)ppGpp and PolyP-dependent systems in the regulation of development of prokaryotes with a complicate life cycle, *Myxococcus coralloides* (Gonzales *et al*., 1989) and *Myxococcus xanthus* (Singler and Kaiser, 1995; Harris *et al*., 1998), has also been obtained.

Generation of high levels of (p)ppGpp in response to amino acid starvation in *E. coli* results in a significant accumulation of PolyP (Kuroda *et al*., 1997). This accumulation can be attributed to the inhibition by pppGpp and/or ppGpp of PolyP hydrolysis by exopolyphosphatase. PolyP accumulation under stress required high levels of ppGpp, independent of whether they are generated by RelA (active during stress response) or SpoT (expressed during P_i starvation) (Rao *et al*., 1998). Accumulation of PolyP requires the functional *PHOB* gene and higher levels of (p)ppGpp (Rao *et al*., 1998; Ault-Riche *et al*., 1998). In *E. coli*, the genes *ppk* and *ppx* are in the same operon, which results in a coordinated regulation of their activities (Rao *et al*., 1998).

Various mechanisms, providing the participation of PolyP in gene expression regulation processes, have been proposed (Figure 7.8). First, polyphosphate kinase may be involved in regulation of the level of nucleoside triphosphates and deoxynucleoside triphosphates, while this enzyme can convert GDP and other nucleoside diphosphates to nucleotide triphosphates using PolyP. Secondly, this enzyme may influence mRNA stability, regulating RNA degradation in degradosomes. Thirdly, PolyP is directly involved in the regulation of RNA polymerase expression and activity. Finally, the (p)ppGpp and PolyP metabolism and *rpoS* expression are closely interrelated. In addition, there are some genes which are regulated by ppGpp or PolyP, independent of the *rpoS* network.

It should be noted that different bacteria might have different predominant mechanisms of PolyP participation in survival under stress and in the stationary-growth phase, or other mechanisms that have not been studied yet. For instance, in *Helocobacter pilory* the pppGpp level does not rise as a result of amino acid starvation (Scoarughi *et al*., 1999). Completely sequenced genomes of several obligately parasitic organisms (*Treponema pallidum*, *Chlamydia species* and *Rickettsia prowazekii*), as well as the known archaea genomes, do not contain rel-like genes, and the role of ppGpp in these organisms may probably be diminished (Mittenhuber, 2001).

These recent data provide good evidence for the essential role of PolyPs in regulation of biochemical processes and the overcoming of stress and starvation by prokaryotic cells.

7.7.2 In Lower Eukaryotes

For eukaryotic microorganisms, the involvement of PolyPs in biochemical regulation under stress has also been observed. For example, the involvement of vacuolar PolyP in survival under osmotic or alkaline stress has been shown in algae and fungi. In the alga *Dunaliella salina*, alkalinization of the cytoplasm results in a massive hydrolysis of PolyP, resulting in pH stat. Various authors have suggested that the hydrolysis of PolyP provides the pH-stat mechanism to counterbalance the alkaline stress (Bental *et al*., 1990; Pick *et al*., 1990; Pick and Weis, 1991).

The role of PolyP as a buffer was demonstrated in *N. crassa* under osmotic stress where the hypoosmic shock produced a rapid hydrolysis of the PolyP with an increase in the concentration of cytoplasmic phosphate (Yang *et al*., 1993).

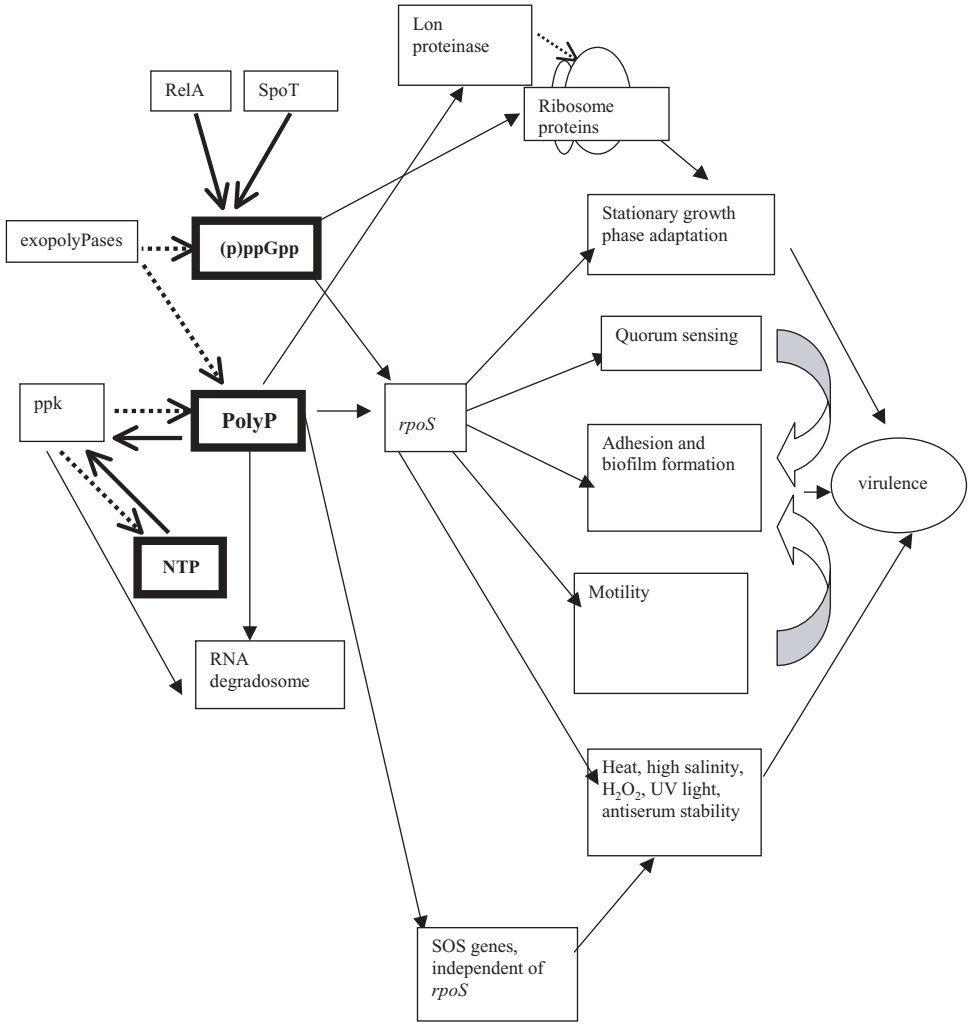

Figure 7.8 The participation of PolyPs in the regulatory processes in bacterial cells: (⟶) biosynthesis of compounds; (--->) utilization of compounds; (⟶►) regulatory effects.

Yeast showed an accumulation of $PolyP_3$ following PolyP hydrolysis induced by amines and basic amino acids (Greenfeld *et al*., 1987). The degradation of 'NMR-observable', probably vacuolar, PolyP to short-chain polymers in the cells of Chemostat-cultivated *S. cerevisiae* contributed to neutralizing the added alkalinity (Castro *et al*., 1995, 1999). In contrast, when the vacuolar vph1-1 mutant, lacking 'NMR-visible' PolyP, was subjected to alkalinization, the absence of a vacuolar source of phosphate slowed re-acidification (Castro *et al*., 1999). Anaerobiosis resulted in the complete hydrolysis of PolyP to P_i (Castro *et al*., 1995).

It was suggested that the accumulation of amines within vacuoles (in response to amine-induced alkaline stress) activates a specific exopolyphosphatase which hydrolyses long-chain PolyPs to $PolyP_3$ (Pick and Weis, 1991). This enzyme appears to be activated at neutral or mild alkaline pH levels and repressed at the physiological intravacuolar acidic pH level. To date, two enzymes, which could catalyse this process, have been purified. An exopolyphosphatase, which hydrolyses long-chain PolyPs but not $PolyP_3$, and has an optimal pH at 7–7.5, was purified from yeast vacuoles (Andreeva *et al*., 1998b). An endopolyphosphatase (Kumble and Kornberg, 1996) could also be involved in this process.

One of the possible ways for the involvement of PolyPs in stress overcoming and biochemical regulation is their interactions with the second messengers. In eukaryotes, second messengers such as phosphoinositides (Mitchell *et al*., 1996; Wera *et al*., 2001) and diadenosinetetra-, penta- and hexapolyphosphates (Kisselev *et al*., 1998) are probably interrelated with PolyPs by analogy with the (p)ppGpp and PolyP interactions in bacteria.

Diadenosine tetraphosphate was found to accumulate in yeast cells under stress caused by exposure to cadmium or heat shock (Baltzinger *et al*., 1986, Rubio-Texeira *et al*., 2002). The diadenosine hexa- and pentaphosphates and hydrolases have an additional function in *S. cerevisiae*, namely, the efficient hydrolysis of diphosphorylated inositol polyphosphates (Safrany *et al*., 1999). Thus, the above second messengers may effectively interact with one another. In addition, their metabolism may be related to PolyP metabolism in some cases. The yeast exopolyphosphatase PPX1 is capable of hydrolysing adenosine–5′-tetraphosphate and guanosine–5′-tetraphosphate (Kulakovskaya *et al*., 1997; Guranowsky *et al*., 1998), while diadenosine–5′,5‴-P^1,P^4-tetraphosphate α,α-phosphorylase (Booth and Guidotti, 1995) may bind the metabolism of the above compounds and PolyP. In addition, the chloroplasts of the eukaryotic alga *Chlamydomonas reinhardtii* was found to possess a gene encoding a putative guanosine–3′,5′-bispyrophosphate (ppGpp) synthase–hydroladase (Kasai *et al*., 2002). This gene exhibited a marked similarity to eubacterial members of the RelA-SpoT family of proteins and the authors suggested that eubacterial stringent control mediated by ppGpp has been conserved during evolution of the chloroplast from a photosynthetic bacterial symbiont (Kasai *et al*., 2002). It is probable that some of the regulatory mechanisms in which PolyPs are involved may be similar in eubacteria and chloroplasts or the mitochondria of eukaryotes.

The participation of PolyPs in development processes and regulation of gene activity is, probably, one of the most important functions of these compounds in eukaryotic microorganisms. The mechanism of this involvement is as yet still little studied; however, many facts confirm this concept. The first data which provide evidence for the involvement of PolyPs in switching on and off large groups of genes were obtained for fungi. It was shown that during the process of sporulation in the fruiting bodies of the fungus *Agaricus bisporus* very large amounts of relatively low-molecular-weight PolyPs accumulate at the actual site of the basidia (Kritsky *et al*., 1965a,b). Significant changes in nuclear PolyPs were observed during this process. Degradation of high-molecular-weight PolyPs to low-molecular fragments in the nuclei during sporulation was observed in the fungi *Agaricus bisporus, Neurospora crassa* (Kritsky and Kulaev, 1963, Kritsky and Belozerskaya, 1968; Kritsky *et al*., 1965a,b, 1970, 1972) and *Physarum polycephalum* (Pilatus *et al*., 1989). The relationship between PolyPs and nucleic acids metabolism in the cells of lower eukaryotes was discovered many years ago, although the precise mechanisms of this relationship is still obscure. The utilizing of PolyP primarily for the biosynthesis of RNA was demonstrated

for the fungus *Aspergillus niger* (Kulaev and Belozersky, 1958; Mudd *et al*., 1958; Stahl *et al*., 1964). It was shown that PolyPs are utilized primarily for the biosynthesis of RNA in *Penicillium chrysogenum* (Kulaev *et al*., 1959; Kritsky *et al*., 1968) *Lenthius tigrinus* (Kritsky and Belozerskaya, 1968; Kritsky *et al*., 1968). A clear correlation between the content of salt-soluble PolyPs and that of RNA was observed. The participation of PolyPs in nucleic acid metabolism was proposed to be connected with the consumption of the activated phosphorus of the PolyP for nucleic acid biosynthesis (Kulaev, 1979; Kulaev and Vagabov, 1983). In discussing the interaction of PolyP metabolism with that of nucleic acids in eukaryotic cells, it is pertinent to mention that PolyPs may also form complexes with RNA (Kulaev and Belozersky, 1958). $PolyP_{60}$ was found in DNA preparations from filamentous fungal species of *Colleotrichum* (Rodriguez, 1993).

The polyP content and polymerization degree have a intricate dynamic during culture growth in yeast and fungi (see details in Chapter 8). This dynamic correlates with the growth stage and indicates the interrelation of PolyP metabolism and culture development. In yeast, the double mutation in exopolyphosphatase PPX1 and endopolyphosphatase genes results in a diminished ability to survive in the stationary-growth phase (Sethuraman *et al*., 2001). The above genes was concluded to be essential for stationary-phase adaptation in yeast (Sethuraman *et al*., 2001).

One of the developmental processes in lower erucaryotes is antibiotic synthesis. Some of the data obtained indicated the interaction between PolyP metabolism and the above process (Kulaev and Vagabov, 1983). It was shown that the levels of PolyP (fractions PP1, PP2 and PP3) in the strain of *Penicillium chrysogenum* intensively producing penicillin were two to three times higher than those in the low-productive strain during the period of penicillin production (Telesnina *et al*., 1985). In contrast, strains of *Tolypocladium sp*. differing in cyclosporine production levels were similar in their PolyP contents (Sotnikova *et al*., 1990). The level of PolyP was lowered two- to threefold during the period of intensive growth and at the beginning of antibiotic synthesis (Sotnikova *et al*., 1990). In *Fusidium coccineum*, the PolyP level was lower in the high-fusidic-acid-producing strain than that in the strain with a low production of the antibiotic (Navashin *et al*., 1983). These authors proposed that PolyP was used as an energy source for antibiotic biosynthesis. Thus, while the mechanisms of the interactions of PolyP metabolism and antibiotics biosynthesis in eukaryotes are still unknown, the interactions between both processes probably depend on the microorganism species, culture conditions and P_i content in the medium.

To summarize, in lower eukaryotes the participation of PolyPs in development processes, gene activity control and overcoming stress is confirmed at present by much indirect evidence and thus establishment of the background for such functions is one of the major future tasks in PolyP biochemistry.

7.8 The Functions of Polyphosphates in Higher Eukaryotes

The cells of higher eukaryotes posesses PolyPs but in smaller amounts than those found in microorganisms. The main function of these biopolymers is probably their participation in regulatory processes.

Few data are available on the PolyP function in higher plants. PolyPs were first observed in maize roots (Vagabov and Kulaev, 1964), while Niemeyer studied PolyPs and their interactions with the inositol phosphate pools in plants (Niemeyer, 1975, 1976, 1999; Niemeyer and Selle, 1989). Some data on the dynamics of PolyPs during the development of cotton plants were obtained (Valikhanov *et al*., 1980). It is probable that some plants can use extracellular PolyP as a phosphorus source (Igamnazarov and Valikhanov, 1980).

Possibly, higher plants possess sufficient amounts of PolyPs only at specific development stages and in certain tissues, and investigation of their role is a difficult task. Genetic methods may be an effective tool for study of the influence of PolyP accumulation on plant cell metabolism. For example, it was demonstrated that the transformation of potato plants with the ppk gene from *E. coli* introduced a new phosphate pool in the chloroplasts of green tissues. PolyPs accumulated during leaf development from 0.06 mg P per g of dry biomass in juvenile leaves to 0.83 mg per g of dry biomass in old leaves and had an average chain length of around 18 residues. Leaves of transgenic plants contained less starch but higher concentrations of soluble sugars when compared to control plants (Van Voorthuysen *et al*., 2000).

As for animals, PolyPs were first found in their cells by Gabel and Thomas (1972). The role of PolyPs in animal cells is still little studied, but some important facts indicate their importance in development and regulatory processes.

Relatively high amounts of PolyPs were found in the freshwater sponge *Ephydatia muelleri*, particularly in the gemmules (Imsiecke *et al*., 1996). Germination and hatching of the gemmules were accompanied by a decrease (by 94 %) in the PolyP level and a rise in the exopolyphosphatase activity. An increase in the PolyP content and decrease in exopolyphosphatase activity also occurred during tissue regression, when the hatched sponges were exposed to polluted river water. Non-ionic organic compounds extracted from this water were identified as the contaminants responsible for the rise in the PolyP content of this organism (Imsiecke *et al*., 1996).

Recently, interest has grown in the study of PolyP functions in the cells of higher animals. PolyPs ($n = 50$–800) were found in the tissues of rodents (brain, liver, lungs and kidneys) and in practically all of the sub-cellular fractions, namely nuclei, mitochondria, plasma membranes and microsomes (Kumble and Kornberg, 1996). PolyPs were also found in human blood and bone tissue (Schröder *et al*., 1999, 2000). Age-dependence studies show that the amount of PolyP in rat brain increases dramatically after birth (Lorenz *et al*., 1997b). The maximal levels were found in 12-month-old animals. Thereafter, the total concentration of PolyPs decreases to about 50 %. This decrease in the total PolyP concentration is due to a decrease in the amount of insoluble long-chain PolyPs, as the amount of soluble long-chain PolyPs does not change significantly with ageing. In rat embryos and newborns, mainly soluble PolyPs could be detected. In rat liver, the age-dependent changes are less pronounced. Changes in the PolyP level were accompanied by those in exopolyphosphatase activity. The highest enzyme activities were found at low PolyP levels. Induction of apoptosis resulted in degradation of long PolyP chains to shorter ones, while the total PolyP content does not change significantly (Lorenz *et al*., 1997b).

The activity of endopolyphosphatase was also found in animal brain. An endopolyphosphatase had been partially purified from rat and bovine brain, where its abundance was about 10 times higher than that in their other tissues but less than a tenth of that in yeast; the end product of digestion of a partially purified brain enzyme is tripolyphosphate (Kumble and Kornberg, 1996).

The nuclei of animal cells contain PolyPs (Griffin *et al*., 1965; Mansurova *et al*., 1975a; Penniall and Griffin, 1984; Kumble and Kornberg, 1995). In the nuclei of rat liver, PolyP is related to the fraction of non-histone proteins (Kulaev and Vagabov, 1983; Offenbacher and Kline, 1984). PolyP may interact with DNA-histone binding in chromatin and this binding has been shown to inhibit the activity of some nuclear enzymes, including topoisomerases (Schröder *et al*., 1999). These data support the idea that PolyPs are involved in the regulation of chromatine functioning in animals.

One example of the probable regulatory function of PolyPs in animals is the ability of these polymers when added to culture media to enhance the proliferation of normal human fibroblast cells. PolyPs also enhanced the mitogenic activities of acidic fibroblast growth factor (FGF-1) and basic fibroblast growth factor (FGF-2). A physical interaction between PolyP and FGF-2 was observed, which stabilized this protein. Furthermore, PolyPs facilitated the FGF-2 binding to its cell surface receptors (Shiba *et al*., 2003).

PolyPs stimulated mammalian TOR, a kinase involved in the proliferation of mammary cancer cells (Wang *et al*., 2003).

The functions of PolyPs associated with their anionic nature were also retained. P_i transported into the lysosomes of human fibroblasts incorporates high-polymer PolyPs synthesized in these organelles (Pisoni and Lindley, 1992). Lysosomes are a storage compartment for bioactive amines, and PolyPs might be able to form complexes with these compounds. A similar process of PolyP synthesis was also observed in granulocytes (Cowling and Birnboim, 1994).

Complexes of PolyP and PHB, similar to those in bacteria, were found in the membranes of the endoplasmic reticulum and mitochondria of animal cells (Reusch, 1989, 1999a, 2000; Reusch and Sadoff, 1988), which suggests their participation in the processes of transmembrane transfer. The most intriguing report was that the Ca–ATPase purified from human erythrocytes contains PolyPs and PHBs and that the plasma membrane Ca^{2+}–ATPase may function as a polyphosphate kinase; this exhibits ATP–polyphosphate transferase and polyphosphate–ADP transferase activities. These findings suggest a novel supramolecular structure for the functional Ca^{2+}–ATPase and a new mechanism of 'uphill' Ca^{2+} extrusion coupled with ATP hydrolysis (Reusch *et al*., 1997).

It was revealed that PolyPs possess antiviral activity by preventing the binding of a virus to a cell (Lorenz *et al*., 1997c). Human blood plasma, serum, peripheral blood mononuclear cells and erythrocytes contain significant amounts of PolyPs (ranging from 53 to 116 μM in terms of phosphate residues). At higher concentrations, the PolyPs may exhibit cytoprotective and antiviral activities. Sodium tetrapolyphosphate and longer polymers, with average chain lengths of 15, 34 and 91 phosphate residues, significantly inhibited the infection of cells by the human immunodeficiency virus type 1 (HIV-1), *in vitro* at concentrations higher than or equal to 33.3 $\mu g\,ml^{-1}$, whereas $PolyP_3$ was ineffective. Over the tested concentration range, these compounds had no effect on cell growth. PolyPs with average chain lengths of 15 and 34 P_i residues, but not $PolyP_3$ and $PolyP_4$, also inhibited HIV-1-induced syncytium formation at a concentration of 160 $\mu g\,ml^{-1}$. The results obtained in the syncytium assay and cell–virus binding experiments indicate that the anti-HIV effect of these non-toxic polyanions may be caused by the binding of these compounds to both the host cell surface and the virus, thereby inhibiting attachment of the virus (Lorenz *et al*., 1997c). Significant amounts of PolyPs and of exopolyphosphatase activity were detected in human-mandibule-derived osteoblast-like cells. The amounts of both soluble and insoluble long-chain PolyPs in unstimulated osteoblast-like cells were higher than in human gingival cells, erythrocytes,

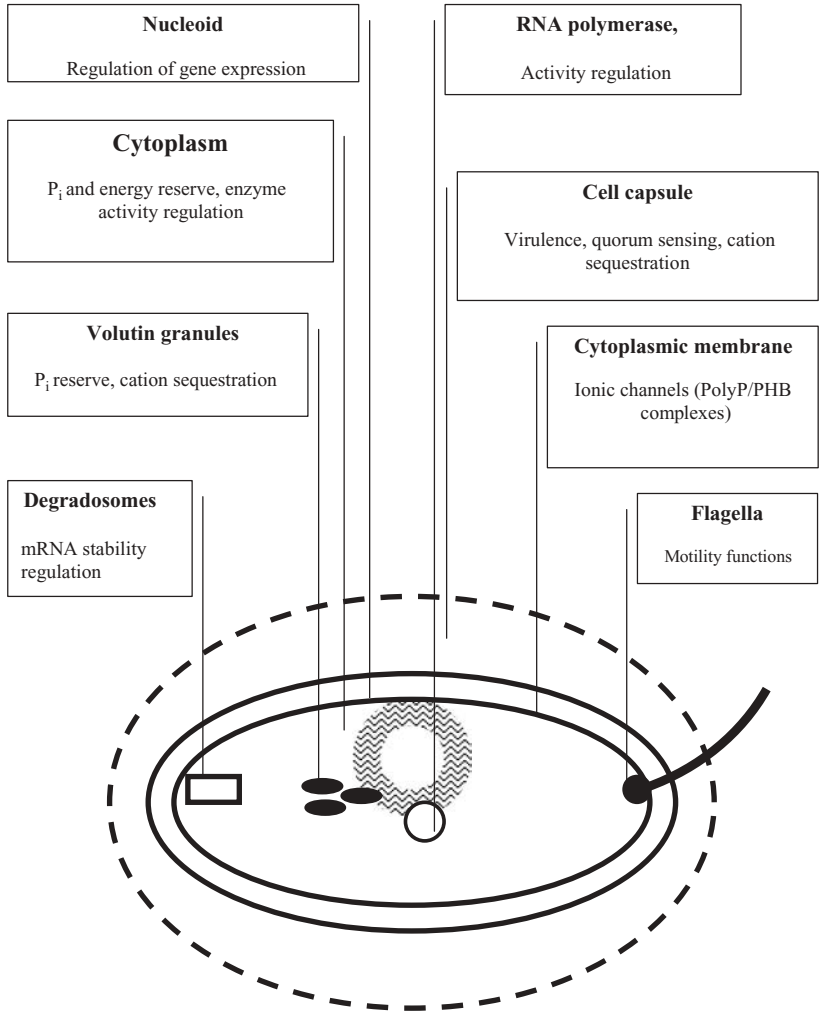

Figure 7.9 The localization and functions of PolyPs in prokaryotes.

peripheral blood mononuclear cells and human blood plasma. The cellular content of PolyPs in osteoblast-like cells significantly decreased after the combined treatment of cells with stimulators of osteoblast proliferation and differentation (Leyhausen *et al*., 1998; Schröder *et al*., 1999, 2000). These authors assume that PolyPs may be involved in the modulation of the mineralization process in bone tissue.

$PolyP_3$ was found to be a phosphodonor for the phosphorylation of some proteins in rat liver microsomes (Tsutsui, 1986) and for nucleoside kinases, in particular human deoxyribonucleoside kinases (Krawiec *et al*., 2003). This gives an additional possibility for the involvement of PolyPs in the regulatory processes in animal cells.

Thus, in spite of the fact that animal cells, probably with the exception of bone tissue cells, do not need PolyPs as phosphate reserves, the functions of these biopolymers are still

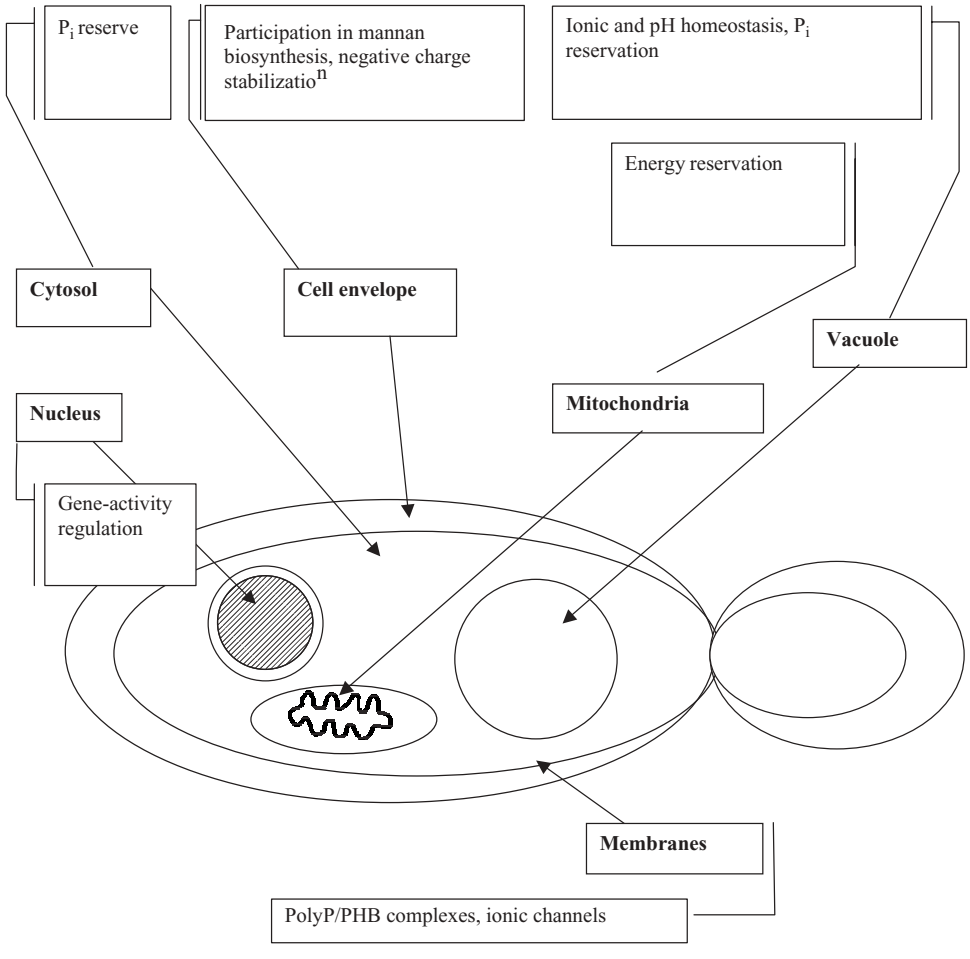

Figure 7.10 The localization and functions of PolyPs in lower eukaryotes.

quite significant. This is confirmed by the presence of such compounds in nearly all tissues and organs.

In conclusion, it should be noted that PolyPs are polyfunctional compounds. Their most important functions are as follows: phosphate and energy reservation, sequestration and storage of cations, formation of membrane channels, participation in phosphate transport, involvement in cell-envelope formation and function, gene activity control, regulation of enzyme activities, and, as a result, an important role in stress response and stationary-phase adaptation.

The functions of PolyPs have changed greatly during the evolution of living organisms. Figures 7.9–7.11 summarize, respectively, data on PolyP functions in prokaryotes, lower eukaryotes and animals. In microbial cells, they play a significant role, increasing cell resistance to unfavourable environmental conditions and regulating different biochemical

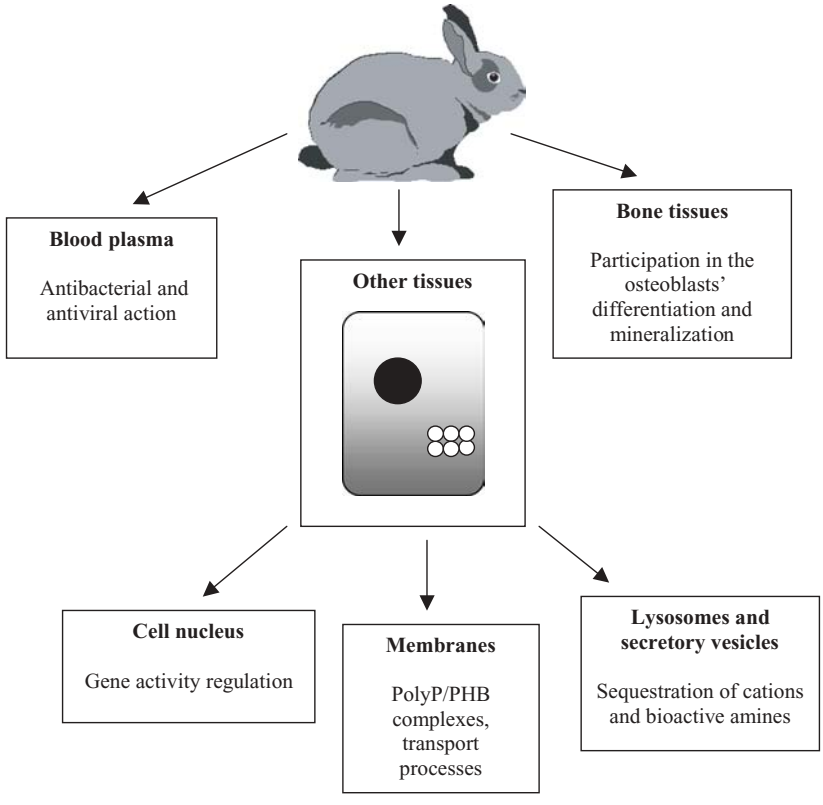

Figure 7.11 Functions of PolyPs in animals.

processes, both as a regulatory factor and as an energy source and phosphate reserve. In higher eukaryotes, the regulatury functions predominate. There is a great difference between prokaryotes and eukaryotes in their PolyP-metabolizing enzymes (see Chapter 6). Eukaryotes do not possess some key prokaryotic enzymes but have developed some new PolyP-metabolizing enzymes lacking in prokaryotes. The synthesis and degradation of PolyPs in each specialized organelle and compartment of eukaryotic cells are mediated by different sets of enzymes. This is consistent with the endosymbiotic hypothesis of eukaryotic cell origin. Despite the great differences in PolyP metabolism and functions in different living organisms, participation in the regulatory processes in the cell is observed for these biopolymers in organisms belonging to different stages of evolution.

8

THE PECULIARITIES OF POLYPHOSPHATE METABOLISM IN DIFFERENT ORGANISMS

It has become clear in recent years that the metabolism of PolyPs in different organisms, although partially involving similar pathways, nevertheless possesses certain characteristic features. In this section, we shall consider the principal features of the more typical aspects of PolyP metabolism in those organisms, which have been subjected to the closest and most thorough examination, and for which it is possible to depict, at least in outline, the general features of PolyP metabolism. Special attention will be paid to the particularly complex, and as yet little investigated, problem of the intracellular control of PolyP metabolism in various groups of organisms. In this part of the review, therefore, we shall consider some specialized aspects of PolyP biochemistry.

8.1 *Escherichia coli*

8.1.1 The Dynamics of Polyphosphates under Culture Growth

PolyP metabolism in *E. coli* is interesting first of all due to the very intensive investigations of its phosphorus metabolism both in the biochemical and genetic aspects.

The earliest work with this bacterium showed that PolyPs occurred in it in extremely low amounts, if at all, and were present not continually and often under specific conditions, usually under growth limitation by some nutrient sources. Indeed, study of the dynamics of PolyP accumulation during the growth of the wild-type strain *E. coli* K12 on a mineral

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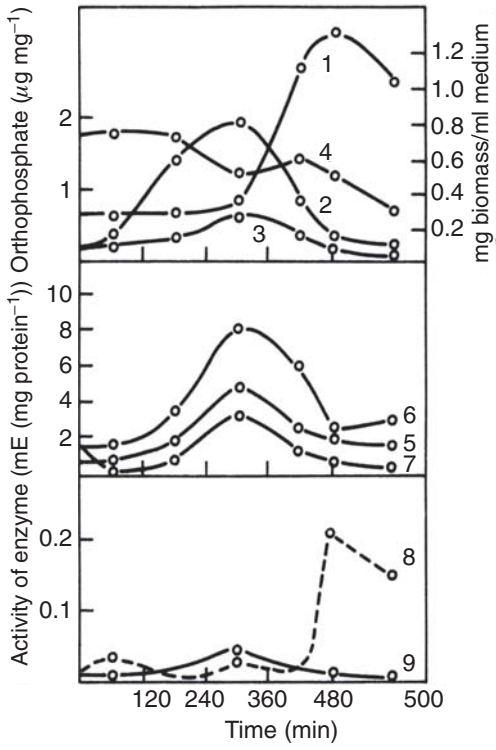

Figure 8.1 PolyP and PolyP-metabolizing enzymes during the growth of *E. coli* K-12: (1) biomass; (2) PolyP; (3) intracellular P_i; (4) exogenous P_i; (5) exopolyphosphatase; (6) tripolyphosphatase; (7) alkaline phosphatase; (8) polyphosphate kinase; (9) 1,3-diphosphoglycerate:polyphosphate phosphotransferase (Nesmeyanova *et al*., 1973).

medium supported such a suggestion, simultaneously closing the question of PolyP occurrence in *E. coli* (Nesmeyanova *et al*., 1973, 1974a,b, 1975a,b; Nesmeyanova, 2000). A high-molecular-weight acid-insoluble PolyP was found in this bacterium and identified by a specific product of its partial acidic hydrolysis, i.e. cyclotriphosphate, using the method of Thilo and Wieker (1957). The highest amount of this high-polymer PolyP, reaching 0.2–0.4 % of dry bacterial weight (in yeast, its amount may reach 20 %), occurs in the cells of this bacterium only at the end of the latent and the beginning of the logarithmic growth phase (Nesmeyanova *et al*., 1973). When the culture passes to exponential growth, the level of intracellular PolyP dramatically decreases 5–10-fold (Figure 8.1). Thus, the accumulation of PolyP precedes intensive culture growth, and then it is utilized by the growing cells (Nesmeyanova *et al*., 1973).

The accumulation of PolyPs in cells results from two processes: synthesis and utilization of PolyP. The dynamics of the known PolyP-synthesizing enzymes, i.e. polyphosphate kinase and 1,3-diphosphoglycerol-polyphosphate phosphotransferase, showed that these enzyme activities weakly correlate with the dynamics of PolyP accumulation under standard growth conditions (see Figure 8.1). The main peak of polyphosphate kinase activity was

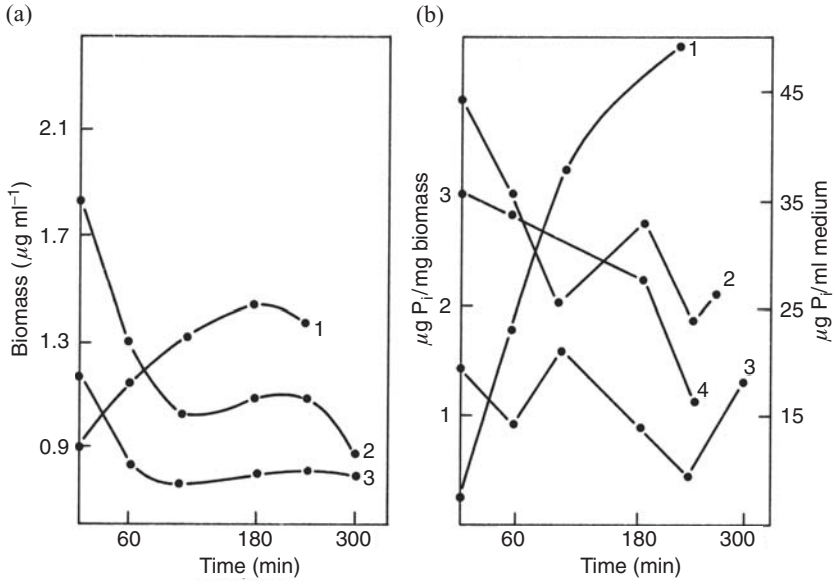

Figure 8.2 Effects of exogenous P_i on the levels of intracellular P_i and PolyP in *E. coli*: (a) deficiency of exogenous P_i; (b) excess of exogenous P_i: (1) biomass; (2) PolyP; (3) intracellular P_i; (4) exogenous P_i (Nesmeyanova *et al*., 1974a).

observed, for instance, during the stationary culture growth, when the content of PolyP in the cells significantly decreased. On the contrary, the activity of an enzyme of PolyP degradation, exopolyphosphatase, directly correlated with PolyP accumulation and with the activity of the alkaline phosphatase (non-specific phosphohydrolase) of *E. coli*.

8.1.2 The Effects of P_i Limitation and Excess

Similar dynamics of the PolyP content in *E. coli* cells was observed under external P_i limitation and excess (Figure 8.2). The culture growth continues during P_i deprivation, but less rapidly than in a P_i-containing medium. In a medium without P_i, there was a greater consumption of PolyP. The level of intracellular orthophosphate actually remained the same, supporting the regulatory role of PolyP in the maintenance of the phosphate level in the cell (Figure 8.2(a)). Rapid growth of the culture on a complete PolyP-containing medium was also accompanied by a fall in the level of both phosphorus compounds in the cells. In the final analysis, however, the level of P_i and PolyP remained higher than in a deprived culture (Nesmeyanova *et al*., 1974a; Nesmeyanova, 2000).

In many organisms, the addition of P_i to a culture previously deprived of phosphorus results in a rapid accumulation of PolyP to an extent many times exceeding the level which is characteristic of normal growth on a complete medium. This phenomenon is called 'hypercompensation' or 'phosphate overplus'. In *E. coli*, this effect was not found. The addition of P_i to a starved culture resulted in a rapid restoration of biomass accumulation.

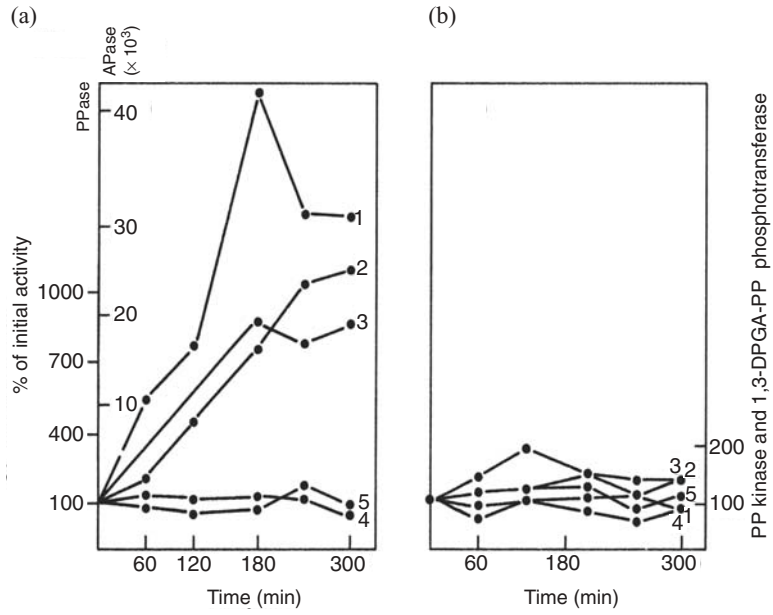

Figure 8.3 Effects of exogenous P_i on PolyP-metabolizing enzymes in *E. coli:* (a) deficiency of exogenous P_i; (b) excess of exogenous P_i: (1) alkaline phosphatase; (2) tripolyphosphatase; (3) exopolyphosphatase; (4) polyphosphate kinase; (5) 1,3-diphosphoglycerate:polyphosphate phosphotransferase (Nesmeyanova *et al*., 1974b).

As regards the PolyP and P_i levels, the only thing observed was a replenishment of the reserves of PolyP and P_i to the levels typical of those in cells growing on a complete medium with P_i (Nesmeyanova *et al*., 1973, 1974a,b).

The regulation of some enzymes of PolyP metabolism in *E. coli* by exogenous P_i was studied first by Nesmeyanova *et al*. (1973, 1974a,b, 1975a,b). Both alkaline phosphatase and exopolyphosphatase appeared to be co-regulated by P_i (Figure 8.3). Their activities appreciably increased under P_i starvation, whereas polyphosphate kinase and 1,3-diphosphoglycerate-PolyP phosphotransferase activities did not depend on the content of P_i in the medium. The greatest derepression was observed in cells during exponential and even latent growth under phosphate starvation. Cells from the stationary growth phase actually showed no de-repression of exopolyphosphatase under the same conditions (Nesmeyanova *et al*., 1974a). If P_i was added to the medium during the synthesis of phosphohydrolases, the latter completely stopped (Figure 8.4). It is evident that the exopolyphosphatase in *E. coli* is strictly regulated by exogenous P_i. Obviously, this enzyme plays the leading role in PolyP utilization under phosphate starvation, which proceeds much quicker than on the medium with P_i. Other authors also observed that the addition of excess phosphate to P_i-starved *E. coli* cells resulted in decreased exopolyphosphatase activity, increased polyphosphate kinase activity and accumulation of PolyP (Sharfstein and Keasling, 1994).

It should be noted that *E. coli* exopolyphosphatase is a surface protein of the cytoplasmic membrane, localized on its periplasmic side (Nesmayanova *et al*., 1975b; 1976). In contrast

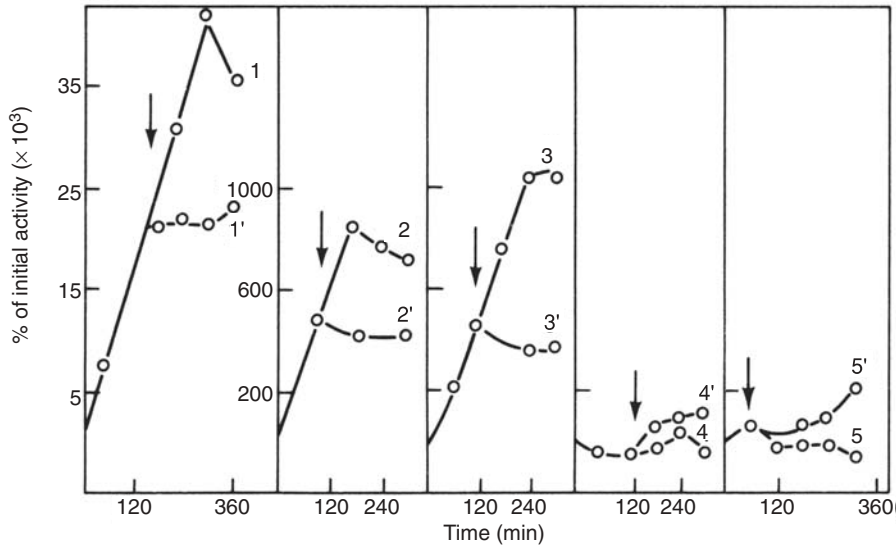

Figure 8.4 Effects of addition of P_i to a previously starved culture on the activity of phosphorus-metabolizing enzymes in *E. coli*: (1) alkaline phosphatase; (2) exopolyphosphatase; (3) tripolyphosphatase; (4) 1,3-DPGA-polyphosphate phosphotransferase; (5) polyphosphate kinase (all in an P_i-free medium): (1′–5′) with the addition of P_i. The time of P_i addition is shown by the arrows (Nesmeyanova *et al*., 1974a).

to alkaline phosphatase, which is completely released from cells during cell wall lysis, exopolyphosphatase remains membrane-bound and is released from the membrane only after a long washing with buffer. The strength of its binding with the membrane depends on the presence of P_i in the medium. Under phosphate starvation, i.e. under de-repression of the enzyme, this binding is weaker and the enzyme is more easily released into the periplasm (Nesmayanova *et al*., 1975b).

The wild-type *E. coli* utilized PolyP with a chain length of 100 phosphate residues as a sole source of phosphate in the growth medium (Rao and Torriani, 1988). The mutation in the *phoA* (alkaline phosphatase) gene prevented growth on this medium, while the mutation in the gene encoding the periplasmic acid phosphatase did nor affect PolyP utilization (Rao and Torriani, 1988).

8.1.3 The Effects of Mutations on Polyphosphate Levels and Polyphosphate-Metabolizing Enzyme Activities

First, polyphosphate kinase is the main enzyme of PolyP synthesis in *E. coli* and the *ppk1*-deficient mutants have virtually no PolyP content (Kornberg, 1995, 1999; Kornberg *et al*., 1999). Overexpression of *ppk1* results in a high level of intracellular PolyP (Ohtake *et al*., 1994; Keasling *et al*., 1998). Overexpression of both *ppk* and *ppx* results in a lower PolyP level and excretion of P_i from the cells (Keasling *et al*., 1998). A strain deficient

in exopolyphosphatase (ppx) has more PolyP than the parent strain (Keasling and Hupf, 1996).

The *ppk1* gene has many pleiotrophic effects on *E. coli* viability and cell functions (Kornberg, 1995, 1999; Kornberg *et al*., 1999), which were briefly described earlier in Chapter 7.

In *E. coli*, a Pho regulon controls the biosynthesis of a number of enzymes participating in phosphorus metabolism and other proteins (Torriani-Gorini, 1994; Wanner, 1994). A very low level of P_i in the medium induces the Pho regulon, comprising the Pst-pathway of using P_i and the PhoB regulator of response, which induces the genes of proteins of this regulon also participating in phosphorus metabolism.

One of the first attempts to elucidate the interrelations of the Pho regulon and PolyP metabolism was made by Nesmeyanova *et al*. (1975a). The effect of mutation of the regulatory gene *phoR*, resulting in a non-inducible synthesis of proteins of the Pho regulon, was studied. Neither alkaline phosphatase nor exopolyphosphatase were induced under phosphate starvation in this mutant strain, indicating that exopolyphosphatase was co-regulated with alkaline phosphatase under the control of the same regulatory system.

The *phoU* mutant is constitutive for alkaline phosphatase and is able to synthesize five times more PolyP than the parent strain under anaerobiosis in a rich medium (Rao *et al*., 1985). Later, the accumulation of a high level of PolyP in the *phoU* mutant ($\sim$ 100-fold higher than in the parent strain) was confirmed (Morohoshi *et al*., 2002). This mutant was able to remove fourfold more P_i from the medium than the parent strain. By using this mutant and a combined method of chemical extraction and ^{31}P NMR spectroscopy, the anaerobiosis-induced PolyP accumulation in *E. coli* was studied (Rao *et al*., 1985). Under these conditions, the total PolyP amount was maximal at the early stationary phase of growth. Both trichloroacetic acid- and NAOH-soluble PolyPs were found in the cells. The acid-soluble fraction contained polymer of about 20 ± 5 phosphate residues, whereas the alkali-soluble fraction had a higher chain length. The ^{31}P NMR spectroscopic analysis revealed PolyP of more than 200 residues (Rao *et al*., 1985). It was observed that under these experimental conditions *E. coli* cells accumulate at first acid-soluble low molecular-weight PolyP, and high-molecular-weight PolyP is synthesized once the growth has ceased (Rao *et al*., 1985).

High levels of PolyP accumulation were obtained by increasing the dosage of *E. coli* genes encoding polyphosphate kinase (*ppk1*), acetate kinase and phosphate-inducible transport systems (*PSTS, PSTC, PSTA, and PSTB*), and by genetic inactivation of *ppx* encoding exopolyphosphatase (Kato *et al*., 1993a; Hardoyo *et al*., 1994; Ohtake *et al*., 1994). All these data support the idea that the massive accumulation of PolyP in *E. coli* may be obtained by genetic modification in the regulatory systems, which provides P_i uptake and its regulation in this bacterium.

PhoB, the response regulator, turns on several genes, among them alkaline phosphatase and the proteins involved in P_i uptake. Pho-regulon mutants affected in PhoB synthesis were tested for PolyP accumulation in a minimal medium containing low levels of P_i (0.1 mM) and amino acids (2 μg ml^{-1}) (Rao and Kornberg, 1999). A large amount of PolyP (48 nmol per mg of protein) accumulated in wild-type cells under these conditions. The mutants lacking PhoB accumulated low levels of PolyP (0.3–1.9 nmol per mg of protein). Inactivation of the protein kinases PhoR and CreC, which activate PhoB (Wanner, 1995), led to a lower level of PolyP (0.1 nmol per mg of protein). The mutants with constitutive expression of the Pho regulon or *phoB* mutants with multicopy *phoB* plasmid accumulated PolyPs to a level comparable with those in wild-type cells (Rao and Kornberg, 1999).

It should be noted that the exopolyphosphatase gene of *E. coli*, i.e. *ppx*, was found 'downstream' of the gene for polyphosphate kinase *ppk1* (Akiyama *et al*., 1993). Transcription of the *ppx* gene depended on the *ppk1* promoters, indicating a single operon of *ppk1* and *ppx* (Akiyama *et al*., 1993). This proposes a coordinate regulation of these enzymes. The fact that one of the promoters has a homology with the Pho box indicated that PolyP metabolism might be regulated by P_i. The effects of some mutations indicated above support this suggestion.

The effects of mutations and overexpression of *ppk1* and *ppx* were studied by Keasling and co-workers (Keasling *et al*., 1998, 2000; Sharfstein *et al*., 1996; Van Dien *et al*., 1997; Van Dien and Keasling, 1998). If the PolyP operon of *E. coli* was overexpressed on a high-copy plasmid under the control of its native promoter, the ppk activity increased during phosphate starvation and dropped after the P_i shift, while ppx activity was the highest when P_i was in surplus. Thus, in such a transformant, the Pho regulon was probably not involved in the expression of ppk and ppx, while utilization of PolyP during starvation and its partial replenishment after the P_i shift was retained (Sharfstein *et al*., 1996; Van Dien *et al*., 1997; Keasling *et al*., 1998; Van Dien and Keasling, 1998). Thus, the interaction of the genes directly involved in PolyP metabolism and the Pho regulon is very complicated and needs further investigation. A mathematical model was proposed to study the multiple aspects of the phosphate-starvation response of *E. coli* (Van Dien and Keasling, 1998).

8.1.4 The Effects of Nutrition Deficiency and Environmental Stress

The influence of nutrition limitation and stress conditions on the PolyP content in *E. coli* was studied by Kornberg and co-workers and described in detail in a number of reviews (Kornberg, 1995, 1999; Rao and Kornberg, 1999; Kornberg *et al*., 1999), and earlier in Chapter 7. Here, we will point out only the most important facts. First, *E. coli*, subjected to nutritional or osmotic stress in a rich medium or to nitrogen exhaustion, had a large and dynamic accumulation of PolyP (Rao and Kornberg, 1996; Kuroda *et al*., 1997; Rao *et al*., 1998; Ault-Riche *et al*., 1998). *E. coli* accumulated large amounts of PolyPs in media deficient in both P_i and amino acids. For example, Figure 8.5 shows PolyP accumulation in the presence of serine hydroxamate inducing amino acid starvation (Kuroda *et al*., 1997). This accumulation is explained by the high level of ppGpp under these conditions and the inhibition of PolyP hydrolysis by this compound (Kuroda *et al*., 1997). The accumulation of PolyPs under stresse seems to be a reaction for overcoming the unfavourable growth conditions.

Based on the available data, a tentative model (Figure 8.6) of the mechanisms responsible for PolyP accumulation in *E. coli* has been proposed (Ault-Riche *et al*., 1998; Rao and Kornberg, 1999).

8.2 *Pseudomonas aeruginosa*

In *Pseudomonas aeruginosa*, a specific regulation of PolyP accumulation by the regulatory protein AlgR2 was revealed. This protein positively regulated the production of alginate,

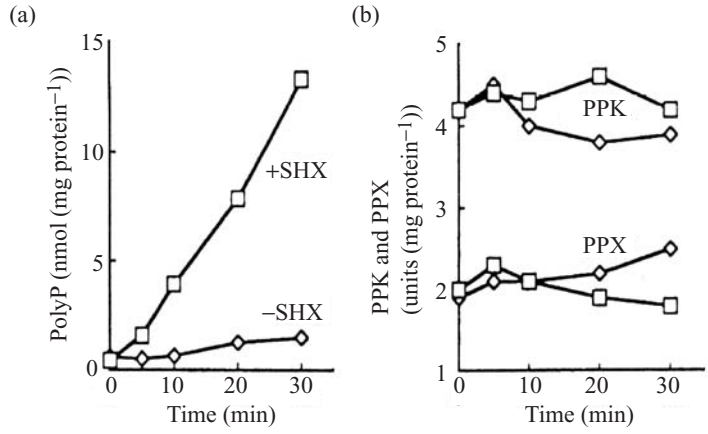

Figure 8.5 PolyP accumulation, and polyphosphate kinase (PPK) and exopolyphosphatase (PPX) activities, under stringent conditions. *E. coli* MG1655 was grown on a MOPS medium containing 0.4 mM P_i. At A_{540} near 0.2, serine hydroxamate (SHX) was added (0.5 mg ml^{-1}) for induction of amino acid starvation and accumulation of (p)ppGpp. Symbols represent with (□) and without (◇) serine hydroxamate: units of PPK and PPX in (b) are 1 nmol P_i min^{-1} (Kuroda *et al*., 1997). Reproduced with permission from Kuroda, A., Murphy, H., Cashel, M. and Kornberg, A., *J. Biol. Chem.*, **272**(34), 21240–21243 (1997). Copyright (1997) American Society for Biochemistry and Molecular Biology.

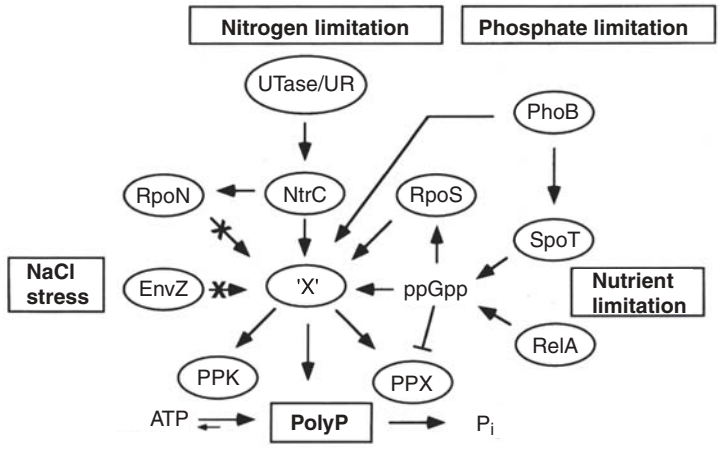

Figure 8.6 Model for stress-induced polyP accumulation in *E. coli*. NtrC (a member of the signal cascade for nitrogen metabolism), together with RpoS and PhoB, is needed for polyP accumulation in response to nitrogen limitation. Involvement of a 'sigma factor' (RpoS) implies activation of an additional factor ('X') which could lead to PolyP accumulation by direct interaction with PolyP, inhibition of PPX, stimulation of PPK, or a combination of all three. Under nutrient limitation, ppGpp accumulated by RelA and SpoT actions, can lead to PolyP accumulation by PPX inhibition and/or RpoS activation. Failure to accumulate PolyP, even when ppGpp and RpoS levels are high (as in carbon starvation), implies the presence of additional regulator(s). In addition, osmotic stress triggers PolyP accumulation through a mechanism that does not involve EnvZ, the osmotic sensor (Ault-Riche *et al*., 1998; Rao and Kornberg, 1999).

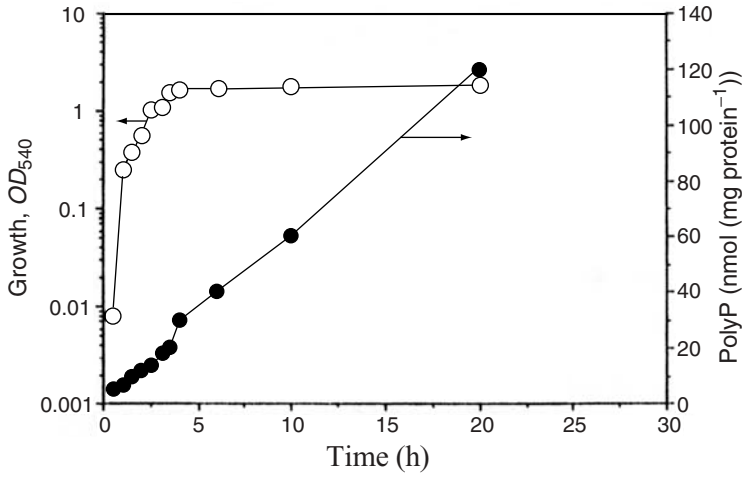

Figure 8.7 Growth and PolyP accumulation by the *Pseudomonas aeruginosa* strain 8830 in L broth (Kim *et al*., 1998).

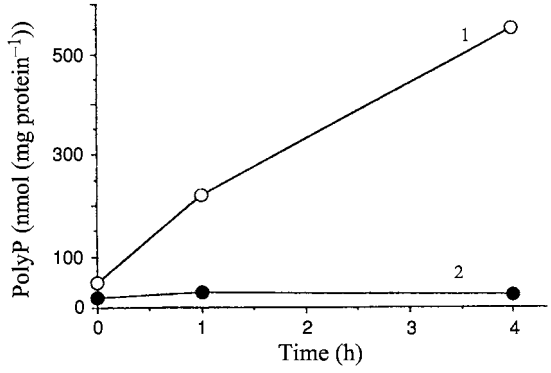

Figure 8.8 PolyP accumulation in *Pseudomonas aeruginosa*, with accumulation induced in a low-phosphate MOPS medium by the addition of serine hydroxamate: (1) *algR2* mutant complemented with plasmid GWS95 with the *ndk* gene, encoding the nucleoside diphosphate kinase; (2) *algR2* mutant (Kim *et al*., 1998).

GTP, ppGpp and PolyP (Kim *et al*., 1998). During the growth in L broth, the bulk of PolyP accumulation in a stable mucoid alginate-producing strain took place at the onset of the stationary phase and continued into the late stationary phase (Figure 8.7). This strain accumulated a large amount of PolyP under stringent response evoked by the addition of serine hydroxamate (Figure 8.8). However, the non-mucouid mutant with AlgR2 deficiency accumulated about 10 % as much PolyP under similar conditions (Kim *et al*., 1998). Thus, the genetic switch that turns on alginate synthesis in *Pseudomonas aeruginosa* also turns on PolyP synthesis. A hyperexpression of the nucleotide phosphate kinase (NDK) gene

restores alginate, GTP, ppGpp and PolyP synthesis (see Figure 8.8) in AlgR2 mutants (Kim *et al*., 1998).

The *ppk* (Ishige *et al*., 1998; Zago *et al*., 1999) and *ppx* (Miyake *et al*., 1999; Zago *et al*., 1999) genes of *P. aeruginosa* were cloned. In contrast to *E. coli*, where the *ppx* and *ppk1* genes are organized in an operon, in *P. aeruginosa ppx* is located in the opposite direction from the *ppk* gene and therefore they do not constitute an operon (Miyake *et al*., 1999; Zago *et al*., 1999). Thus, the independent regulation of *ppk1* and *ppx* in this bacterium may explain the high level of PolyP, since it is possible to regulate the exopolyphosphatase level independently of the polyphosphate kinase level. No coregulation between the *ppk* and *ppx* promoters has been demonstrated in response to osmotic shock and oxidative stress (Zago *et al*., 1999). It was proposed that PolyP accumulation in *P. aeruginosa* is regulated at the enzymatic level through ppx activity inhibition by the stress response molecules of ppGpp without any modulation of the transcription rate of these two genes (Kim *et al*., 1998; Zago *et al*., 1999).

After *ppk1* inactivation, the knockout mutants show no growth defects when compared with the parent strain. One of the remarkable defects in these mutants was the loss of motility (Rashid and Kornberg, 2000; Rashid *et al*., 2000a,b). A low-residual polyphosphate kinase activity was detected in these mutants (Zago *et al*., 1999) and attributed to the activity of the *ppk2* gene (Zhang *et al*., 2002). However, one cannot exclude the existence of other pathways of PolyP synthesis in this bacterium.

8.3 *Acinetobacter*

PolyP metabolism has been intensively studied in *Acinetobacter,* because this organism may be responsible for enhanced biological phosphorus removal (EBPR) at many wastewater treatment plants and serves as a good model organism for developing molecular techniques to characterize metabolism and genetic control in potential EBPR organisms. The ability of this organism to accumulate PolyP and the peculiarities of this process have been effectively studied (Deinema *et al*., 1980, 1985; Van Groenestijn *et al*., 1989; Bonting *et al*., 1991, 1993a,b; Van Veen *et al*., 1994; Geissdorfer *et al*., 1998). The PolyP metabolism of these bacteria has been described in detail in many reviews, for example, Kortstee and Van Veen (1999) and Kortstee *et al*. (2000). A number of *Acinetobacter* species have been isolated, and many of them accumulate large amounts of PolyP under certain conditions (Vasiliadis *et al*., 1990; Kim *et al*., 1997; Gavigan *et al*., 1999).

Polyphosphate kinase is important for PolyP metabolism in *Acinetobacter* (Van Groenestijn *et al*., 1989). The *ppk* gene from the *Acinetobacter* sp. strain ADP1 was cloned (Geissdörfer *et al*., 1998) and the polyphosphate kinase was purified and characterized (Trelstad *et al*., 1999). The induction of *ppk* transcription by P_i starvation was revealed (Gavigan *et al*., 1999). The polyphosphate kinase showed an interesting behaviour when the *Acinetobacter* cultures were subjected to a cycle of P_i starvation and surplus (Kortstee and Van Veen, 1999). Although the *ppk* gene was strongly induced under P_i-limited conditions, the net PolyP-synthesis activity declined and the PolyP levels became almost negligible. In addition, a strong PolyP-degrading activity, which seemed to be due to the presence of exopolyphosphatase but not the reverse work of polyphosphate kinase, was detected in cultures grown under low-P_i conditions. The exopolyphosphatase and AMP–PolyP

phosphotransferase activities declined upon the addition of P_i, while both the PolyP-synthesis activities and PolyP levels rose. Indeed, under most conditions the *Acinetobacter* polyphosphate kinase works poorly in reverse. If polyphosphate kinase does not work (or works poorly) in reverse *in vivo*, then the cell would be forced to use exopolyphosphatase and the phosphate–inorganic (Pit) system to recover energy from the PolyP (Kortstee *et al*., 2000). It was suggested that the formation of PolyP-producing enzymes is linked to the formation of PolyP-degrading enzymes. Thus, the same conditions (here, P_i starvation) which trigger the induction of exopolyphosphatase lead to the induction of polyphosphate kinase. When the conditions change (e.g. P_i is added), polyphosphate kinase is ready and available to form PolyP. This type of regulation could also occur with other nutritional stresses, such as carbon starvation to surplus and anaerobic to aerobic shifts, which occur in EBPR systems. When high-phosphate-grown cells of the strictly aerobic *A. johnsonii* were incubated anaerobically, their PolyP content was degraded and P_i was excreted. The bacterium have two enzymes catalysing PolyP degradation, i.e. polyphosphate:AMP phosphotransferase and exopolyphosphatase (Kortstee *et al*., 2000). In *A. johnsonii*, PolyP serves as an energy source during anaerobiosis by (i) direct synthesis of ATP via the polyphosphate:AMP phosphotransferase/adenylate kinase pathway, and (ii) generation of a proton motive force by the coupled excretion of $MeHPO_4$ and H^+. Exopolyphosphatase may enhance the latter energy recycling mechanism by providing the efflux process with a continuous supply of P_i and divalent metal cations (Kortstee *et al*., 2000).

8.4 ***Aerobacter aerogenes*** **(*Klebsiella aerogenes*)**

Aerobacter aerogenes (Klebsiella aerogenes) does not accumulate PolyPs in a sufficient amount under normal growth conditions but sometimes begins to accumulate PolyPs under unfavourable growth conditions (Smith *et al*., 1954). A detailed investigation has been carried out into the effects of different conditions and growth phases on the PolyP content in this organism (Wilkinson and Duguid, 1960). Quite a substantial accumulation of PolyP occurred at pH 4.5 with sulfur deficiency, after P_i had been added to the phosphorus-starved culture. For PolyP accumulation by this bacterium, in addition to the P_i and energy source, the presence of K^+ and Mg^{2+} ions in the culture medium was essential. The interrelation between PolyP and nucleic acid metabolism has also been observed (Wilkinson and Duguid, 1960). The total amount of PolyP in the cells increased when the growth and nucleic acid synthesis ceased, but the accumulated PolyP (in the form of volutin granules) disappeared after the growth had been resumed.

The fundamental work on PolyP metabolism in *K. aerogenes* has been carried out by Harold and co-workers (Harold, 1963a,b, 1964, 1966; Harold and Harold, 1963, 1965; Harold and Sylvan, 1963). It was shown that the effect of growth conditions on PolyP metabolism is mediated by two entirely different mechanisms.

The first mechanism was realized when the growth was ceased by nutrient deprivation or stress conditions. For example, Figure 8.9(a) shows the gradual PolyP accumulation during sulfur deprivation, while the growth and nucleic acid biosynthesis are suppressed (Harold, 1966). A similar observation was made (Harold, 1963b) during the examination of auxotrophic mutants, which required uracil or methionine for normal growth. PolyP was accumulated in the culture medium in the absence of these components, while on addition

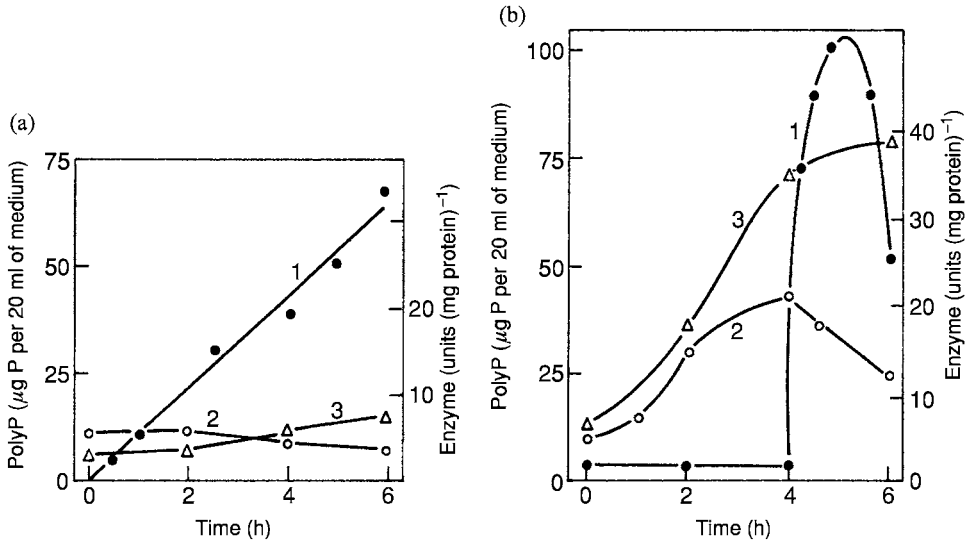

Figure 8.9 Accumulation of PolyPs in *Aerobacter aerogenes* (Harold, 1966): (a) sulfur deprivation, cells placed at 0 h in a P_i- free medium; (b) phosphate overplus, cells placed at 0 h in a P_i-free medium, with P_i was added after 4 h: (1) PolyP; (2) polyphosphate kinase activity; (3) exopolyphosphatase activity.

of the latter the growth was restored and PolyP was utilized for biosynthetic processes. It is probable that such mechanisms are realized by the same way as in other bacteria by the ppGpp and rpoS controlling genes (see earlier in Chapter 7).

In contrast to *E. coli*, *K. aerogenes* exhibits rapid and extensive PolyP accumulation (known as 'PolyP overplus') when P_i is added to cells previously subjected to P_i starvation (Harold, 1966). The changes in PolyP content, polyphosphate kinase and exopolyphosphatase activities under phosphate overplus are presented in Figure 8.9(b). It was shown that the absence of P_i in the medium de-repressed the polyphosphate kinase (Harold, 1964; Harold and Harold, 1963, 1965).

The mutant, which was devoid of polyphosphate kinase, had no PolyP accumulation independent of the growth conditions (Harold, 1964). Later, using specific gene constructions with a cloned *ppk* gene, it was confirmed that in *K. aerogenes*, as in *E. coli*, polyphosphate kinase is responsible for the synthesis of the most part of PolyP and for PolyP overplus (Kato *et al.*, 1993b; Ohtake *et al.*, 1999; Kuroda and Ohtake, 2000). A *ppk* mutant of *K. aerogenes* showed no PolyP overplus. Like the *E. coli ppk-ppx* operon, the *ppx* gene is located immediately 'downstream' of the *ppk* gene (Kato *et al.*, 1993b). As expected, the polyphosphate kinase activity increased in response to P_i starvation and decreased upon addition of P_i. However, unlike polyphosphate kinase, the exopolyphosphatase activity did not increase but rather slightly decreased under conditions of P_i starvation, although the *ppx* mRNA was induced (Kuroda and Ohtake, 2000). Earlier, it had been shown that PolyP degradation was completely inhibited in the mutant without exopolyphosphatase (Harold, 1966).

Harold and Harold (1963, 1965) also obtained mutants with defects in the regulation of phosphorus metabolism. One of them could not de-repress the PolyP-metabolizing enzymes by P_i deprivation and did not display phosphate overplus, while the other had these enzymes constitutively de-repressed and accumulated PolyP during the exponential phase (Harold and Harold, 1965). Later it was established that there is a putative Pho box in the promoter region of the *K. aerogenes ppk-ppx* operon (Kato *et al*., 1993b; Kuroda and Ohtake, 2000). Unlike the *E. coli ppk–ppx* operon, the *K. aerogenes ppk–ppx* operon seems to be under a stronger control of the PhoB and PhoR proteins (Kato *et al*., 1993b). The existence of the PolyP operon in this bacterium indicates that both increased PolyP synthesis and decreased PolyP degradation are responsible for regulation of the PolyP content in *K. aerogenes*.

8.5 *Azotobacter*

PolyP metabolism in several species of *Azotobacter* has been investigated by Zaitseva and co-workers (Zaitseva and Belozersky, 1958, 1960; Zaitseva and Li Tszyun-in, 1961; Zaitseva and Frolova, 1961; Zaitseva *et al*., 1959, 1960a,b, 1961). These investigations were initially concerned with the changes in PolyP content during the development of cultures of various species of *Azotobacter*. It was shown that, as in *E. coli,* acid-insoluble PolyPs accumulated during the latent phase of development and was subsequently utilized actively during the exponential growth of the culture. In the case of *Azotobacter agile,* it disappeared completely at this growth stage, reappearing only in the stationary phase. Acid-insoluble PolyPs accumulated in the early stationary phase, followed later by accumulation of acid-soluble PolyPs (Zaitseva and Belozersky, 1958; Zaitseva *et al*., 1959, 1960a,b). The behaviour of PolyPs in a synchronized culture of *Azotobacter* was also studied (Zaitseva *et al*., 1961). It was revealed that acid-insoluble PolyP attained its highest level in synchronously growing cells immediately prior to the onset of cell division, and during cell division it degraded to acid-soluble PolyP and then to P_i. These results indicate possible participation of PolyPs in cell-cycle regulation.

The dependence of PolyP metabolism in *Azotobacter* on the nutrient medium composition was investigated. The greatest interest was connected with possible participation of PolyPs in nitrogen fixation. However, no specific features were observed, which could indicate a direct participation of PolyPs in this process. In a medium containing ammonium salts, PolyPs accumulated in larger amounts than under nitrogen-fixation conditions or in the absence of a nitrogen source in the medium. The inhibitory effect of Ca^{2+} ions on both nitrogen fixation and PolyP accumulation was established (Esposito and Wilson, 1956; Zaitseva *et al*., 1960b). However, Zaitzeva *et al*. (1960b) showed that these two phenomena were not directly related and that PolyP did not provide the source of phosphorus and energy in nitrogen fixation. It was revealed that a high Ca^{2+} concentration had an inhibitory effect on glycolitic phosphorylation, which was the common energy source for both processes in *Azotobacter*.

In *Azotobacter*, the main enzyme of PolyP metabolism was shown to be polyphosphate kinase (Zaitseva and Belozersky, 1958, 1960), which was capable of PolyP synthesis and of a reverse reaction. This enzyme was isolated and purified to a considerable extent (Zaitseva and Belozersky, 1960).

8.6 *Cyanobacteria* (Blue–Green Algae) and other Photosynthetic Bacteria

The great interest in PolyP metabolism in *Cyanobacteria* is connected with the ability of PolyPs to grow rapidly under P_i and heavy metal excesses in the water. In many studies, special attention was paid to a possible use of cyanobacteria as assimilators of substantial amounts of phosphate in the form of PolyP. This problem arose from severe pollution of inland waters with various detergents, among which $PolyP_3$ is the most abundant pollutant.

It should be noted that an important contribution to the study of PolyP metabolism in cyanobacteria was made by Jensen and co-workers (Jensen, 1968, 1969; Jensen and Sicko, 1974; Sicko-Goad *et al*., 1975; Sicko-Goad and Jensen, 1976; Lawry and Jensen, 1979; Baxter and Jensen, 1980a,b). In these experiments, a special emphasis was laid on the accumulation of PolyP granules by cyanobacteria under conditions similar to those of inland waters. Normally, the conditions of phosphorus and sulfur starvation occur in these waters. When large amounts of industrial and domestic detergents enter inland waters, an intensive bloom of cyanobacteria occurs, leading to contamination of vast water reservoirs.

Using electron microscopy with the cyanobacteria *Nostoc puriforme* (Jensen, 1968), *Plectonema boryanum* (Jensen, 1969; Jensen and Sicko, 1974; Sicko-Goad *et al*., 1975) and *Anacystis nidulans* (Lawry and Jensen, 1979), Jensen and his colleagues investigated the accumulation of PolyP granules under various cultivation conditions. From these studies, in particular with *Plectonema boryanum* cultured under phosphate starvation followed by phosphate overplus, Jensen drew the following conclusions (Jensen and Sicko, 1974). Under normal growth conditions, PolyP granules were found mainly on DNA fibrills and in a zone enriched in ribosomes. Under conditions of P_i starvation, an additional zone was formed in the region of nucleoplasm. Under phosphate overplus, PolyP granules accumulated in nucleoplasm and appeared in the polyhedral bodies involved in the dark reactions of photosynthesis in cyanobacteria (Stewart and Codd, 1975). In certain cells, PolyP granules formed near thylakoids. Similar reports for cyanobacteria have been made by other authors (Vaillancourt *et al*., 1978; Barlow *et al*., 1979).

In *Anacystis nidulans*, the intacellular PolyP level, which was manipulated by growth in the presence of various P_i concentrations in the medium (0.3–3 mM), increased with the P_i concentration up to 2.1 mM and decreased thereafter (Keyhani *et al*., 1996). Thus, the PolyP accumulation in cyanobacteria depended on the phosphorus content in the medium, as in other bacteria. The growth rate of cyanobacteria under phosphate starvation has been shown to be a function of the amount of previously accumulated PolyPs in the cells (Rhee, 1973). PolyP storage is a survival strategy under conditions of fluctuating phosphate supply characteristic of the environmental conditions, in which the cyanobacteria live (Falkner *et al*., 1995).

The above studies also give evidence of multiple localization of PolyP in the cells of cyanobacteria. This conclusion was confirmed by a ^{31}P NMR spectroscopic study. In the cyanobacterium *Synechocystis sp*., two pools of soluble PolyP were identified *in vivo* by ^{31}P NMR spectroscopy (Lawrence *et al*., 1998). One of these (PolyP–cation complexes) lost their association cations after EDTA treatment, while the other did not.

The increase of PolyP accumulation in cyanobacteria was observed under conditions of sulfur deficiency, which diminished the growth (Lawry and Jensen, 1979; 1986). This

fact indicated that in cyanobacteria, as in other bacteria, there are mechanisms of PolyP involvement in the overcoming of nutrition stresses.

The distribution of PolyPs between different fractions in cyanobacteria depends on culture age and growth conditions. For example, in *Anabaena flos-aquae* phosphorus is stored in different fractions depending on the nitrogen source. Under N_2 fixing conditions, P is stored as sugar P, whereas with nitrate as the N source it is stored as PolyP (Thompson *et al*., 1994).

X-ray dispersive microanalysis (Sicko-Goad *et al*., 1975; Baxter and Jensen, 1980a) combined with electron microscopy, established the phosphate nature of granules and showed that appreciable amounts of K^+ and comparatively low quantities of Ca^{2+} and Mg^{2+} were present in the PolyP granules of cyanobacteria under ordinary cultivation conditions. Under special conditions, when the medium contained an excess of some metals such as Mg^{2+}, Ba^{2+}, Mn^{2+} or Zn^{2+} and some heavy metals, they accumulate in large quantities in the PolyP granules (Jensen *et al.,* 1982). Str^{2+} was found to accumulate in the cells of cyanobacteria, but in other inclusions containing sulfur instead of phosphorus.

X-ray microanalysis of thin cryosections of *Anabaena cylindrica* showed that aluminium was rapidly taken up and accumulated in PolyP granules (Pettersson *et al*., 1985). In addition, aluminium was found in the cell walls but could not be detected in the cytoplasm. The concentration of phosphorus in the medium affected the accumulation pattern. More aluminium was bound with PolyP granules and with the cell walls after growth in a P_i-rich medium.

Some evidence was obtained that the cells of *Anacystic nidulans* with a high PolyP content showed a greather tolerance to Cd^{2+} than those cells with a small PolyP reserve (Keyhani *et al*., 1996). Thus, the accumulation of metal cations in the PolyP granules of cyanobacteria may function as a detoxification mechanism.

Some cyanobacteria possess cyanophycin, which is a copolymer of aspartic acid and arginine (Lawry and Simon, 1982). This copolymer is accumulated in the granules, and its localization in complexes with PolyP is not improbable.

As regards other photosynthezing bacteria, PolyP metabolism was investigated in *Chlorobium thiosulfactophillum* (Fedorov, 1959, 1961; Shaposchnikov and Fedorov, 1960; Hughes *et al*., 1963; Cole and Huges, 1965). The accumulation of PolyP in the course of culture development was studied (Fedorov, 1959; Shaposhnikov and Fedorov, 1960). It was shown that the maximal accumulation of PolyP occurred in the stationary phase. During culture growth, the proportion of acid-soluble to acid-insoluble PolyPs shifted towards the latter. Large amounts of acid-soluble PolyPs were accumulated in this bacterium when cells were illuminated in the absence of CO_2 (Fedorov, 1959). It was suggested that PolyPs were used as an energy store in the absence of CO_2 fixation.

In another phototrophic bacterium, *Rhodospirillum rubrum*, a massive pyrophosphate (PP_i) biosynthesis by photosynthetic phosphorylation was shown (Baltscheffsky, 1967a,b,c, 1969; Baltscheffsky *et al*., 1966; Keister and Yike, 1967a,b; Keister and Minton, 1971, 1972; Kulaev *et al*., 1974a). It was demonstrated that, when *Rh. rubrum* was grown anaerobically in light, its chromatophores accumulated salt-soluble PolyPs in addition to PP_i (Kulaev *et al*., 1974). The PolyPs in chromatophores may be synthesized from ATP (Shadi *et al*., 1976) or from PP_i (Ok Duck-Chenn and Lee Hynn-Soon, 1987).

It should be pointed out (Kulaev *et al*., 1974a) that in *Rh. rubrum*, both in the dark and in the light, the accumulation of PolyP took place not only in salt-soluble but also in alkali-soluble and hot-perchloric-acid-extractible fractions. The total amounts of PolyP

Table 8.1 The content of P_i and PolyPs in the early stationary growth phase in *Halobacterium salinarium* and *Halorubrum distributum* (μmol of P_i per g of wet biomass).

	Initial P_i concentration in medium			
	H. salinarium		*H. distributum*	
Component	2.3 mM	11.5 mM	2.3 mM	11.5 mM
P_i	170	1000	100	760
Acid-soluble PolyP	30	100	30	90
Alkali-soluble PolyP	9	4	5	8
Acid-insoluble PolyP	1	1	0.7	1.2

in this bacterium differ little in the light and in the dark. However, under conditions of phototrophic nutrition, the ratio of salt-soluble to total PolyPs in the cells at all stages of growth was much higher than in the control cells grown in the dark.

These data are in good agreement with the investigations on the purple bacteria carried out by Weber (1965). However, *Rh. rubrum* and *Rhodospheromonas spheroides* differed substantially in PolyP distribution between variuos fractions. In *Rh. rubrum*, the greater part of PolyP was found in the acid-insoluble fraction, whereas in *Rhodospheromonas spheroides* the acid-soluble PolyPs constituted a much greater part of the total content in the cells. PolyPs were also identified in other photosynthesizing bacteria, namely *Chromatium okenii* (Schlegel, 1962), *and Rhodopseudomonas palustris* (Fedorov, 1961).

As to the enzymes of PolyP metabolism in photosynthetic bacteria, polyphosphate kinase activity was revealed in the cyanobacteria *Anacystis nidulans* (Vaillancourt *et al*., 1978) and *Oscillatoria redekei* (Zaiss, 1985). The mutant in this enzyme had no PolyP granules observable by electron microscopy (Vaillancourt *et al*., 1978). The alignment analysis revealed the genes encoding putative polyphosphate kinase (*ppk1* and *ppk2*) in several genomes of cyanobacteria (Zhang *et al*., 2002) (see Table 6.1 above).

The cyanobacterium *Synechocystis sp* possesses the *ppx* gene encoding exopolyphosphatase, which was induced by P_i starvation. The ppx mutant exhibited lower growth rates under P_i-sufficient conditions, hence indicating the importance of exopolyphosphatase in the phosphorus metabolism of this organism (Gomez-Garcia *et al*., 2003).

8.7 *Mycobacteria* and *Corynebacteria*

The metabolism of PolyP in these two genera of bacteria has many features in common, and it would be better to discuss them together. These bacteria, under normal growth conditions, accumulate substantial amounts of volutin granules and possess the widest range of PolyP-metabolizing enzymes known so far (Muhammed *et al*., 1959; Muhammed, 1961; Hughes and Muhammed, 1962; Szymona, 1957, 1962, 1964; Phillips *et al*., 1999).

The most detailed investigations into PolyP metabolism of the *Mycobacteria* and *Corynebacteria* have been carried out by Drews (1960a,b), Mudd and co-workers (Mudd

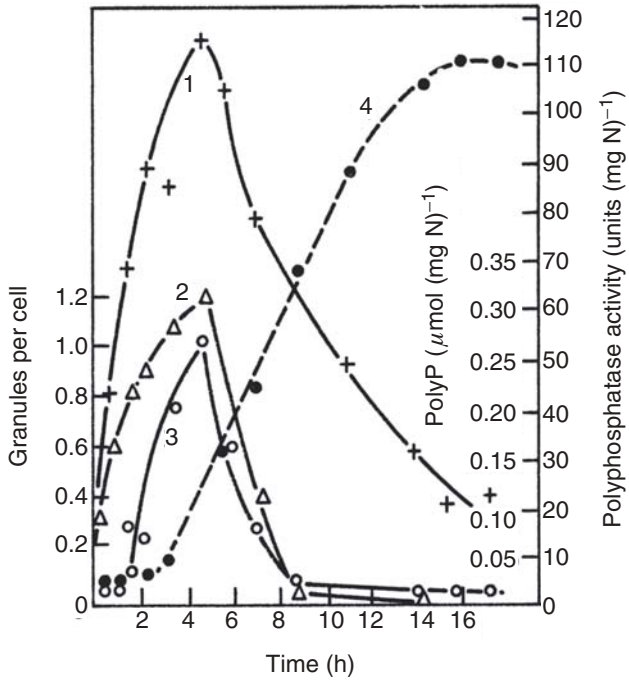

Figure 8.10 Changes in the amount of PolyP, granules and exopolyphosphatase activity in *Corynebacterium xerosis* (Hughes and Muhammed, 1962): (1) exopolyphosphatase activity; (2) PolyP; (3) granules; (4) biomass.

et al., 1958), Winder and Denneny (1957) and Szymona (1964). These workers have shown that rapidly dividing cells of *Mycobacteria* accumulate minimal amounts of PolyPs. When the cells enter the stationary growth phase, the more rapid biosynthetic processes cease and the PolyP content increases rapidly. When culture development is inhibited by nitrogen starvation (Sall *et al*., 1956), antimetabolites azaserine (Mudd *et al*., 1958) or ethionine (Ebel, 1952d), or deficiency of Zn^{2+} in the medium, PolyP accumulates rapidly. Thus, the accumulation of PolyPs under stress is also characteristic of this group of bacteria.

When the normal grouwth conditions are restored, PolyP is actively utilized for the biosynthesis of nucleic acids and phospholipids as a source of phosphorus (Mudd *et al*., 1958; Winder and Denneny, 1957).

PolyP metabolism under various growth conditions and at different growth stages has also been investigated in *Corynebacterium* (Hughes and Muhammed 1962; Dirheimer and Ebel, 1962, 1964b, 1965, 1968). Hughes and Muhammed (1962) showed that the PolyP content of *Corynebacterium xerosis* was a function of the phase of growth (Figure 8.10). When the cells of this bacterium were placed in a fresh medium, PolyP accumulation was observed during the latent period. On entering the logarithmic growth phase, PolyP was actively utilized and accumulated again in the stationary phase. As in *Mycobacteria*, rapid accumulation of PolyP occurred under nitrogen starvation. In *Corynebacterium diphteriae*, it was shown that acid-insoluble PolyP accumulated in the greatest amounts at a stage

preceding cell division and was consumed rapidly in the process of division (Dirheimer and Ebel, 1962, 1964b, 1965, 1968).

Using ^{31}P NMR spectroscopy, the accumulation of soluble cytosolic PolyP under aeration and its breakdown under anaerobiosis have been observed in *Corynebacterium glutamicum* (Lambert *et al*., 2002). Under 60–80 % saturation with oxygen, PolyP accumulation was detected when P_i and glucose or acetate were added to a cell suspension. This PolyP was apparently of a high molecular weight, but at the initial stages of PolyP formation its chain length was $\sim$ 40 phosphate residues. The PolyP level rose after the addition of carbon sources and declined again when the oxygen level was recovered. Both processes, the increase of PolyP during aeration and supply with carbon source and P_i and the decrease during anaerobiosis, occurred within minutes (Lambert *et al*., 2002). Thus, PolyP occurs in *Corynebacterium glutamicum* not only as a granular store material, but also as a very dynamic compound that may play a decisive role in this bacterium.

The presence of some specific PolyP-dependent enzymes is characteristic of these bacteria. First, it is polyphosphate glucokinase that was found in *Mycobacterium phlei* (Szymona, 1957), *Corynebacterium xerosis* (Dirheimer and Ebel, 1962, 1964b, 1968) and *Mycobacterium tuberculosis* (Hsieh *et al*., 1993a,b; 1996a,b), and other representatives of this systematic group (Dirheimer and Ebel, 1962, 1964b; Kulaev and Vagabov, 1983). This enzyme, purified from *Mycobacterium phlei* (Girbal *et al*., 1989) and cloned from *Mycobacterium tuberculosis* (Hsieh, 1996; Hsieh *et al*., 1996a), is well described in a recent review (Phillips *et al*., 1999). Secondly, in *Mycobacterium tuberculosis* a polyphosphate/ATP–NAD kinase was characterized (Kawai *et al*., 2000). Such activity was found in *Corynebacterium ammoniagenes* (Fillipovich *et al*., 2000).

Thirdly, in *Mycobacterium phlei* in media containing fructose, mannose or gluconate, enzymatic activities were found forming fructose-6-phosphate, mannose-6-phosphate or gluconate-6-phosphate through PolyP utilization (Szymona and Szumilo, 1966; Szymona *et al*., 1969). Finally, AMP phosphotransferase activity in *Corynebacterium and Mycobacterium* was revealed (Winder and Denneny, 1957; Szymona, 1964; Dirheimer and Ebel, 1965). It is likely that the abilities to utilize PolyPs directly for the phosphorylation of NAD, glucose and other sugars provide considerable energetic advantages for these bacteria.

Polyphosphate kinase (Muhammed, 1961; Robinson and Wood, 1986; Robinson *et al.,* 1987) and exopolyphosphatase (Muhammed *et al*., 1959) are presented as well, while putative genes for these activities have also been found (Zhang *et al*., 2002; Cardona *et al*., 2002). The exopolyphosphatase of *C. xerosis* was studied by Muhammed *et al*. (1959). This activity changes during the culture growth, in parallel with the accumulation of PolyP and PolyP granules (see Figure 8.10), hence indicating the importance of this enzyme in PolyP metabolism. The possible pathways of PolyP metabolism in *Mycobacteria* are shown schematically in Figure 8.11 (Szymona, 1964).

8.8 *Propionibacteria*

PolyP metabolism has been most studied in *Propionibacterium shermanii*. Konovalova and Vorob'eva (1972) have examined the PolyP content in this bacterium. In this study, 70–80 % of the total PolyP was found in the fraction extracted by hot perchloric acid at all

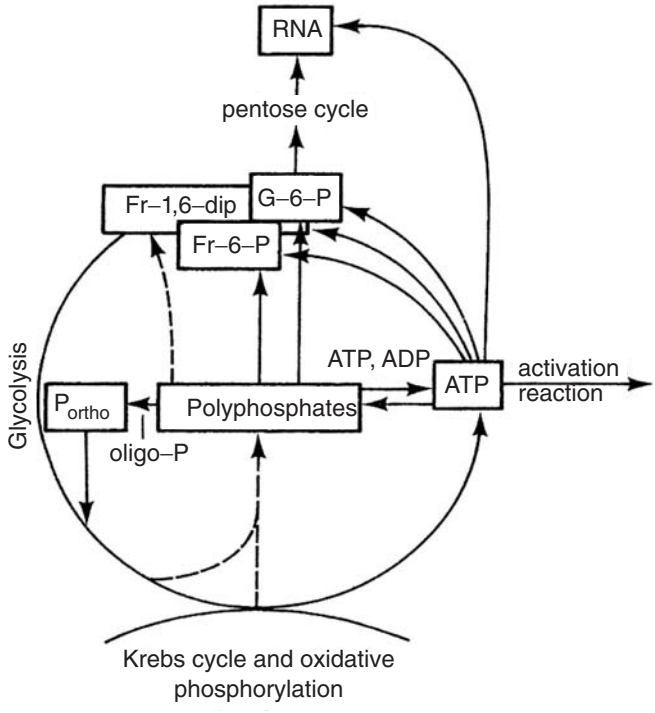

Figure 8.11 Possible pathways of PolyP metabolism in *Mycobacteria* (Szymona, 1964).

growth stages, using lactate as a carbon source. The remaining 20–30 % of the PolyP was evenly distributed between the salt-soluble and alkali-soluble fractions. Low-molecular-weight acid-soluble PolyP in the propionic bacteria at all growth stages on lactate was not found. The total amount of PolyP increases during the culture growth (Figure 8.12). The PolyP content increased sixfold under logarithmic growth whereas it remained at the same level during the stationary growth phase (Kulaev *et al*., 1973a).

It was shown that the accumulation of PolyP fractions in *P. shermanii* was strongly inhibited by adding 50 μg ml^{-1} of the antibiotic polymyxin M to the medium (Konovalova and Vorob'eva, 1972). The presence of this antibiotic in the culture medium substantially retarded the accumulation of this compound (Figure 8.12). This observation is of great interest in view of the fact that the site of attack of this antibiotic in the bacterial cell is the cytoplasmic membrane. The close link between PolyP metabolism and the functioning of the cytoplasmic membrane may be explained by inhibition of the P_i uptake or by possible inhibition of PolyP-synthesizing enzymes or, conversely, activation of PolyP-cleaving enzymes. In order to resolve this question, an attempt was made in our laboratory to determine the activities of most of the enzymes currently known to be involved in the synthesis or utilization of PolyPs in this microorganisms (Kulaev *et al*., 1973). The activities of some PolyP-dependent enzymes were examined at different growth stages of *P. shermanii* (see Figure 8.12). It can be seen that under normal growth conditions, 1,3-diphosphoglycerate–polyphosphate phosphotransferase is more active in old culture. Polymyxin M has no

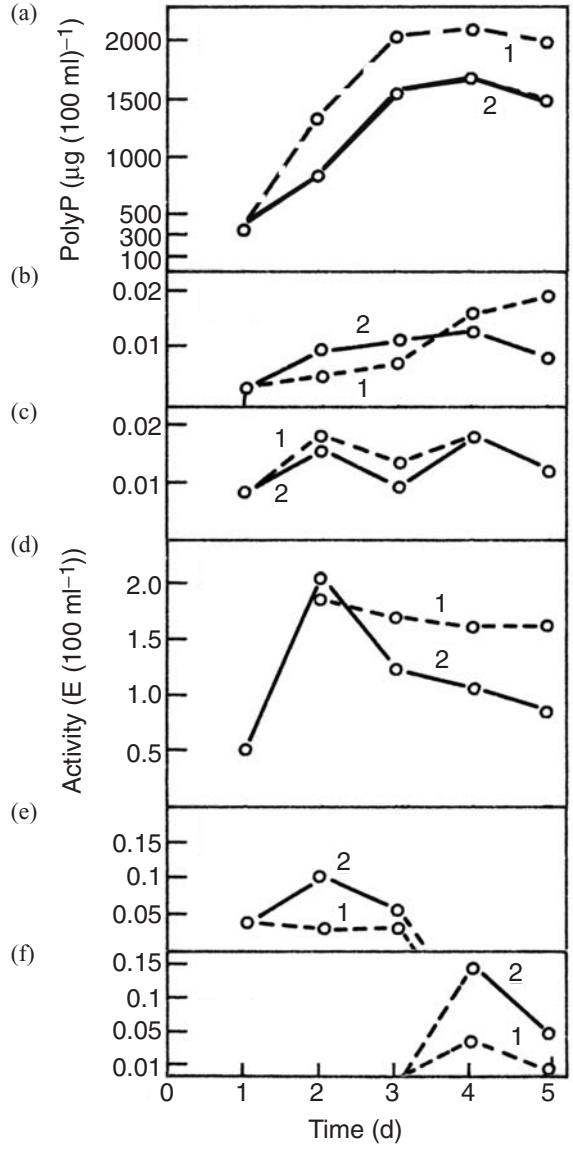

Figure 8.12 Changes in polyphosphate and polyphosphate-metabolising enzymes during the development of a culture of *Propionibacterium shermanii* under normal conditions, and in the presence of polymyxin M (Kulaev *et al*., 1973a): (1) control conditions; (2) polymyxin M: (a) total PolyP; (b) 1,3-diphosphoglycerate–polyphosphate phosphotransferase; (c) polyphosphate kinase; (d) polyphosphate–glucokinase; (e) tripolyphosphatase; (f) exopolyphosphatase with $PolyP_{290}$.

effect on polyphosphate kinase activity and only a slight, but nevertheless definite, effect on 1,3-diphosphoglycerate–polyphosphate phosphotransferase activity. The polyphosphate glucokinase activity remained at a high level during the whole time of cultivation and was not affected by polymyxin. Polymyxin M, when added to the growth medium, significantly increases (by a factor of 2–7) the activity of exopolyphosphatase and tripolyphosphatase. This is an indication that the functioning of these enzymes may be closely bound (as in other microorganisms) with the cytoplasmic membrane, which is the site of action of polymyxin M. The polyphosphatase evidently plays an important part in PolyP degradation. As soon as this enzyme activity begins to appear, PolyP accumulation ceases. In the presence of polymyxin, the increase of this activity correlated with the reduction in the PolyP level.

Great contributions to the studies of PolyP metabolism were made by Wood and coworkers (Clark *et al*., 1986; Clark and Wood 1987; Wood and Clark, 1988). These workers obtained three PolyP fractions from *P. shermanii*, i.e. short-chain PolyPs soluble in trichloroacitic acid, long-chain PolyPs soluble at neutral pH, and long-chain PolyPs present in volutin granules. Cells grown on lactate did not contain short-chain PolyPs but did contain a high amount of long-chain PolyPs, which accumulated to 3 % of the dry cell biomass (Clark *et al*., 1986). At least 70 % of this PolyP was present in volutin granules. The PolyPs ranged from 250 to 725 phosphate residues and were of the same average size as those synthesized *in vitro* by polyphosphate kinase from this bacterium. In contrast to the cells grown on lactate, the glucose-grown cells did not contain volutin granules, but did contain short-chain PolyPs with an average chain length of 25–75 residues. It has been proposed that the amounts and chain lengths of the PolyPs are lower during growth on glucose, because they are utilized as substrates in the phosphorylation of glucose by polyphosphate glucokinase present in this organism (Wood and Clark, 1988). Later, polyphosphate glucokinase from this organism was purified, cloned and characterized (for reference, see Chapter 6).

Data indicating participation of PolyPs in overcoming stress were obtained in *Propionibacteria*. The PolyP component in the ^{31}P NMR spectra of *Propionibacterium acne* increased after ultraviolet light irrardiation (Kjeldstad and Johnson, 1987) and after hyperthermia treatment (Kjeldstad *et al*., 1988). Such treatments, carried out in triplicate, induced an increase in the PolyP content, as observed by ^{31}P NMR spectroscopy. One of the explanations for this might be that hyperthermia and ultraviolet light induce an oxidative stress in the cells, which increases the amount of PolyP (Kjeldstad and Johnson, 1987; Kjeldstad *et al*., 1988).

To summarise, it should be said that the PolyP content and chain length in *Propionibacteria* are strongly dependent on the carbon source. These bacteria possess polyphosphate glucokinase and are able to directly utilize PolyP for glucose phosphorylation.

8.9 *Archae*

PolyP metabolism in Archae, a very ancient and heterogenic domain of prokaryotes, has been little studied. PolyP and PolyP-dependent enzymes were observed in some representatives of this domain (Scherer and Bochem, 1983; Skorko, 1989; Trotsenko and Shishkina, 1990; Rudnick *et al*., 1990; Andreeva *et al*., 2000; Smirnov *et al*., 2002a,b; Cardona *et al*., 2002).

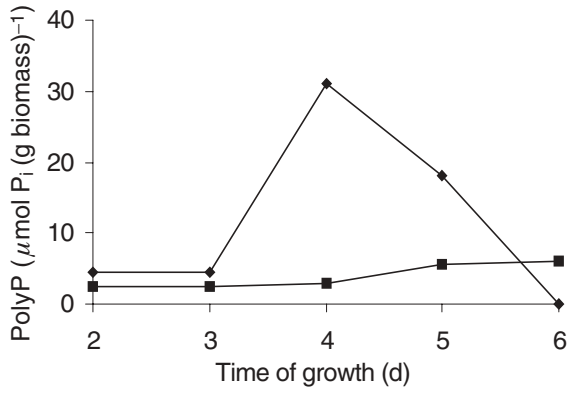

Figure 8.13 Changes in PolyP content during growth of *Halobacterium salinarium* on a medium with 2.3 mM of P_i (Smirnov *et al*., 2002a): (♦) acid-soluble PolyP; (■) alkali-soluble PolyP.

Methanosarcina frisia accumulates phosphate up to a level of 14 % of its dry weigh (Rudnick *et al*., 1990). The phosphate is stored as PolyP, as shown by ^{31}P NMR spectroscopy. This archaeon accumulate more phosphate in the presence of methanol as the carbon source, when compared with CO_2 and H_2 as the only carbon and energy sources (Rudnick *et al*., 1990).

Halobacterium salinarium and *Halorubrum distributum,* extremely halophilic archae, were capable of consuming up to 95 % of the phosphate from the culture medium at P_i concentrations of 2.3 and 11.5 mM. These archae possess PolyPs of acid-soluble and alkali-soluble fractions, and the contents of these PolyPs changed abruptly during growth (Andreeva *et al*., 2000; Smirnov *et al*., 2002a,b). The dynamics of the PolyP content during the growth of *H. salinarium* on the medium with 2.3 mM P_i is shown on Figure 8.13. However, the phosphorus of the PolyP was no more than 10 % of all of the phosphorus accumulated in the culture (Table 8.1). The greater part of the P_i was present in biomass as magnesium orthophosphate, the amount of which by the early stationary phase might have reached nearly 90 % of the phosphate consumed by the cells (Smirnov *et al*., 2002a,b). The excess accumulation of P_i evoked changes in cell morphology, and a part of the cell population lost viability. The accumulation of phosphate as its inorganic soluble salt is an unfavourable factor for the vital functions of individual cells but may be useful for the survival of a population as a whole at further growth on a phosphate-deficient medium. It was shown that *H. salinarium* cells grown on a medium with P_i excess can use the P_i-phosphate reserve in a P_i-limited medium (Smirnov *et al*., 2002a,b). Obviously, *H. salinarium* has insufficiently developed regulatory mechanisms, which might regulate phosphate utilization and reservation as PolyP. Although this organism had an appreciable PolyP pool, its exopolyphosphatase activity was very low (Andreeva *et al*., 2000). This activity did not depend on the phosphate content in the medium, the amount of PolyP, or the culture age. Thus, in contrast to yeast and a number of bacteria where PolyPs play an important role in maintaining homeostasis of phosphorus compounds in a cell under unfavorable conditions, the PolyP function and metabolism in *H. salinarium* are not directly connected with P_i reservation. *H. salinarium* obviously shows a rather ancient and primitive form of phosphate reservation as inorganic P_i.

It should be noted that no *ppx* or *ppk* similar genes were found in *Halobacterium* genome (Cardona *et al*., 2002). There are little data on exopolyphosphatase activity in other Archae. In *Sulfolobus solfactaricus* (Cardona *et al*., 2002) this was very low – much less than in bacteria. In *Sulfolobus solfactaricus*, however, a functionally active gene of exopolyphosphatase was found (Cardona *et al*., 2002) with a similarity to bacterial *ppx*. In other archaeal genomes, putative genes similar to the yeast *PPX1* or bacterial *ppx* genes have been revealed (Cardona *et al*., 2002). However, the functional activity and significance of the proteins encoding by these genes are still unclear.

8.10 Yeast

Yeasts are the microorganisms where PolyPs were first discovered (Liebermann, 1888). Many papers and reviews summarize the available data on PolyP metabolism and functions in these organisms (Schmidt *et al*., 1946; Hoffman-Ostenhof and Weigert, 1952; Wiame, 1947a,b, 1948, 1949; Hoffmann-Ostenhof *et al*., 1955; Yoschida, 1955a,b; Langen and Liss, 1958a,b, 1959; Kulaev and Belozersky, 1962a,b; Harold, 1966; Weimberg and Orton, 1964, 1965; Weimberg, 1970; Dawes and Senior, 1973; Matile, 1978; Kulaev, 1971, 1974; Kulaev and Vagabov, 1983; Kornberg, 1995, Kornberg *et al*.,1999; Kulaev *et al*., 1999; Kulaev and Kulakovskaya, 2000), and here we will cite only some of these.

The content of PolyPs in yeast cells strongly depends on the culture conditions and growth stage and can be as much as 10 % of the total dry weight of a yeast cell (Salhany *et al*., 1975). In P_i complete media, the highest values of PolyPs were observed in the stationary phase. PolyPs with chain lengths of as low as 3–8 to as high as 200–260 were obtained from the cells of these microorganisms by chemical extraction (Langen and Liss, 1959; Schuddemat *et al*., 1989a).

It should be noted that yeast possesses PolyPs in nearly all cell compartments (see Chapter 5) and the compartmentation of these biopolymers should be taken into consideration when analysing their accumulation and utilization.

8.10.1 Yeast Cells Possess Different Polyphosphate Fractions

The content of PolyP in yeast cells was determined by various techniques, including chemical extraction, ^{31}P NMR spectroscopy, enzymatic, and electrophoretic methods (see Chapter 2). One of the most suitable methods for the study of PolyPs in yeasts is chemical extraction (Langen and Liss, 1958a,b; Chernyscheva *et al*., 1971; Vagabov *et al*., 1998), which made it possible to isolate five PolyP fractions.

The acid-soluble fraction, PolyP(I), was extracted with 0.5 M $HClO_4$ (or 10 % trichoroacetic acid) at 0 °C for 30 min. The salt-soluble fraction, PolyP(II), was extracted with a saturated solution of $NaClO_4$ at 0 °C for 1 h. The weak alkali-soluble fraction, PolyP(III), was extracted with weak NaOH, pH 9–10, at 0 °C for 30 min. The alkali-soluble fraction, PolyP(IV), was extracted with 0.05 M NaOH at 0 °C for 30 min. The last fraction, PolyP(V), was assayed by the amount of P_i which appeared after the hydrolysis of biomass in 0.5 M $HClO_4$ at 90 °C for 40 min.

The synthesis and degradation of these fractions are closely related to metabolic processes in individual cell compartments, and their dynamics are affected in different ways by changes in the culture conditions.

8.10.2 The Dynamics of PolyP Fractions during the Cell Cycle

The relationship between the metabolism of various PolyP fractions, RNA and DNA in synchronous cultures of the yeast *Schizosaccharomyces pombe* (synchronicity index, 0.7–0.8) has been investigated (Kulaev *et al*., 1973b). As shown in Figure 8.14, there were substantial changes in the amounts of a wide variety of phosphorus compounds in the interval between the two episodes of division, i.e. during the growth of dividing cells. The shape of the curve for total phosphorus accumulation during this period is mainly determined by accumulation of RNA in the cells. The amount of DNA is doubled over a short time interval ($\sim$ 15 min) and reaches its maximum at the beginning of the next episode of cell division. During the first two thirds of the period of synchronous growth of *S. pombe*, the total PolyP content increased. In the period immediately preceding cell division, a slight fall in PolyP accumulation was observed. Fractions PolyP(III) and PolyP(IV) accumulated rapidly at the beginning of this period. Accumulation of PolyP(IV) appeared first. These data are in agreement with the opinion that these fractions are synthesized in connection with the biosyntheses of the cell wall polysaccharides mannan and glucan (Kulaev *et al*., 1972d). Fraction PolyP(II) accumulated in the cells of the synchronous culture in correlation with the RNA biosynthesis. It is probable that the formation of PolyP(II) and the nucleic acids biosynthesis are linked as shown in Figure 8.15. Using the ^{32}P isotope, it was shown that the phosphorus of the PolyP might incorporate with RNA synthesis to the same extent for all of the nucleosides (Kulaev and Belozersky, 1957). As regards PolyP(I), its behaviour is converse to that of the total RNA. This fraction is apparently a reserve of high-energy phosphate and is probably used during the intensive synthesis of nucleic acids. On the basis of these results, the following outline of the PolyP relationship during the cell cycle in the yeast *S. pombe* may be proposed. During the biosynthesis of RNA, the high-energy phosphate of PolyP(I) is utilized. On the other hand, the PP_i formed in the nucleus during the RNA (DNA) biosynthesis may be a source for the synthesis of PolyP(II). By depolymerization of these fractions, restoration of the PolyP(I) fraction may occur. The PolyP(III) and PolyP(IV) fractions are apparently connected with the formation of the cell wall during cell division and are not involved in nucleic acid biosynthesis. Thus, the data obtained on the synchronous culture of yeast gave evidence of the different roles of PolyP fractions during the cell cycle.

In the *S. cerevisiae* synchronous culture, an increased P_i uptake from the culture medium during DNA synthesis was observed (Gillies *et al*., 1981). At a high level of external P_i, this uptake provided the necessary phosphorus level in cells and the ^{31}P 'NMR-visible' PolyP remained constant. However, if the external P_i content was low, this PolyP was consumed, acting as a substitute for the phosphate reserve (Gillies *et al*., 1981).

A complicated and indirect interaction of the mitosis specific activation of the P_i-responsive gene *PHO5* and the PolyP level in *S. cerevisiae* has been found (Neef and Kladde, 2003). *PHO5* mitotis activation was repressed by P_i addition, which significantly

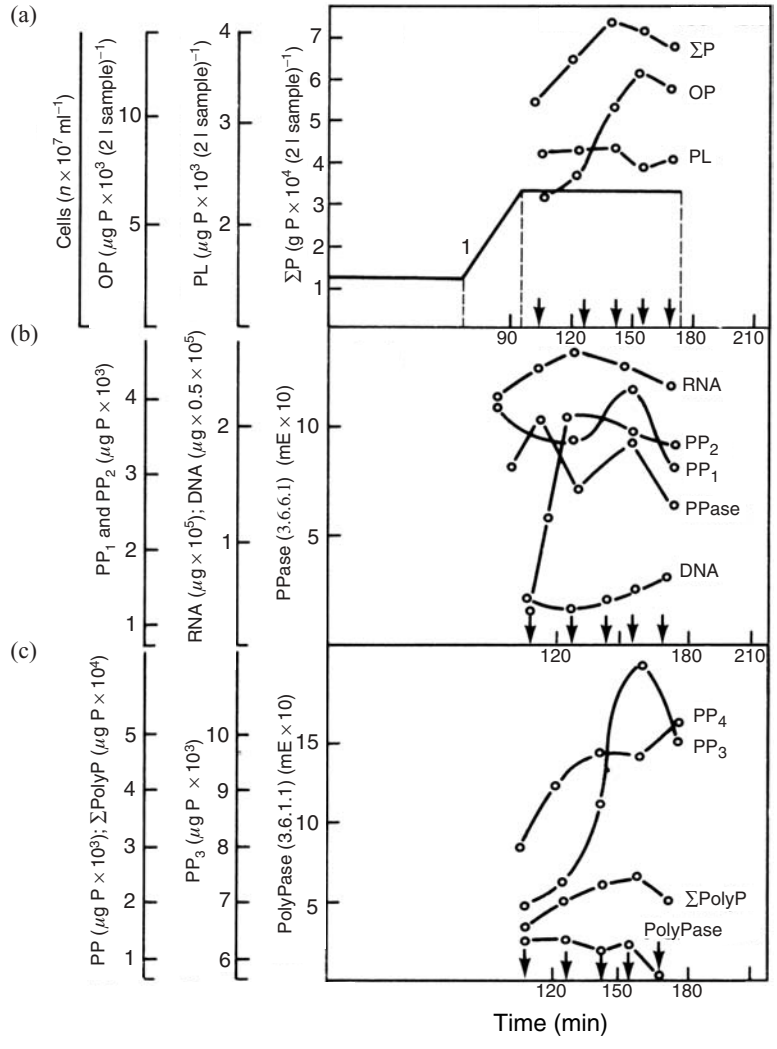

Figure 8.14 Changes in the content of nucleic acids, phospholipids, P_i and PolyPs, and the activities of exopolyphosphatase (PolyPase) and pyrophosphatase (PPase) during synchronous growth of *Schizosaccharomyces pombe* (Kulaev *et al*., 1973b): (a) 1, number of cells; PL, phospholipids; OP, P_i; $\sum$P, total phosphate: (b) PP_1, acid-soluble polyphosphate; PP_2, salt-soluble polyphosphate: (c) PP_3, alkali-soluble polyphosphate; PP_4, hot-perchloric-acid-soluble polyphosphate; $\sum$PolyP, total polyphosphate.

increased the PolyP content. The PolyP level fluctuated inversely with the *PHO5* mRNA during the cell cycle, thus indicating an important link between this polymer and mitotic regulation of *PHO5* (Neef and Kladde, 2003).

All of these observations give evidence for the important role of PolyPs in cell cycle regulation in yeast.

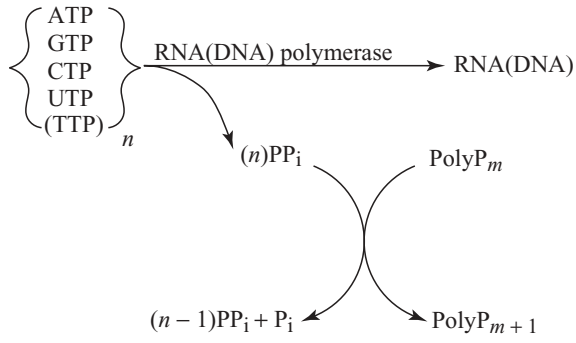

Figure 8.15 The hypothetical linkage of PolyPs and nucleic acids biosynthesis in yeast and fungi.

8.10.3 The Relationship between the Metabolism of Polyphosphates and other Compounds

Besides the correlation between the rates of accumulation of RNA and the PolyP(II) fraction (Kulaev *et al*., 1973b; Kulaev and Belozersky, 1957; Kulaev and Vagabov, 1983), a good correlation between the rates of PolyP(IV) accumulation and the synthesis of cell wall polysaccharides has been revealed (Kulaev *et al*., 1972d; Vagabov *et al*., 1973; Tsiomenko *et al*., 1974; Shabalin *et al*., 1979; 1985; Vagabov, 1988). This correlation can be seen in Figure 8.16. These data suggest a specific interrelation between the metabolisms of these two compounds, which, although quite different in their chemical nature, are nevertheless components of the same organelle, namely the cell envelope. The presence of PolyPs in the cell envelopes of yeast and fungi has been established by using many techniques (Weimberg and Orton, 1965; Weimberg, 1970; Kulaev and Afanas'eva, 1970, Vořišek *et al*., 1982; Tijssen *et al*., 1982, 1983; Tijssen and Van Steveninck, 1984, 1985; Vagabov *et al*., 1990; Ivanov *et al*., 1996). Later, an enzyme dolichyl-diphosphate:polyphosphate phosphotransferase (EC 2.7.4.20) was found in the membrane fraction of yeast cells (Shabalin *et al*., 1979, 1984, 1985; Naumov *et al*., 1985; Kulaev *et al*., 1987), and the putative pathway of joint mannan and PolyP biosynthesis was proposed.

There was some evidence for the possible involvement of PolyPs localized in the cell periphery in the uptake and phosphorylation of sugars as energy and phosphate donors (Van Steveninck and Booij, 1964; Hofeler *et al*., 1987). Later, studies of the mechanisms of transport-associated phosphorylation of 2-deoxy-D-glucose in the yeast *Kluyveromyces marxianus* (Schuddemat *et al*., 1989b) and *Saccharomyces cerevisiae* (Schuddemat *et al*., 1990) resulted in the conclusion that PolyPs seem to replenish the P_i pool and therefore had an indirect role in sugar transport.

8.10.4 Polyphosphate Fractions at Growth on a P_i -Sufficient Medium with Glucose

Glucose is the most common carbon substrate for many yeasts, and the PolyPs contents at different growth stages using this energy source has been analysed in detail. A ^{31}P NMR

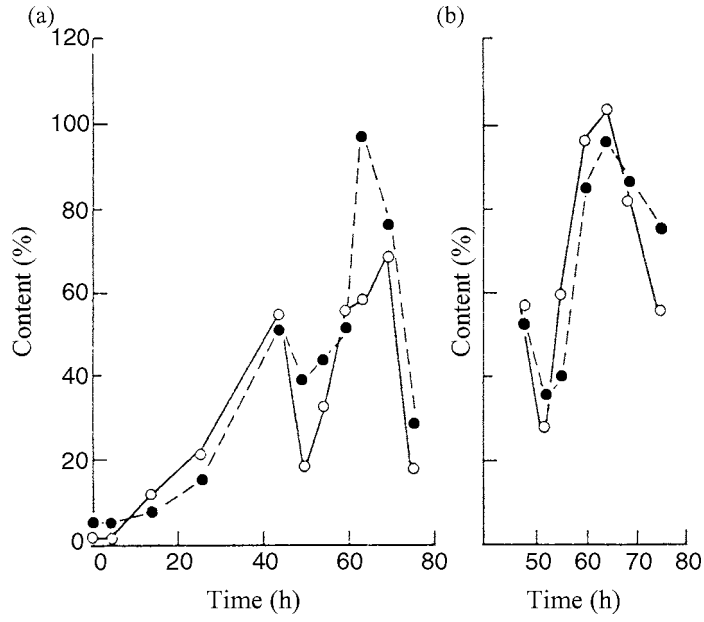

Figure 8.16 Changes in the content of mannan (○) and PolyP(IV) fraction (●) during growth of *Saccharomyces cerevisiae* in a Reader medium in the presence (a) and absence (b) of a nitrogen source (Kulaev *et al.*, 1972d).

spectroscopic study showed that the logarithmic cells contained substantially shorter PolyPs than the stationary cells (Greenfield *et al.*, 1987).

The contents and chain lengths of the PolyPs in different fractions from *S. cerevisiae* growing on a 'Reader medium' (Reader, 1927) with glucose and a sufficient amount of P_i were studied by a combination of chemical extraction and ^{31}P NMR spectroscopy (Vagabov *et al.*, 1998). Before glucose was consumed from the medium (11 h of culture growth), the biomass and total cellular PolyP content had increased in parallel (Figure 8.17.). After glucose depletion, the content of PolyP in the cells fell sharply and then increased again in a 24 h culture. The significant decline in the content of intracellular PolyP, while the P_i concentration in the growth medium is high may imply that in this growth phase PolyP is an energy rather than a P_i source.

The changes in the contents of the PolyP(I) and PolyP(II) fractions were minimal during yeast growth (Figure 8.17). The PolyP(III) fraction increased almost threefold after 3 h of growth and then decreased by about fivefold by the time of glucose exhaustion (10.5 h of culture growth). In contrast, the content of the PolyP(IV) fraction diminished noticeably in a 3 h culture. It is known that the PolyP(IV) fraction is located at the peripheries of the yeast cells and their synthesis is coupled with the synthesis of mannoproteins of the cell wall (Shabalin *et al.*, 1979, 1985). It can be assumed that the changes in the cellular content of these PolyP fractions are associated with the formation of the cell wall in *S. cerevisiae*.

The PolyPs belonging to different fractions differ to a greater extent in their states or localizations in the cells rather than in their degrees of polymerization (Vagabov *et al.*, 1998). The chain lengths of the PolyPs belonging to these fractions are not

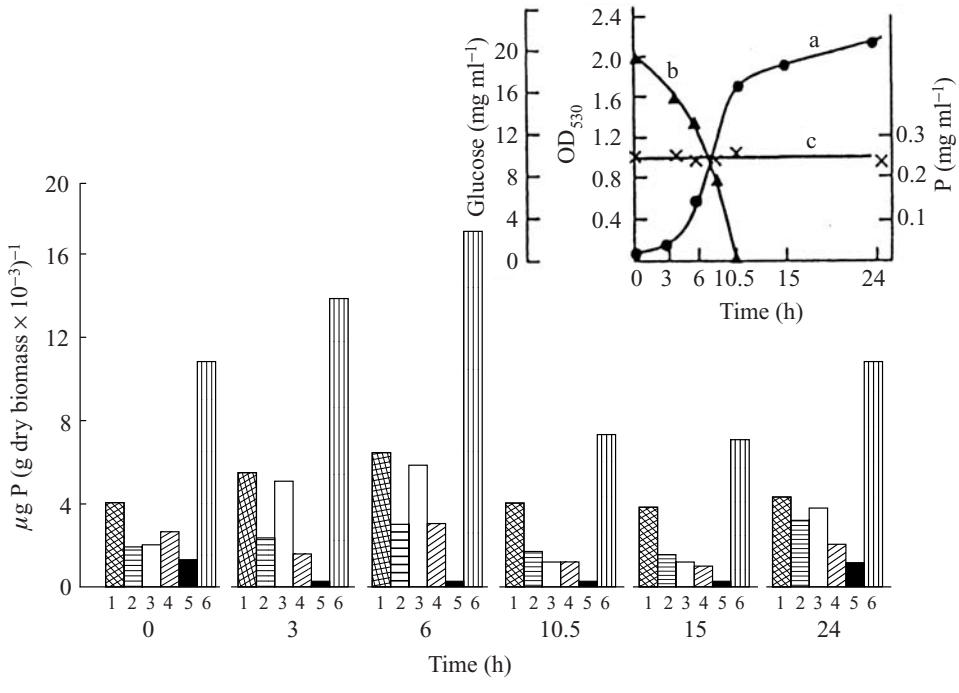

Figure 8.17 Changes in the content of different PolyP fractions during growth of *Saccharomyces cerevisiae* on glucose (Vagabov *et al*., 1998): (1) PolyP(I); (2) PolyP(II); (3) PolyP(III); (4) PolyP(IV); (5) PolyP(V); (6) total PolyP content. Inset: (a) cell growth; (b) glucose concentration in the medium; (c) P_i concentration in the medium.

always significantly different and depend on the growth stage (Figure 8.18). This becomes especially evident when comparing the changes in the degree of polymerization of the fractions of PolyP(II), PolyP(III) and PolyP(IV). In all of these fractions, the PolyP chain is drastically shortened during the first 3 h of yeast growth. In the case of PolyP(III) and PolyP(IV), this shortening took 6 h of culture growth in a complete medium. The degrees of polymerization of these fractions were found to increase again only in a 24 h culture (Figure 8.18). These data support the assumption that in a P_i-sufficient growth medium PolyPs may act as reserves of energy, which are replenished when the carbon sources have been depleted.

The accumulation of PolyPs (accompanied by their dramatic shortening) in actively growing yeast cells indicates that the processes of PolyP synthesis and depolymerization may occur in parallel. Exopolyphosphatases (Lichko *et al*., 2003a) and endopolyphosphatase (Kumble and Kornberg, 1995) could be involved in the depolymerization of PolyPs. The active synthesis of PolyPs, accompanied by dramatic decreases in their lengths in the logarithmic phase of *S. cerevisiae* growth in a carbon- and phosphorus-sufficient medium, suggests that the energy derived from PolyP hydrolysis is necessary to maintain the high rate of yeast growth.

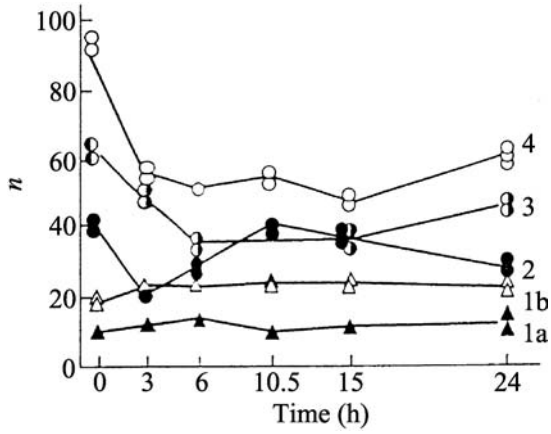

Figure 8.18 Changes in the degree of polymerization (n) in PolyP fractions during growth of *Saccharomyces cerevisiae* on glucose (Vagabov *et al*., 1998): (1a) PolyP(I), precipitation by barium salt at pH 8.2; (1b) PolyP(I), precipitation by barium salt at pH 4.5; (2) PolyP(II); (3) PolyP(III); (4) PolyP(IV).

8.10.5 The Effects of P_i Limitation and Excess

The P_i limitation causes a sharp decline of PolyPs in yeast cells (Liss and Langen, 1962; Kulaev and Vagabov, 1983). Under P_i limitation, yeast cells have often no ^{31}P 'NMR-visible' PolyPs (Hofeler *et al*., 1987). When P_i-starved yeast cells are placed on a complete medium, the PolyP content rises sharply, i.e. the so-called 'phosphate overplus' (hypercompensation) effect occurs (Liss and Langen, 1962).

The content (Figure 8.19) and degree of polymerization (Figure 8.20) of PolyPs were determined in the course of growth of the yeast *Saccharomyces cerevisiae* in a medium with glucose, which contained varying P_i amounts at a constant level of all necessary components (Vagabov *et al*., 2000). After 7 h of phosphate starvation, the yeast was shown to use almost the complete phosphate reserve in the form of PolyPs to support its vitality (Figure 8.19). The PolyP drop was followed by a considerable shortening of the polymer chain length of acid-soluble (PolyP(I)) and two alkali-soluble (PolyP(III) and PolyP(IV)) fractions (Figure 8.20). Under the same conditions, the content of a salt-soluble fraction (PolyP(II)) decreased almost 20-fold with a simultaneous increase of the chain length of nearly twofold.

Re-inoculation of yeast cells after phosphate starvation to a complete P_i- and glucose-containing medium resulted in the accumulation of PolyP within 2 h, mainly in PolyP(III), and, to a lesser extent, in the PolyP(I), PolyP(II) and PolyP(V) fractions. In the PolyP(IV) fraction localized on the cell surface, PolyP 'super-accumulation' was not detected. Increase in the PolyP amount in the above fractions turned out not to be accompanied by simultaneous elongation of their chain lengths and occurred at the lowest level, which is characteristic of a polymer level for each fraction (Figure 8.20). Further cultivation of the yeast on the complete medium over 2 h had little or no effect on the PolyP content in the cells but led to elongation of the PolyP chain, especially in the PolyP(III) and PolyP(IV) fractions. This phenomenon of considerable elongation of the PolyP chain on the background of

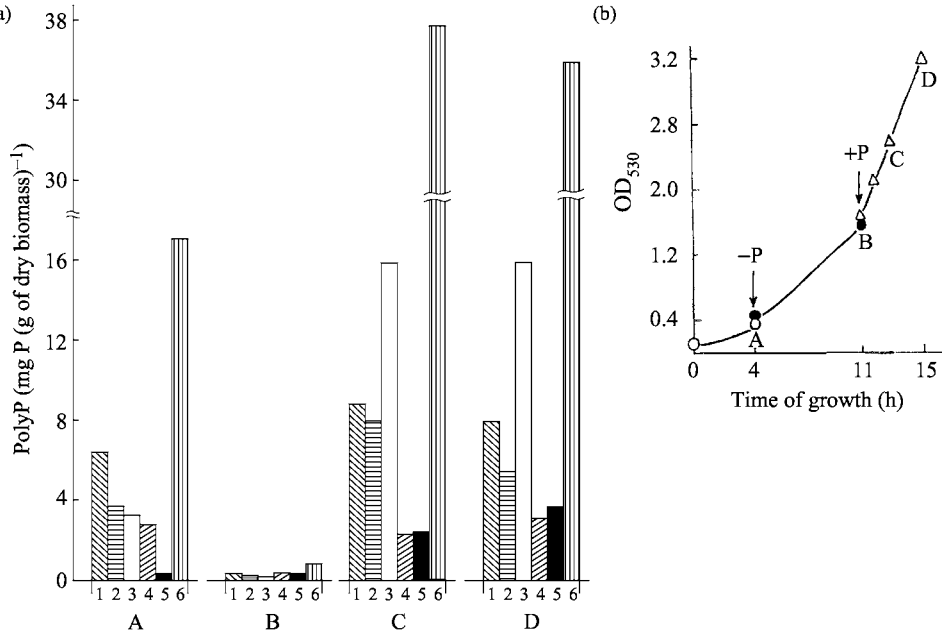

Figure 8.19 The PolyP content (a) and cell growth (b) of *Saccharomyces cerevisiae* under different culture conditions (Vagabov *et al.*, 2000): (1) PolyP(I); (2) PolyP(II); (3) PolyP(III); (4) PolyP(IV); (5) PolyP(V); (6) total PolyP content. The points indicate the following: (A) re-inoculation from complete medium to the medium without P_i; (B) re-inoculation to the complete medium after growth on the medium without P_i; (C) and (D) growth in the complete medium for 2 and 4 h, respectively (after re-inoculation from the medium without P_i).

its fixed content suggests that yeast cells possess an unknown discrete pathway of PolyP biosynthesis, which results in the formation of comparatively low-molecular-weight chains and then of high-molecular-weight polymers (Figure 8.20). The different behaviours of the separate PolyP fractions during phosphate overplus suggests that the mechanism of the synthesis of distinct PolyP fractions may be different or at least regulated in different ways.

The phosphate overplus phenomenon is achieved in *S. cerevisiae* not only after the complete absence of P_i in the culture medium, but also after P_i limitation (Kulakovskaya *et al.*, 2004) (Figure 8.21). The dynamics of changes in the PolyP content of separate fractions was studied at re-inoculation of late-logarithmic cells from P_i-limited to complete medium. The PolyP(I), PolyP(II) and PolyP(III) fractions increased more significantly during the first 2 h of cultivation. After further cultivation, some redistribution of PolyPs between the fractions took place. The content of the PolyP(I) fraction decreased while those of the PolyP(II), PolyP(III) and PolyP(V) fractions increased in the stationary phase, although the content of PolyP(IV) changed insignificantly (Figure 8.21). Its twofold increase in the stationary phase was mainly due to the PolyP(III) and PolyP(V) fractions (Figure 8.21).

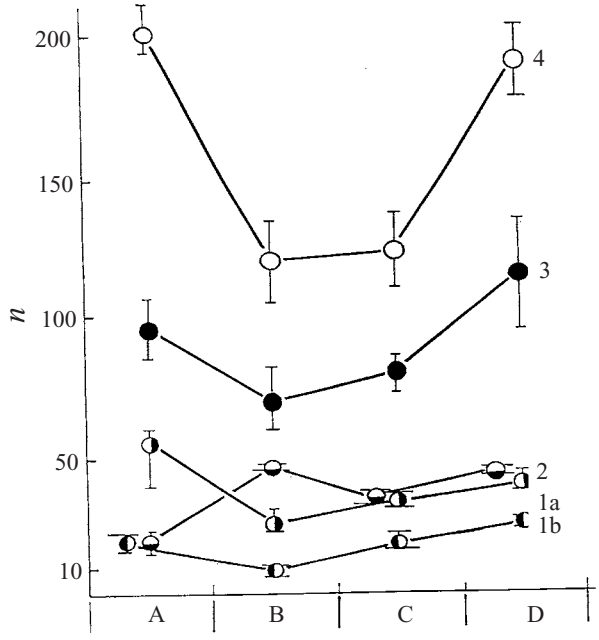

Figure 8.20 Changes in the degree of polymerization (n) in PolyP fractions of *Saccharomyces cerevisiae* in dependence of P_i concentration in the culture medium (Vagabov *et al*., 1998): (1a) PolyP(I), precipitation by barium salt at pH 8.2; (1b) PolyP(I), precipitation by barium salt at pH 4.5; (2) PolyP(II); (3) PolyP(III); (4) PolyP(IV). The points indicate the following: (A) re-inoculation from complete medium to the medium without P_i; (B) re-inoculation from the medium without P_i to the complete medium; (C) and (D) growth in the complete medium after re-inoculation from the medium without P_i for 2 and 4 h, respectively.

At a control re-inoculation from complete to fresh medium, the total PolyP content did not change during 6 h of cultivation.

The cytosol posesses about ~ 60 % of the total exopolyphosphatase activity of *S. cerevisiae* cells. This activity is represented by two enzymes, the 40 kDa exopolyphosphatase 1 splitting $PolyP_3$ off most actively and the 830 kDa exopolyphosphatase 2 which is specific to long-chain PolyPs (see Chapter 6). Therefore, the effect of P_i limitation and excess on these exopolyphosphatases was examined. During the growth of *S. cerevisiae* using a low initial culture density (Figure 8.22), the activities of the cytosol exopolyphosphatases 1 and 2 were the same, both in the complete and P_i-limited media. Low-molecular-weight exopolyphosphatase 1 (PPX1) predominated in the cytosol independent of P_i concentration in the medium (Figure 8.22).

Under re-inoculation of late-logarithmic or stationary phase cells on the fresh medium with a high initial culture density (Figure 8.23(c)), the activities of two exopolyphosphatases in the cytosol essentially changed. Under phosphate overplus, the activity of exopolyphosphatase 2 increased, while the activity of exopolyphosphatase 1 decreased (Figure 8.23(a)). After a control re-inoculation from complete to fresh medium, the total activity of

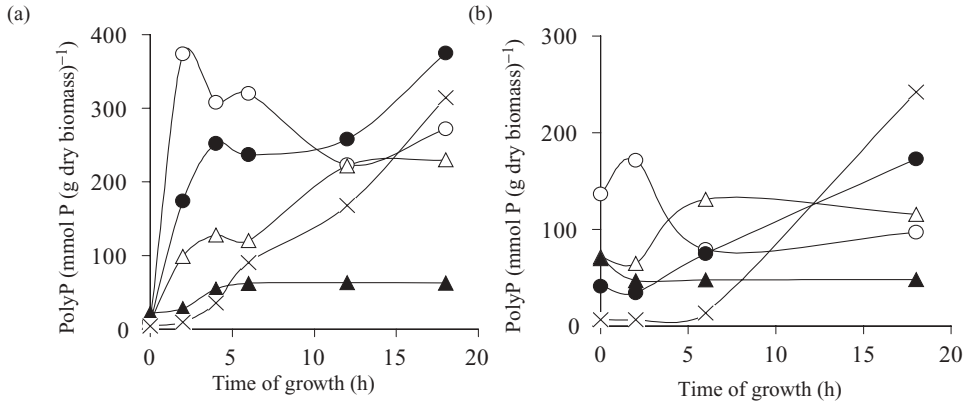

Figure 8.21 The content of different PolyP fractions in *Saccharomyces cerevisiae* cells in the process of growth after re-inoculation of late-logarithmic cells on the fresh medium with a high initial culture density: (a) re-inoculation from a P_i-limited medium (with 1 mM P_i) to a complete Reader medium (with 18 mM P_i); (b) re-inoculation from a complete Reader medium to a fresh one: (○) polyP(I); (Δ) polyP(II); (●) polyP(III); (▲) polyP(IV); (×) polyP(V). The growth curves are shown below in Figure 8.23(c).

exopolyphosphatase 2 increased as well, although to a lesser extent (Figure 8.23(b)). The increase of exopolyphosphatase 2 activity was completely blocked by cycloheximide, indicating that the enzyme was synthesized *de novo*, while the inhibitor had little effect on PolyP accumulation (Figure 8.24). Thus, no direct interrelation between accumulation and utilization of PolyP and the activities of exopolyphosphatase 1 and 2 was observed. The role of these enzymes in the dynamics of PolyPs needs further investigation.

While PolyPs are localized in different compartments of the yeast cell, it is important to determine the effects of P_i starvation and P_i overplus on PolyPs in organelles. The content of PolyP in vacuoles of the yeast *S. cerevisiae* was ∼ 15 % of the total cellular PolyP. Over 80 % of vacuolar PolyPs were represented by the acid-soluble fraction. It was established by ^{31}P NMR spectroscopic studies that the polymeric degrees (n) of two subfractions obtained by precipitation with Ba^{2+} ions in succession at pH 4.5 and 8.2 were approximately 20 ± 5 and 5 ± 2 residues of orthophosphoric acid, respectively. Under the deficit of phosphate (P_i) in the culture medium, the PolyP content in vacuoles decreased ∼ sevenfold at the same drastic reduction of its content in the cell. Unlike the intact yeast cells where PolyP overcompensation is observed after their transfer from phosphate-free to phosphate-containing medium, the vacuoles do not show this effect (Table 8.2). The data obtained indicate the occurrence of special regulatory mechanisms of PolyP synthesis in vacuoles differing from those in the whole cell.

Mitochondria possess a PolyP pool, which is strongly influenced by the P_i content in the medium (Pestov *et al*., 2003). Table 8.3 shows that the PolyP content in mitochondria increases sufficiently under phosphate overplus. This PolyP, represented by the acid-soluble fraction, had a chain length of ∼ 25, estimated by electrophoresis under phosphate overplus (Pestov *et al*., 2003) and was shorter (∼ 15) under the control conditions.

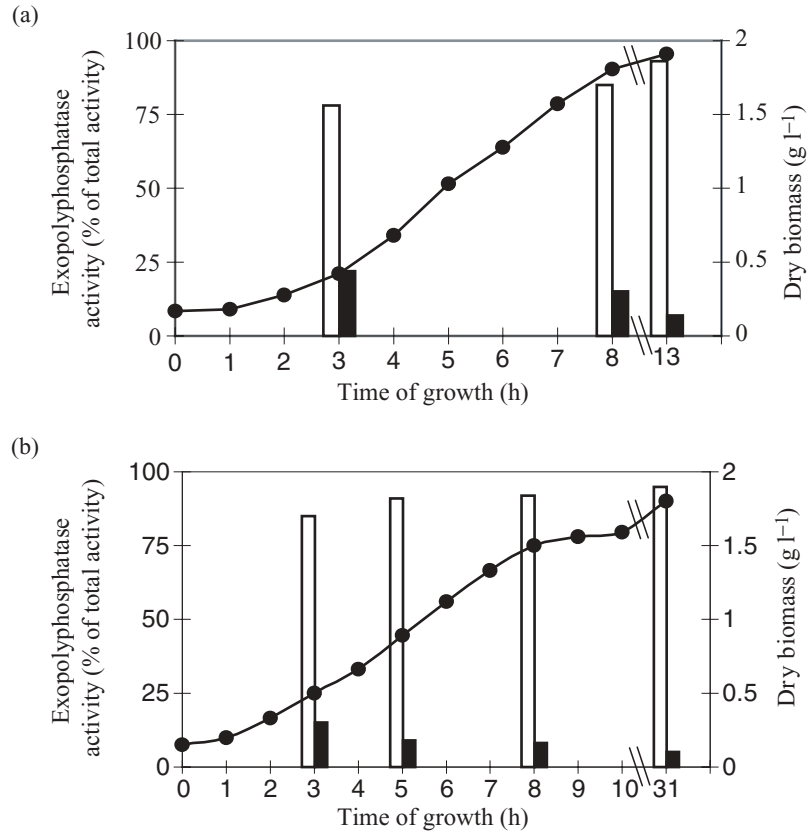

Figure 8.22 The activities of exopolyphosphatases 1 (□) and 2 (■) in the cytosol fraction of *Saccharomyces cerevisiae* during the process of growth (●) at a low initial cell density without re-inoculation: (a) complete Reader medium; (b) P_i-limited medium. The cytosol fraction was subjected to gel filtration on a Sephacryl S-300 column. The exopolyphosphatase activities were estimated separately in the fractions corresponding to the molecular masses of ~ 40 kDa (exopolyphosphatase 1) and ~ 830 kDa (exopolyphosphatase 2).

8.10.6 The Effects of other Conditions on the Polyphosphate Content in Yeast Cells

The dependence of the PolyP content in yeast on the carbon and nitrogen sources has not been studied systematically. In some yeasts, which were able to grow on alkanes, much more rapid accumulation of PolyP (2–3 times) during growth on these carbon sources was observed when compared with growth on glucose (Levchuk *et al*., 1969; Grigor'eva *et al*., 1973). In contrast, PolyP synthesis with ethanol as an energy source was slower than with glucose in the cells of *S. cerevisiae* and *K. marxianus* (Schuddemat *et al*., 1989a).

When young *S. cerevisiae* cells were incubated in Tris buffer, at pH 7.5, with 5 % of ethanol, a rapid breakdown of the 'NMR-visible' PolyP into smaller fragments occurred

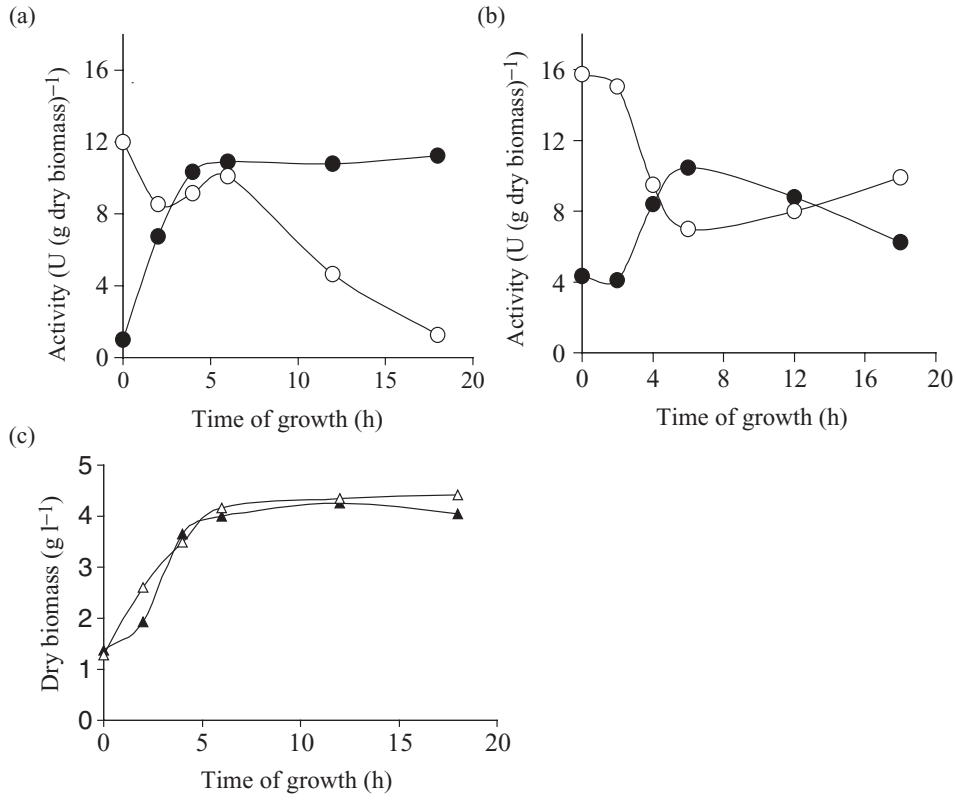

Figure 8.23 (a,b) The activities of exopolyphosphatases 1 (○) and 2 (●) in the cytosol fraction of *Saccharomyces cerevisiae* during the process of growth at a high initial cell density: (a) re-inoculation of late-logarithmic cells from a P_i-limited to a complete Reader medium; (b) re-inoculation of late-logarithmic cells from a complete medium to a fresh one. (c) The growth of *Saccharomyces cerevisiae* after re-inoculation from a P_i-limited medium to a complete Reader medium (▲) and from a complete Reader medium to a fresh one (△).

(Loureiro-Dias and Santos, 1990), in concordance with the ability of high ethanol concentrations for the de-energization of cytoplasmic and vacuolar membranes (Loureiro-Dias and Santos, 1990; Petrov and Okorokov, 1990).

The NMR spectroscopic study showed that the addition of 20 mM of NH_4^+ to *S. cerevisiae* cells caused a rapid (within 10 min) substantial increase in the cytoplasmic and vacuolar P_i and a breakdown of long-chain PolyP to short-chain PolyP and P_i (Greenfeld *et al*., 1987). The effect did not depend on the anion used and was observed in both the logarithmic and stationary phase cells. Earlier, it was reported that the addition of ammonia or amino acids to nitrogen-starved cells caused an immediate (1-5 min) increase in the $PolyP_3$ level (Lusby and McLaughlin, 1980). The $PolyP_3$ was thought to derive from a breakdown of longer PolyPs, on the basis of metabolic labelling studies. In contrast, removal of nitrogen from the medium halted the $PolyP_3$ accumulation within 10 min (Lusby and McLaughlin,

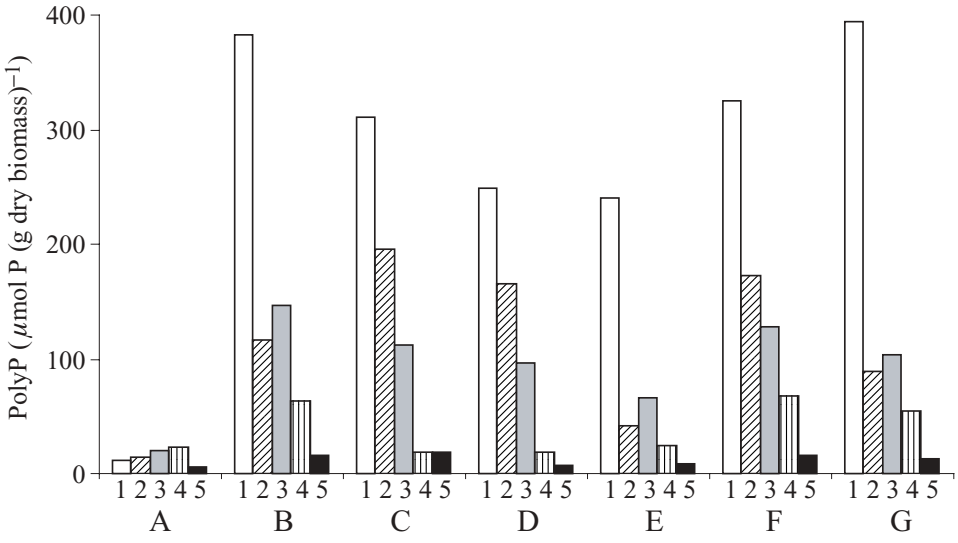

Figure 8.24 The effect of inhibitors on the content of certain PolyP fractions in *Saccharomyces cerevisiae* cells under phosphate overplus (Trilisenko *et al*., 2003). (A) PolyP in P_i-starved cells; (B–G) PolyP in cells grown at 2 h after re-inoculation on the complete medium (phosphate overplus): (B) control conditions; (C) 10 mg ml^{-1} of cycloheximide; (D) 10 μM FCCP; (E) 20 μM FCCP; (F) 250 μM iodacetamide; (G) 50 nM bafilomycin A. PolyP fractions: (1) PolyP(I); (2) PolyP(II); (3) PolyP(III); (4) PolyP(IV); (5) PolyP(V).

1980). It is possible that the ammonia-initiated PolyP hydrolysis provided the mechanisms for maintenance of pH homeostasis in vacuoles.

The degradation of 'NMR-visible', probably vacuolar, PolyPs to short-chain polymers in *S. cerevisiae* was observed under conditions where it was necessary to neutralize the alkalization of cytoplasm (Castro *et al*., 1995, 1999).

Decrease in the PolyP level was observed under anaerobic conditions, probably because PolyP biosynthesis requires a great deal of energy (Den Hollander *et al*., 1981; Beauvoit *et al*., 1991; Castro *et al*., 1995). In energy-limited *S. cerevisiae* cells, a competition between ion transport and PolyP biosynthesis was revealed: ion transport was only observed in anaerobic cells without exogenous glucose with low ATP synthesis, whereas the addition of glucose supported PolyP synthesis (Hofeler *et al*., 1987).

In a continuous culture of *Candida utilis* (glucose-containing medium), a direct and almost linear relationship between the specific growth rate and the PolyP content in the cells and vacuoles was observed. The relationship of the growth rate and chain length of vacuolar PolyPs was an inverse one. At a low growth rate, two peaks with $\sim$ 35 and $\sim$ 5 P_i residues were observed, while at an intermediate growth rate, a peak of 15–25 units appeared with shorter chains of 5 units. When the growth rate was maximal, the short-chain PolyPs ($\sim$ 5 residues) prevailed (Nunez and Callieri, 1989).

Table 8.2 PolyP content (mg of Pi per g of dry cell biomass) in the cells, spheroplasts and vacuoles of *S. cerevisiae*. The yeast was grown for 4 h in a medium with 9 mM P_i (+P), then for 7 h in P_i-free medium (−P) and finally for 2 h in a medium with 9 mM P_i (+P, phosphate overplus) (Trilisenko *et al.*, 2002).

Culture conditions	Cells	Spheroplasts	Vacuoles
+P	16.85	12.27	2.29
−P	2.01	1.81	0.28
+P, phosphate overplus	38.32	27.21	0.65

Table 8.3 The content of acid-soluble PolyPs in isolated mitochondria of *S. cerevisiae* under P_i-limitation and excess in the culture medium: (−P) P_i-limited medium with 1.3 mM P_i; (+P) complete medium with 18 mM P_i, stationary growth stage.

Culture conditions	PolyP (μmol (mg protein)$^{-1}$)
(−P)	0.006
(+P)	0.25
Re-inoculation of the cells from (−P) to (+P) medium, phosphate overplus for 2 h of growth	0.85

All of these experiments suggested that the PolyP content in yeast cells depended strongly on the energetic status of the cells, including the ionic gradients on the membranes.

8.10.7 The Effects of Inhibitors on the Polyphosphate Content in Yeast Cells

The PolyP content in yeast cells depends on many factors, including the P_i concentration in the culture medium, the energetic state of the cells, and the activity of P_i uptake. Thus, all compounds that affect the above functions may influence the PolyP metabolism. While glucose is the main energy source in most studies of PolyPs in yeast, desoxyglucose is an effective inhibitor of PolyP accumulation. The amount of PolyPs in *S. cerevisiae* decreased by a factor of four upon the addition of 5 mM desoxyglucose and became undetectable in the presence of 10 mM desoxyglucose in a medium with 25 mM glucose (Herve *et al.*, 1992). PolyP accumulation under phosphate overplus was inhibited by 0.2 % desoxyglucose in the presence of 2 % glucose to a half of the control level (Kulakovskaya *et al.*, 2003).

Many workers have studied the effects of uncouplers and ionophores on the PolyP content under different growth conditions. All of these experiments showed that such reagents decreased the PolyP content in yeast cells.

The syringomycin caused P_i efflux from the cells of *Rhodotorula pillimanae*, with the efflux being accompanied by a decrease in the PolyP content and acidification of the cytoplasm (Reidl *et al*., 1989). ^{31}P NMR spectroscopy performed on xylose-grown whole cells of *Candida tropicalis* showed that azide lowered the intracellular pH, inhibited the P_i uptake, and decreased the building of PolyP (Lohmeier-Vogel *et al*., 1989). A similar result was obtained with the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Lohmeier-Vogel *et al*., 1989).

It was shown that the P_i uptake and PolyP accumulation in *S. cerevisiae* is suppressed by antimycin A if ethanol is used as an energy source and is not suppressed in the presence of glucose (Schuddemat *et al*., 1989a). This fact indicates that the miotochondrial function may be important for PolyP accumulation when oxidative phosphorylation is the main energy source.

The effect of CCCP on 'NMR-detected' PolyP was investigated in *S. cerevisiae* grown on lactate (Beauvoit *et al*., 1991). The cells were incubated in a resting medium in aerobiosis with lactate or glucose or in anaerobiosis with glucose. For each case, *in vivo* ^{31}P NMR spectroscopy was used to measure the levels of phosphorylated compounds. A spontaneous PolyP breakdown occurred in anaerobiosis and in the absence of CCCP. In aerobiosis, PolyP hydrolysis was induced by the addition of either CCCP or a vacuolar membrane ATPase-specific inhibitor, bafilomycin A1 (Beauvoit *et al*., 1991).

It is important to note that selection of the concentrations of the uncouplers, which are inhibitory for PolyP accumulation but not for P_i uptake, is a difficult task. In many cases, the decrease of PolyP content in the presence of membrane-damaging agents and ionophores may be due to a break in P_i uptake and a lack of P_i for PolyP synthesis. The latter may decrease independent of the form of P_i-uptake inhibition. For example, *Candida humicola* accumulated 10-fold more PolyP during active growth in a complete glucose–mineral salt medium, pH 5.5, than at pH 7.5. This is probably due to the high P_i uptake rate from the culture medium at pH 5.5, whereas a 4.5-fold decrease in P_i uptake occurred at pH 7.5 (McGrath and Quinn, 2000).

Despite the above circumstances, we have attempted to analyse the effects of some inhibitors on PolyP accumulation in glucose-grown *S. cerevisiae* during phosphate overplus (Trilisenko *et al*., 2003). The protonophore FCCP suppressed PolyP accumulation, indicating the dependence of this process on the $\Delta\mu H^+$ on the membranes (see Figure 8.24). The PolyP(IV) fraction was shown to be the most sensitive, with the PolyP(I) fraction the next in sensitivity to FCCP (Figure 8.24). Iodoacetamide, a well-known inhibitor of glycolysis, had little effect despite the effective growth inhibition. In the presence of bafilomycin A1, the increases in the contents of PolyP(I), PolyP(IV) and PolyP(V) during phosphate overplus were the same as in the control, whereas the contents of PolyP(II) and PolyP(III) were lower than in the control. The synthesis of some parts of these fractions probably depends on the $\Delta\mu H^+$on the vacuolar membranes. In the presence of cycloheximide, an inhibitor of protein synthesis, only the accumulation of the PolyP(IV) fraction appeared to be disrupted. The insignificant effect of cycloheximide on the accumulation of other PolyP fractions indicates that the corresponding enzymatic system was already induced during phosphorus limitation. The unequal effects of the inhibitors on the accumulation of certain PolyP fractions confirms the idea that these fractions have specific pathways of biosynthesis and a specific function in yeast cells.

8.10.8 The Effects of Mutations on the Content and Chain Lengths of Polyphosphate in Yeast

Kornberg and co-workers (Kornberg, 1995, Wurst *et al*., 1995; Kornberg *et al*., 1999; Sethuraman *et al*., 2001) have made a great contribution to identification of the genes involved in PolyP metabolism in yeast.

Many genes are probably involved in the regulation of PolyP metabolism in yeast. The most studied enzymes of PolyP metabolism in yeast are exopolyphosphatases and endopolyphosphatase (see Chapter 6). The genes of one of the exopolyphosphatases, *PPX1* (Wurst *et al*., 1995), and endopolyphosphatase, *PPN1* (Sethuraman *et al*., 2001), were disrupted and their effects on the dynamics and chain lengths of PolyPs were studied. The *PPX1*-defficient mutant had more PolyP at early growth stages than the parent strain, and the double *PPX1* and *PPN1*-defficient mutant had approximately three times more PolyP than the parent strain (Figure 8.25). In addition, both mutants contained mostly medium-chain-length PolyPs when compared with the predominance of short chains in the parent strain (Figure 8.26). It was proposed that the loss of viability of the *PPN1* mutants may be a result of accumulation of a large amount of PolyP, which could affect the concentration of important divalent cations such as Ca^{2+} and Mg^{2+} in the cells (Sethuraman *et al*., 2001).

When analysing the involvement of new genes in PolyP metabolism, it should be taken into account that PolyP accumulation strongly depends on the availability of P_i for yeast cells. In *S. cerevisiae*, the PHO system includes many genes involved in the P_i uptake (Oshima, 1997) and it is not unexpected that the products of these genes may influence PolyP accumulation and utilization. DNA microarray analysis was used to identify 22 PHO-regulated genes (Ogawa *et al*., 2000a). Some of these genes, e.g. PHM1, PHM2, PHM3 and PHM4, are 32–56 % identical. The PHM3 or PHM4 single mutants and the PHM1/PHM2 double mutant are deficient in accumulation of P_i and PolyP. It is probable that the proteins encoded by these genes are involved in vacuolar transport (Cohen *et al*., 1999). The disruption of another gene, PHM5, gives a phenotype with an essentially long PolyP with no effect on its content in cells (Ogawa *et al*., 2000a). The PHM5 protein has a similarity with the yeast endopolyphosphatase, which was characterized by Kumble and Kornberg (1996).

The pleiotrophic effect of the genes involved in phosphorus metabolism is not a surprise, because this element is vital for living organisms. PolyP as a phosphate and energy reserve may be involved in different regulatory processes, and mutations in the genes related to PolyP metabolism might influence many aspects of cellular regulation. For example, disruption of the gene *YOL002c* results in accumulation of PolyP to a much higher level than in the wild-type cells. In addition, this mutant shows the induction of many genes involved in fatty acid metabolism, phosphate-signaling pathways and nystatin resistance (Karpichev *et al*., 2002).

Two ‘bursts’ in the production of acid-soluble PolyPs were shown to occur during the growth of some *S. cerevisiae* strains on a medium containing glucose and galactose under aerobic conditions (Solimene *et al*., 1980). The respiratory deficient mutant, however, had only one PolyP ‘burst’, which indicated that the accumulation of PolyP produced in the first ‘burst’ depended on the active mitochondrial function (Solimene *et al*., 1980).

Many publications have reported the absence of PolyPs in mutants with disturbed vacuolar functions (Westenberg *et al*., 1989; Beauvoit *et al*., 1991; Shirahama *et al*., 1996). It

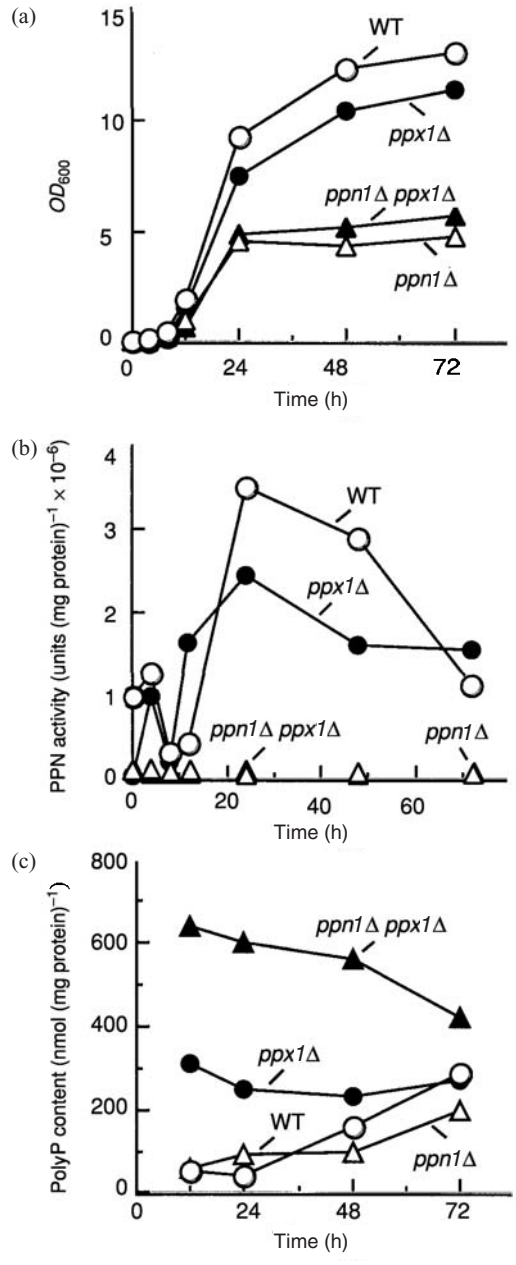

Figure 8.25 The effects of mutations in the genes *PPX1* and *PPN1* on the cell growth, endopolyphosphatase activity and PolyP content in cells of *Saccharomyces cerevisiae* (Sethuraman *et al.*, 2001): WT, parent strain; *ppx1*Δ, mutant with inactivated *PPX1* gene; *ppn1*Δ, mutant with inactivated *PPN1* gene; *ppn1*Δ *ppx1*Δ, double-mutant. (a) growth at 30 °C in a glucose–peptone–yeast-extract-containing (YPD) medium. (b) Endopolyphosphatase *PPN1* activity (10^6 units per mg of protein) in samples from (a). (c) PolyP levels in samples from (a). Frozen cells were thawed and suspended in equal volumes of extraction buffer (50 mM Tris–HCI, pH 7.4, 100 mM KCI, 1 mM EDTA). PolyP was extracted from the lysate with a buffer of phenol–chloroform, saturated with 10 mM Tris–HCI, pH 7.5, and 1 mM EDTA, followed by chloroform and ether extractions.

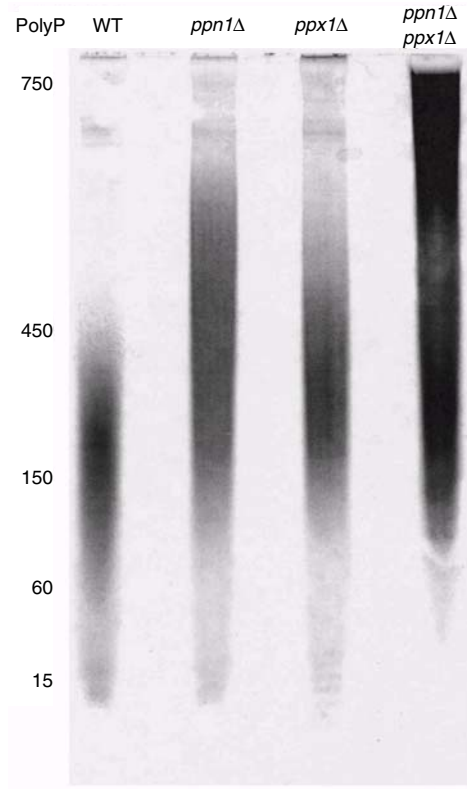

Figure 8.26 PolyP chain lengths in mutant strains determined by electrophoresis in PAGE (Sethuraman *et al*., 2001). Cells were grown in a synthetic medium containing 7.35 mM P_i; WT, parent strain; *ppx1*Δ, mutant with inactivated *PPX1* gene; *ppn1*Δ, mutant with inactivated *PPN1* gene; *ppx1*Δ *ppn1*Δ, double-mutant.

should be noted, however, that in most cases there are 'NMR-visible' PolyPs (Beauvoit *et al*., 1991; Shirahama *et al*., 1996), which represent only a part of the yeast cell PolyP. In the work of Westenberg *et al*. (1989), the yeast *S. cerevisiae* was grown on a specific culture medium with arginine as a nitrogen source. As was shown earlier, under these growth conditions the greater part of the cellular PolyP is localized in vacuoles (Matile, 1978).

There is an example of another effect of mutation of the vacuolar PolyP. *S. cerevisiae*, with a defect of the *SPT7* gene, became less sensitive to nickel and had a highly elevated amount of PolyPs in the vacuoles (Nishimura *et al*., 1999).

Therefore, the effects of mutations in the vacuolar functions on PolyP metabolism in the whole cell need further investigation.

The genes of the PHO system, including acid and alkali phosphatases, are regulated by the P_i content in the medium. Under P_i starvation, the activity of the acid phosphatase increases $\sim$ 500-fold and that of the alkali phosphatase $\sim$ 60-fold (Yoshida *et al*., 1987). It was assumed that low-molecular-weight PolyP might also participate in the regulation of expression of the genes encoding multiple yeast phosphatases (Bostian *et al*., 1983). A possible interrelation between the vacuolar PolyP, exopolyphosphatase and the PHO system

was proposed (Vagabov, 1988). However, the ways of involving phosphatases in PolyP metabolism in yeast are still obscure. For example, the strains of *Schizosaccharomyces pombe* with mutations in the structural genes for three different phosphatases, i.e. PHO1, PHO2 or PHO3, degraded PolyPs at the same rate as the wild-type strain during phosphate starvation and showed the same type of over-compensation when phosphate was added again (Müller *et al*., 1992).

To summarize, it must be said that mutational analysis and modern genetic methods have proved to be a great success in studies of PolyP metabolism in yeast and will provide new knowledge in this field in future. Data on the role of P_i transport systems in PolyP metabolism of yeast have been summarized in a recent review (Persson *et al*., 2003).

In conclusion, it should be noted that PolyP accumulation and utilization in yeast depends strongly on the culture conditions and cell development stage. A great difference has been observed in the dynamics of separate PolyP fractions and in the effects of some culture conditions on the PolyP content in some cellular organelles. Each compartment of the yeast cell possesses its own exopolyphosphatases (see Chapter 6) and probably its own endopolyphosphatases and other PolyP-metabolizing enzymes. One of the intriguing questions in the study of PolyP metabolism in yeast is the pathways of its biosynthesis. Despite many reports that have shown polyphosphate kinase activity in these organisms (Felter and Stahl, 1973; Shabalin *et al*., 1979; Kornberg *et al*., 1999; McGrath and Quinn, 2000), this enzyme was not purified and no gene ecoding it was found in yeast genomes (Kornberg, 1999; Zhang *et al*., 2002). The role of this activity in PolyP accumulation in yeast is still obscure. The activity of other PolyP-synthesizing enzymes, such as 1,3-diphosphoglucerate kinase and dolychil polyphosphate kinase, is not so significant for providing the synthesis of all PolyPs in yeast cells. An assumption was made that exopolyphosphatases or endopolyphosphatase may synthesize PolyP in a similar way to ATPases or pyrophosphatases by a reverse reaction (Kulaev and Vagabov, 1983; Vagabov *et al*., 2000; Kulaev *et al*., 1999). However, no evidence for the actual existence of such a process has been obtained. There is no doubt, however, that PolyP synthesis in yeast is dependent on the energetic status of the cell, in particular, on the ionic gradients on the membranes.

8.11 Other Fungi (Mould and Mushrooms)

The metabolism of PolyP has been investigated during onthogenetic development in many fungi (Bajaj *et al*., 1954; Belozersky and Kulaev, 1957; Nishi, 1961; Harold, 1962a,b; Kulaev *et al*., 1960a-c; 1961; 1966a, 1968; 1970a–d); Kritsky and Kulaev, 1963; James and Casida, 1964; Kulaev and Uryson, 1965; Kritsky *et al*., 1965a,b, 1968, l972; Mel'gunov and Kulaev, 1971; Kulaev, 1973; Okorokov *et al*., 1973a,b; Trilisenko *et al*., 1980, 1982a,b). It was revealed that the rapid synthesis of acid-insoluble PolyPs took place during the germination of fungal spores. Using ^{32}P it was shown that the formation of highly polymerized PolyP at this development stage involves utilization of less polymeric acid-soluble PolyP, resulting in a complete conversion of the latter into an acid-insoluble form (Kulaev and Belozersky, 1962a,b). During this period of development, the endopolyphosphatase was found to be inactive in mould fungi (Kritsky *et al*., 1972). This seems to facilitate to a great extent the accumulation of large amounts of PolyPs. On the other hand, at this time PolyPs began to be utilized for the synthesis of a variety of compounds. Nishi (1961) showed that PolyP utilization for the synthesis of nucleotides, sugar phosphates and RNA had already started

during the first few hours of spore germination in *Aspergillus niger*. The utilization of PolyP during the active RNA synthesis was also demonstrated in other fungi (Kulaev *et al*., 1960a-c; Harold, 1962b, 1966; Kritsky *et al*., 1965a,b, 1968; Kulaev and Vagabov, 1983).

A ^{31}P NMR spectroscopic analysis of the PolyP pool in cellular and nuclear extracts of *Physarum polycephalum* (Pilatus *et al*., 1989) demonstrated that plasmodia and cycts contained PolyP with an average chain length of about 100 residues. During sporulation, this PolyP degrades to a lower one with a chain length of $\sim$ 10 residues. PolyP was degraded at a sufficient amount of P_i, and it was concluded that the PolyP serves to supply energy for biosynthetic processes during sporulation.

Some authors have suggested that PolyP utilization during spore germination provided the required osmotic pressure for the 'explosion' of cysts and penetration of germ cells of pathogenic fungi into the cells of host organisms (Kulaev and Vagabov, 1983). It was proposed that such osmotic pressure developed during PolyP hydrolysis in the lamellae of the fruiting bodies of *Agaricus bisporius* involved in spore dissemination (Kulaev *et al*., 1960a,b; Kritsky *et al*., 1965a,b). Gezelius et al. (1973) showed that large amounts of PolyP were synthesized during the transition of *Dictyostelium discoideum* from the amoeboid to the aggregated stage.

All these data suggested that PolyPs are very important for the development of fungi, especially spore formation and germination. Tables 8.4–8.6 and Figures 8.27 and 8.28 show the changes in PolyP content at different stages of development in some fungi.

Under vegetative growth, fungal cells, like yeast cells, possess PolyPs of different chain lengths, belonging to acid-soluble, salt-soluble, alkali-soluble and acid-insoluble fractions and which are localized in different cell compartments. PolyPs were found in the vacuoles, cell envelope and nuclei of fungi (see Chapter 5). The dynamics of the PolyP content in three different strains of *N. crassa* are illustrated in Figure 8.28. It can be seen that different fractions of PolyP have individual changes during the culture growth. The slime variant without the cell wall is characterized by the lower content of the most high-molecular-weight fractions, while the mutant with the lower exopolyphosphatase activity is characterized by the higher content of PolyP (Trilisenko *et al*., 1980, 1982a,b).

Table 8.4 PolyP content in the cells of *Endomyces magnusii* (Kulaev *et al*., 1967a), *Neurospora crassa* (Kulaev *et al*., 1966a) and in the fruiting bodies of *Giramitra esculenta* (Kulaev *et al*., 1960b), expressed as mg of P per g of dry biomass.

PolyP fraction	Extractant	*E. magnusii* cells, 12 h growth	*N. crassa* mycelia, 17 h growth	*G. esculenta* fruiting bodies
PolyP(I)	0.5 M $HClO_4$, 0–4 °C	1.10	0.62	0.00
PolyP(II)	Saturated $NaClO_4$ solution, 0–4 °C	0.90	1.24	1.52
PolyP(III)	NaOH, pH 9, 0–4 °C	0.20	0.12	0.24
PolyP(IV)	NaOH, pH 12, 0–4 °C	0.90	0.82	0.01
PolyP(V)	10 % $HClO_4$, 100 °C	0.40	0.00	—
Total PolyP	—	3.50	2.80	1.77
Total P	—	17.3	15.6	6.03

Table 8.5 PolyP content in resting spores and mycelia of a 24 h culture of *Penicillium chrysogenum* under different conditions of growth (Kulaev *et al*., 1959), expressed as mg of P per g of dry biomass.

PolyP fraction	Extractant	Spores	Mycelia on synthetic medium	Mycelia on medium containing maize extract
PolyP(I)	1 % TCA, 0–4 °C	4.90	0.09	1.07
PolyP(II)	Saturated $NaClO_4$ solution, 0–4 °C	2.18	1.75	1.01
PolyP(III) + PolyP(IV) + PolyP(V)	10 % $HClO_4$, 100 °C	0.44	8.18	1.96
Total PolyP	—	7.52	10.02	4.04
Total P	—	16.15	28.28	44.27

High-resolution ^{31}P NMR spectroscopy was employed to investigate the effects of growth stage and environmental osmolarity on the changes in PolyP metabolism in intact *Neurospora crassa* cells (Yang *et al*., 1993). The ratio of PolyP to P_i in the vacuoles increased from 2.4 to 13.5 in *N. crassa* as cells grew from the early logarithmic phase to the stationary phase. Hypo-osmotic shock of *N. crassa* initiated growth-dependent changes, including (i) rapid hydrolysis of PolyP with a concomitant increase in the concentration of the cytoplasmic phosphate, (ii) an increase in cytoplasmic pH, and (iii) an increase in vacuolar pH. The early logarithmic-phase cells produced the most dramatic response, whereas the stationary-phase cells appeared to be recalcitrant to the osmotic stress. Thus, 95 and 60 % of the PolyP in the early- and mid-logarithmic-phase cells, respectively, disappeared in response to hypoosmotic shock, but little or no hydrolysis of PolyP occurred in the stationary cells. The osmotic stress-induced PolyP hydrolysis and pH changes in the early- and mid-logarithmic-phase cells were reversible, thus suggesting that these changes to relate to environmental osmolarity (Yang *et al*., 1993).

One of the interesting features of PolyP metabolism in fungi is the interrelation between the metabolism and antibiotic biosynthesis (Kulaev, 1986). It was demonstrated that in high-productive strains, under intensive synthesis of antibiotics, the PolyP content was lower than in low-productive strains at the same growth stage (Figure. 8.29). This fact indicated that PolyP is probably utilized as an energy source in the processes of antibiotic biosynthesis, or there is a competitive relationship between the biosynthetic pathways of antibiotics and PolyP for the energy sources (Kulaev, 1986).

8.12 Algae

8.12.1 Localization and Forms in Cells

Being eukaryotes, algae contain PolyPs in different cell compartments. The intracellular localization of PolyPs in volutine granules of *Chlorella fusca* and *Chlorella pyrenoidosa*

Table 8.6 PolyP content of spores and fruiting bodies of the mushroom *Agaricus bisporus* at various stages of development (Kulaev *et al*., 1960b), expressed as mg of P per g of dry biomass.

		Fruiting bodies			
PolyP fraction	Extractant	1.5 days growth (undifferentiated)	3–4 days growth (fully differentiated)	8 days growth (vigorously sporutating)	Spores leaving the fruiting bodies
PolyP(I)	1 % TCA, 0–4 °C	1.69	1.15	2.38	0.24
PolyP(II)	Saturated $NaClO_4$ solution, 0–4 °C	1.76	1.10	2.56	0.97
PolyP(III)	NaOH, pH 9, 0–4 °C	0.31	0.27	0.61	0.44
PolyP(IV)	NaOH, pH 12, 0–4 °C	0.11	0.10	0.09	0.09
PolyP(V)	10 % $HClO_4$, 100 °C	0.18	0.02	0.03	0.24
Total PolyP	—	4.05	2.64	5.67	1.98
Total P	—	11.44	8.80	11.28	5.03

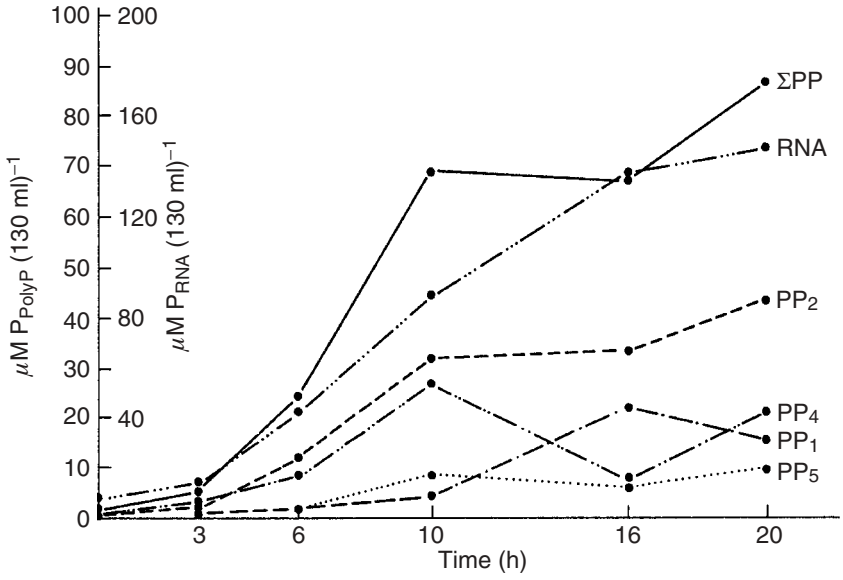

Figure 8.27 Changes in the content of PolyP fractions and RNA during the growth of a culture of *Neurospora crassa* (Chernysheva *et al*., 1971): PP_1, PP_2, PP_4 and PP_5 represent the PolyP(I), PolyP(II), PolyP(IV) and PolyP(V) fractions, respectively.

was demonstrated by electron microscopic methods (Atkinson *et al*., 1974; Peverly *et al*., 1978). By X-ray microanalysis, phosphorus-containing granules were observed in the cytoplasm, vacuoles and chloroplasts of *Scenedesmus quadricauda* (Voříšek and Zachleder, 1984). In *Cosmarium*, PolyP was revealed in cytoplasmic granules (Elgavish and Elgavish, 1980). PolyP was identified in the vacuoles of *Ulva latuca*, and this compartment was also rich in Mg^{2+} (Lundberg *et al*., 1989). The chain length of the PolyP in this organism was determined to be $\sim$ 20 residues (Weich *et al*., 1989).

A ^{31}P NMR spectroscopic study of the living cells of *Chlorella fusca* allowed conclusions concerning the localization and structural features of its particular PolyP. The signal of the core PolyP groups of this organism had a broad width. This indicated that the PolyP may be present in cell compartments under different chemical conditions, may have different chain lengths cation complexations, and may also be subjected to rapid exchange processes (Sianoudis *et al*., 1986). A high concentration of EDTA or adjustment of the pH to 12.9 led to a partial shift of the core PolyP signal. Thus, at least some part of this signal originated from PolyP located outside the cytoplasmic membrane, because this form was easily accessible to environment changes (Sianoudis *et al*., 1986). These some workers also showed the importance of divalent cations for structural organization of the PolyP in *Chlorella fusca* but did not exclude the presence of monovalent complexed PolyP (Sianoudis *et al*., 1986). Peverly *et al*. (1978) concluded that divalent cations played only a minor role in the synthesis of vacuolar PolyP granules in *Chlorella pyrenoidosa*, while K was an essential component.

The alga *Chlamydomonas reinhardtii* contains cytoplasmic vacuoles that are often filled with dense granules. Purified granules contained PolyP complexed with calcium and

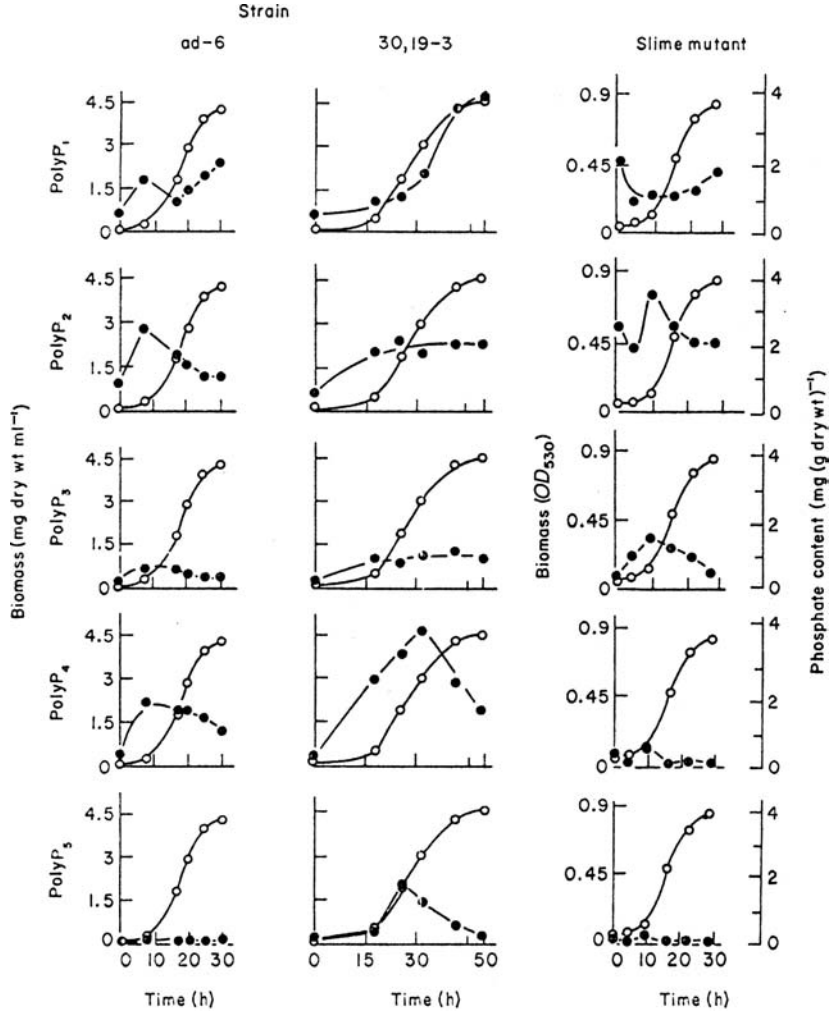

Figure 8.28 Changes in the content of PolyP fractions during growth of *Neurospora crassa* strains ad-6 (parent strain) and 30,19-3 (a leaky mutant in exopolyphosphatase), and a slime mutant devoid of the cell envelope (Trilisenko *et al*., 1980; 1982a,b): (○) growth; (●) PolyP content in different fractions.

magnesium as the predominant inorganic components (Komine *et al*., 2000). These organelles were similar to acidocalcisomes of other microoganisms (Ruiz *et al*., 2001b). X-ray microanalysis of the electron-dense vacuoles or PolyP bodies of *C. reinhardtii* showed large amounts of phosphorus, magnesium, calcium and zinc. Immunofluorescence microscopy revealed a vacuolar-type proton pyrophosphatase (H^+–PPase) in this compartment. Purification of the electron-dense vacuoles using iodixanol density gradients showed preferential localization of H^+–PPase and V–H^+–ATPase activities, in addition to high concentrations of PP_i and short- and long-chain PolyPs (Ruiz *et al*., 2001b).

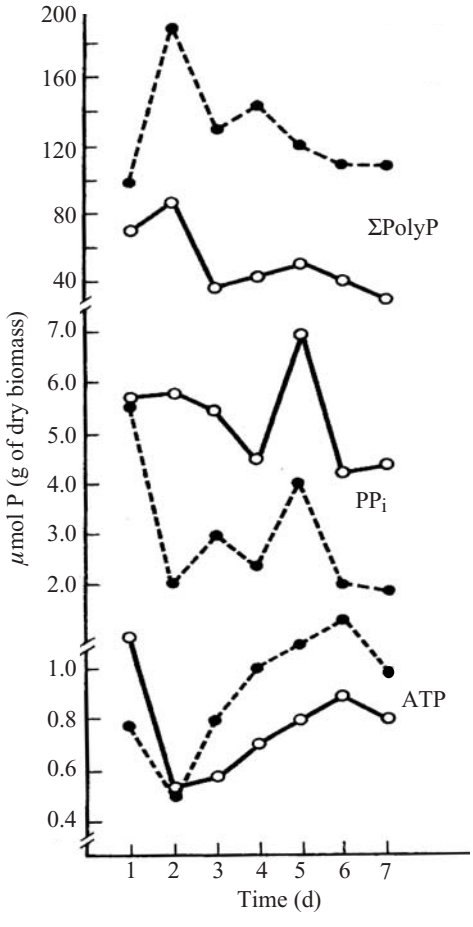

Figure 8.29 Changes in total PolyP, pyrophosphate and ATP content in mycelium of *Fusidium coccineum* during growth of strains with high antibiotic (fusidium acid) productivity (–○–) and with low antibiotic productivity (- -●- -) (Kulaev, 1986).

8.12.2 The Dynamics of Polyphosphates in the Course of Growth

As in other organisms, the PolyP content in algal cells depends a great deal on the growth stage, being the lowest during the exponential phase and the highest in older cultures (Smillie and Krotkov, 1960; Vagabov and Serenkov, 1963). However, the dymanics of PolyP fractions may be diverse during growth and development. For example, Table 8.7 shows the changes in the content of P_i and different PolyP fractions in the gigantic unicellular alga *Acetabularia crenulata* (Kulaev *et al.*, 1975). At early stages of its growth, only acid- and salt-soluble PolyPs were present. At the stages of cyst formation, characterized by intensive synthesis of cell wall components, alkali-soluble and hot-perchloric-acid-extractible PolyPs appeared.

Table 8.7 The contents of PolyPs and P_i in *Acetabularia crenulata* at different stages of development (Kulaev *et al*., 1975). The stages of growth were as follows: (1) young cells, 1.5–2 cm long; (2) cells 2.5–3 cm long, up to 2 mm in diameter; (3) cells with umbellulles filled with secondary nuclei; (4) cells with mature umbellulles filled with cysts.

	Phosphate content (μg P (cell^{-1}))			
	Stages of growth			
Fraction	1	2	3	4
P_i	0.52	1.40	0.76	1.88
PolyP(I) (acid-soluble)	0.67	3.30	10.10	2.41
PolyP(II) (salt-soluble)	0.12	0.46	0.44	1.27
PolyP(III) (alkali soluble)	0.0	0.0	0.25	0.54
PolyP(V) (hot perchloric acid extract)	0.0	0.0	0.0	0.81
Total PolyP	0.79	3.76	10.79	5.03

Algae were shown to have a correlation between PolyP and nucleic acid biosynthesis during growth, similar to that revealed in fungi. Close links between the PolyP and RNA contents during onthogenetic development were demonstrated in *Euglena* (Smillie and Krotkov, 1960) and *Chlorella pyrenoidosa* (Hermann and Schmidt, 1965). During the growth and development of synchronous cultures of *Chlorella pyrenoidosa,* the accumulation of PolyP, RNA and DNA took place in parallel, although PolyP biosynthesis during the first few hours of growth outpaced to some extent the synthesis of nucleic acids (Hermann and Schmidt, 1965). The work of Miyachi and co-workers (Miyachi, 1961; Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi *et al*., 1964) showed that only some of the PolyP fractions with specific cellular localization were involved in RNA and DNA synthesis. These investigations, together with those of Okuntsov and Grebennikov (1977), showed that the metabolism of the various PolyP fractions in *Chlorella* proceeded differently in the dark and in the light, as well as in the presence and absence of P_i in the medium.

8.12.3 The Influence of Light and Darkness

The fact that algae are authotrophs has a profound effect on their PolyP metabolism. The early work of Wintermans (1954, 1955) and subsequently of other researchers (Stich, 1953, 1955, 1956; Nihei, 1955, 1957; Vagabov and Serenkov, 1963; Baslavskaya and Bystrova, 1964; Kulaev and Vagabov, 1967; Kanai and Simonis, 1968; Lysek and Simonis, 1968; Sundberg and Nilshammer-Holmvall, 1975; Ullrich and Simonis, 1969) showed that the formation of PolyP and PolyP-containing granules in algae proceeded much more rapidly in the light than in the dark. It was shown that PolyP synthesis in *Ankistodesmus braunii* (Ullrich and Simonis, 1969) was strongly stimulated as the oxygen concentration in the medium increased. These observations lead to the conclusion that there is a close connection between the formation of PolyP in algae and photosynthesis. However, it is not possible

yet to come to a firm conclusion as to whether the accumulation of PolyP in algae in the light is directly linked to photosynthesis itself, or if their formation is merely promoted by increased ATP (and perhaps pyrophosphate) during photosynthetic phosphorylation.

Kanai and Simonis (1968) showed that, although ^{32}P incorporation in PolyP proceeded more rapidly in the light and decreased in darkness, PolyP synthesis did continue to some extent. It was concluded that PolyP synthesis in algae occured without the involvement of photosynthesis, although it was strongly promoted by the latter process. Similar results were obtained by Domanski-Kaden and Simonis (1972) on *Ankistrodesmus braunii*, and by Overbeck (1961, 1962) on *Scenedesmus quadricauda*. The fact that photosynthesis is not obligatory for PolyP accumulation was demonstrated in experiments with *Euglena* (Smillie and Krotkov, 1960). Substantial amounts of PolyP were found in this organism under heterotrophic growth (Smillie and Krotkov, 1960). Furthermore, it was shown that in *Scenedesmus obliquus* PolyP was produced by glycolytic phosphorylation when this alga was grown in the dark (Kulaev and Vagabov, 1967).

It can therefore be concluded that some part, if not all, of the PolyP formed in the algal cells is produced independent of photosynthesis and photosynthetic phosphorylation. A further contribution to the understanding of this problem was made by the investigations of Miyachi and co-workers (Miyachi, 1961; Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi *et al*., 1964) which has shown that only one of the four PolyP fractions of *Chlorella* was formed in the light. This was fraction C, which was precipitated by neutralization of a 2N KOH extract with $HClO_4$ in the presence of $KClO_4$. This fraction was localized, in the opinion of the authors, either in chloroplasts or in their vicinity (Miyachi *et al*., 1964). Fraction A (extractable by 8 % trichloracetic acid) was found in volutine, and its accumulation depended on photosynthesis only to a certain extent, probably because this fraction was derived from fraction C through degradation. The biosynthesis and degradation of the alkali-soluble fractions B and D (see Chapter 2) were shown to be absolutely unrelated to photosynthesis. Their metabolism depended on the presence of P_i in the medium. Similar results were obtained with *Ankistrodesmus braunii* (Kanai and Simonis, 1968).

Miyach and co-workers (Miyachi, 1962; Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi *et al*., 1964) have shown that utilization of different PolyP fractions for nucleic acid biosynthesis in *Chlorella* is different in the light and in the dark. In the opinion of these authors, PolyPs of different fractions are involved in the biosynthesis of nucleic acids and other compounds in different ways. In the light, fraction C is a phosphorus donor for the biosynthesis of chloroplast DNA, while fraction A is involved in the synthesis of nuclear DNA. RNA is not formed in this alga from PolyP under conditions of P_i sufficiency, although PolyPs of fractions B and D are utilized for RNA biosynthesis when P_i is absent in the medium. In the light, the PolyPs of these fractions are hydrolysed to P_i, which is then utilized for the biosynthesis of RNA and other compounds. In the dark and in the absence of P_i, PolyP seems to be able to provide phosphate for RNA synthesis.

Some authors doubt the possibility of a direct interrelation between PolyP and photosynthesis in algae (Rubtsov *et al*., 1977; Rubtsov and Kulaev, 1977). The following facts support this point of view. No high-molecular-weight PolyP was found in the chloroplasts of *Acetabularia mediterranea* (Rubtsov *et al*., 1977). The inhibitor analysis and detection of polyphosphate kinase activity in this alga (Rubtsov and Kulaev, 1977) point to the fact that PolyP is not directly, but rather indirectly, connected with the photosynthesis through the formation of ATP, which provides energy for P_i transport and PolyP synthesis.

8.12.4 The Effects of P_i Limitation and Excess

Many algae were shown to accumulate and store large amounts of PolyPs when grown under conditions of unlimited available P_i, namely *Scenedesmus* (Rhee, 1973), *Cosmarium* (Elgavish *et al*., 1980), *Chlorella* (Miyachi and Tamiya, 1961) and *Heterosigma* (Watanabe *et al*., 1987; 1988; 1989). Overbeck (1961, 1962) showed that *Scenedesmus quadricauda* was able to accumulate excessive amounts of PolyPs in the dark when grown on a phoshate-containing medium after P_i starvation. In other words, he demonstrated that algae, like heterotrophs, display a hypercompensation (phosphate overplus) effect.

It is probable that PolyP in algae can play the role of phosphorus reserve, as in other organisms. It was observed that the granules disappeared from the vacuoles of *Scenedesmus quadricauda* under phosphate starvation (Voříšek and Zachleder, 1984). If *Chlorella* was grown in the light and on a P_i-containing medium, it did not utilize PolyP for nucleic acid synthesis, although such utilization occurred under P_i starvation (Baker and Schmidt, 1964a,b).

The results of Lundberg *et al*. (1989) concerning P_i uptake and storage demonstrated that PolyP formed the main P_i store in the marine macroalga *Ulva lactuca*. The short-chain 'NMR-visible' PolyP in this alga was synthesized when the organism was grown in seawater supplemented by P_i, and utilized to support the growth when the organism was transferred to a P_i-deficient medium. However, this organism might possess another P_i storage pool, namely an amorphous calcium phosphate in the cell wall (Weich *et al*., 1989).

The effect of starvation and the subsequent addition of phosphate-containing medium on phosphorus-containing compounds was studied by ^{31}P NMR spectroscopy of perchloric acid extracts and intact cells of *Heterosigma akashiro* (Watanabe *et al*., 1987, 1888, 1989). The PolyP content and chain length decreased under starvation and rapidly increased on P_i restoration in the medium (Table 8.8).

This phenomenon, known as 'luxury storage', is important for development of the algae population under P_i-starved conditions, while the P_i concentrations in the coastal zones are influenced by such factors as land run-off and wastewaters. When the phosphate concentration in the water becomes low, the PolyP store may be used for further synthesis. The alga *Heterosigma akashiro* has a specific feature in its phosphate metabolism during its vertical migrations in natural sea water (Watanabe *et al*., 1987) and under simulation of such migrations in a (laboratory) tank (Watanabe *et al*., 1988). At night, this alga migrated to the lower phosphate-rich water layer and took up P_i, which is used for elongation of the PolyP chains. In the daytime, the alga migrated to the P_i-depleted surface water and

Table 8.8 Some phosphate compounds in extracts from the algae *Heterosigma akashiro* under P_i starvation and restoration (Watanabe *et al*., 1987).

Conditions/time	Sugar P (%)	P_i (%)	ATP (%)	PolyP (%)	PolyP (fmol cell^{-1})	PolyP average chain length
Starvation	33.5	21.5	5.6	5.8	76	10
2 h after P_i addition	12.1	10.9	7.4	43.7	108	14
1 d after P_i addition	9.0	9.5	—	59.1	222	20
3 d after P_i addition	9.2	8.4	—	63.3	185	17

utilized the accumulated PolyPs for photophosphorylation by shortening the PolyP chains (Watanabe *et al*., 1988). During P_i uptake, *Heterosigma akashiro* required Mn^{2+}, which was excreted from cells after the PolyP pool had been saturated (Watanabe *et al*., 1989).

8.12.5 Changes in Polyphosphate Content under Stress Conditions

The ammonium-induced cytoplasmic alkalization in the unicellular algae *Dunaliella salina* resulted in degradation of long-chain PolyPs to $PolyP_3$ (Pick *et al*., 1990; Bental *et al*., 1990; Pick and Wess, 1991). The hydrolysis was shown to correlate with the recovery of cytoplasmic pH and might provide the 'pH-stat' mechanism to counterbalance the alkaline stress.

A decrease of the PolyP level in *Ulva lactuca* was observed at high external nitrate concentrations during cultivation in continuous light (Lundberg *et al*., 1989). These authors assumed that either nitrate might inhibit the P_i uptake or, in the case where nitrate was the only nitrogen source, the energy of the PolyP could be used for nitrate uptake and reduction (Lundberg *et al*., 1989).

The alga *Phaerodactilum tricornutum* was found to respond to hyperosmotic stress by a marked elongation of PolyP and a decrease in its total amount, while exposure to hypoosmotic stress resulted in a higher content of shorter PolyPs and an increased total PolyPs content (Leitao *et al*., 1995). It is probable that, such variations might allow the adjustment of the intracellular osmotic pressure to an extracellular one.

In conclusion, it should be said that the PolyP-metabolizing enzymes in algae have been little studied. An activity, which transferred P_i from PolyP to ADP, was observed in cell-free extracts from *Chlorella* (Iwamura and Kuwashima, 1964) and *Acetabularia* (Rubtsov and Kulaev, 1977). Exopolyphosphatase activity was found in *Acetabularia* (Rubtsov and Kulaev, 1977), while polyphosphate glucokinase activity was not found in algae (Uryson and Kulaev, 1970).

It is clear, however, that the intracellular concentration of ATP and P_i, as well as the P_i level in the culture medium, exert a regulatory effect on the biosynthesis and degradation of PolyPs in algae such as heterotrophs (Curnutt and Schmidt, 1964a,b; Miyachi, 1961; Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi *et al*., 1964; Rubtsov and Kulaev, 1977; Kuhl, 1960, 1962, 1976). There is little doubt that PolyP metabolism in algae is strongly influenced by light (Miyachi and Miyachi, 1961; Miyachi *et al*., 1964) and oxygen concentration in the medium (Ullrich, 1970).

8.13 Protozoa

PolyP in protozoa was found long ago (Ebel *et al*., 1958b; Mattenheimer, 1958; Janakidevi *et al*., 1965; Rosenberg, 1966). Its metabolism was studied with the purpose of searching for specific biochemical peculiarities of parasitic representatives of this taxon, which could offer prospects for drug development.

The intracellular levels of short- and long-chain PolyPs were measured by means of polyphosphate glucokinase assay in *Leishmania major* promastigotes incubated in a

phosphate-free medium (Blum, 1989). The short-chain PolyP content did not differ between the cells incubated for 1 h in the absence of exogenous substrate or in the presence of glucose or glycerol. The long-chain PolyP content, however, was lower in cells incubated without glucose than in cells incubated with glucose and was also lower in cells incubated for 1 h with glycerol, as compared with freshly washed cells. The P_i and PP_i increase (up to 61 %) which occurred in promastigotes incubated in the absence of an exogenous substrate could have arisen from the concomitant decrease in the long-chain PolyP content (Blum, 1989).

Leishmania major promastigotes contain electron-dense vacuoles (LeFurgey *et al*., 1990). The elemental compositions of these vacuoles and the cytoplasm were determined by electron probe X-ray microanalysis. The electron-dense vacuoles are rich in P, presumably present as PolyP, while Mg^{2+} was about nine times higher than its cytoplasmic level and its content was enough to neutralize most of the negative charge of PolyP. The electron-dense vacuoles also contain appreciable amounts of Ca^{2+} and Zn^{2+}, which are not detectable in the cytoplasm, as well as Na^+, K^+ and Cl^- (the latter two in concentrations below that in the cytoplasm). These results suggest that the vacuolar membranes have at least one cation transport system. Incubation of the promastigotes for 1 h in the absence of phosphate, independent of the presence or absence of glucose, did not cause any significant changes in the vacuolar contents of phosphorus, magnesium or zinc (LeFurgey *et al*., 1990).

The interaction of *Entamoeba histolytica* with collagen induces the intracellular formation and the release of electron-dense granules containing collagenase activity, which is important for pathogenicity of this parasite. Purified granules are a complex of mainly cationic proteins, which contains numerous proteolytic activities, actin, and small molecules such as P_i, PP_i and cations (Leon *et al*., 1997). It is not improbable that such granules also contain some quantities of PolyP.

An unusual characteristic of some protozoa is the presence in their cells of a specific organelle, named as acidocalcisome (Docampo and Moreno, 2001). Acidocalcisome is an electron-dense acidic organelle, which contains a matrix of pyrophosphate and PolyP with bound calcium and other cations. Its membrane possesses a number of pumps and exchangers for the uptake and release of these components. Acidocalcisome is possibly involved in PolyP and cation storage and in the adaptation of these microoganisms to environmental stress (Docampo and Moreno, 2001). A ^{31}P NMR spectroscopic study revealed the high levels of PP_i, $PolyP_3$ and $PolyP_5$ in perchloric-acid extracts of *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major* (Moreno *et al*., 2000). The ^{31}P NMR spectra of purified acidocalisomes of these organisms indicated the presence of PolyP with an average chain length of 3–4 (Moreno *et al*., 2000).

In *Trypanosoma cruzi*, the PolyP was represented by a millimolar level of PolyP with a chain length of $\sim$ 50 residues and a micromolar level of longer PolyPs with a chain length of about 700–800 residues (Ruiz *et al*., 2001a). The PolyP was mostly localized in acidocalcisomes. The level of PolyP was measured at different development stages of *Trypanosoma cruzi* (Ruiz *et al*., 2001a). A rapid increase in the PolyP level was observed at differentiation of trypomastigote to amastigote and in the lag phase of epimastigotes growth. Both the short- and long-chain PolyPs content increased during these development stages. The PolyP content rapidly decreased after the epimastigotes-resumed growth.

Similar to *Trypanosoma cruzi*, the acidocalcisomes of *Toxoplasma gondii* contained PolyPs with an average chain length $\sim$ 50 residues at the millimolar level and a micromolar

level of longer PolyPs with a chain length of about 700–800 residues (Rodrigues *et al*., 2002b). The level of PolyP in both organisms rapidly decreased upon the exposure of the parasites to a calcium ionphore (ionomycin), to an inhibitor of vacuolar V-ATPase (bafilomycin A1), or to the alkalization of the medium by NH_4Cl (Rodrigues *et al*., 2002b). Thus, the PolyP level in acidocalcisomes may depend on $\Delta\mu H+$ on the membrane of these organelles.

High levels of short-chain ($\sim$ 20 mM) and long-chain ($\sim$ 60 mM) PolyPs were detected in *Leishmania major* promastigotes. An exopolyphosphatase has been purified and cloned from this organism, which resembles the PPX1 enzyme from *S. cerevisiae* in its properties and amino acids sequence (Rodrigues *et al*., 2002a). It was proposed that the characteristics of exopolyphosphatase and PolyP metabolism revealed in pathogenic protozoa might facilitate the development of novel antiparasitic agents (Rodrigues *et al*., 2002a).

8.14 Higher Plants

Despite the fact that PolyPs have been found in a wide range of tissues of the higher plants (see Chapter 3), very little is known about PolyP metabolism in these organisms. For example, Khomlyak and Grodzinskii (1970, 1972) have shown that $^{32}P_i$ introduced into tomato leaves via the steam conductive system is first incorporated into the acid-soluble PolyP fraction and subsequently into the acid-insoluble one. PolyP-metabolizing enzymes have been observed in many higher plants (Pierpoint, 1957a,b; Rotenbach and Hinkelmann, 1954; Jungnickel, 1973) Two different exopolyphosphatases were observed in plants (Jungnickel, 1973). One of these was a constitutive enzyme, while the other appeared when the plant was grown under conditions of phosphorus deprivation, i.e. it was inducible. Some data on the relation of PolyP and nucleic acid metabolisms in plants were obtained by Schmidt and co-workers (Schmidt and Buban, 1971; Schmidt, 1971, 1972).

It should be noted that the role of phosphate reserve in plants belongs not to PolyP but to phytin (Ca–Mg salt of inositol phosphate), which is formed in large amounts during the ripening of seeds, in parallel with the accumulation of reserve substances such as starch and fats (Sobolev, 1962). Such accumulation and the presence of chlorophyll cause additional difficulties for the identification and study of PolyPs in plants. However, PolyP is also present in plants in the sites where large amounts of phytin are accumulated (Asamov and Valikhanov, 1972; Valikhanov *et al*., 1980). The amounts of PolyPs normally present in the tissues of higher plants are very small and may be observed at certain stages of development. For example, fairly large amounts of PolyPs accumulate at an early stage of the ripening of cotton seeds (Assamov and Valikhanov, 1972; Valikhanov *et al*., 1980). At this stage, PolyPs represented more than 10 % of the total phosphorus of the seeds, exceeding the phytin phosphorus by more than twofold (Assamov and Valikhanov, 1972).

8.15 Animals

Although the first evidence for the presence of PolyPs in mammalian cells was obtained long ago (Gabel and Tomas, 1971), the metabolism of this biopolymer in the higher eukaryotes is

Table 8.9 PolyPs in some animal tissues.

Tissue	PolyP (mg P (g wet biomass)$^{-1}$)	Reference
Rat liver, nuclei	100–200	Mansurova *et al*., 1975a
Rat liver	1–2	Mansurova *et al*., 1975a
Rat liver	3–5	Gabel and Thomas, 1971
Rat brain	12.8–15.0	Gabel and Thomas, 1971
Rat brain	10–15	Kulaev and Rozhanets, 1973
Bovine brain	2.95	Gabel and Thomas, 1971
Bovine liver	2.24	Gabel and Thomas, 1971
Bovine pancreas	2.10	Gabel and Thomas, 1971
Bovine kidney	1.18	Gabel and Thomas, 1971
Bovine spleen	1.35	Gabel and Thomas, 1971
Rabbit erythrocytes	0.74	Gabel and Thomas, 1971

Table 8.10 PolyP contents of certain insects (μM P (g body weight)$^{-1}$).

Phosphorus compounds	Mulberry moth larva	Mosquito larva	Image of granary weevil	Tropical cockroach intact insects	Tropical cockroach insects without intestines
P_i	13.30	9.05	13.40	8.40	3.26
Acid-soluble PolyP	0.04	0.09	0.06	0.45	0.51
Acid-insoluble PolyP	0.17	0.01	0.005	0.03	0.03
ATP	2.05	2.00	2.46	—	—

still little studied. One of the reasons for this is the very small amounts of PolyPs in animal cells. The concentrations of PolyPs range from 10 to 100 μM (expressed as P_i), while the chain lengths may be 100 to 1000 residues (Kornberg, 1999). Table 8.9 shows some earlier data reported on the content of PolyPs in some animal tissues.

PolyPs have been found in insects (Kulaev *et al*., 1974), and not only in intact insects but also in those with removed intestines (Table 8.10). Hence, its presence in these organisms does not seem related to intestinal microorganisms. It should be noted that the amounts of PolyPs in insects, as in mammals, are less than the amount of ATP.

The precise and sensitive methods of PolyP assay, including enzymatic methods, have served to confirm the presence of PolyPs in a great variety of animal tissues and cell compartments and to obtain interesting data on the dynamics of PolyP formation and utilization in animal cells (Cowling and Birnboim, 1994; Kumble and Kornberg, 1995; Lorenz *et al*., 1997a,b; Schröder *et al*., 1999). This polymer was found in different sub-cellular fractions, including nuclei (Penniall and Griffin, 1964; Griffin *et al*., 1965; Kumble and Kornberg, 1995), microsomes and mitochondria (Kumble and Kornberg, 1995; Kornberg *et al*., 1999), membranes (Reusch, 1989) and lysosomes (Pisoni and Lindley, 1992). In

Table 8.11 Occurrences of PolyPs in animals (Kumble and Kornberg, 1995; Kornberg, 1999).

System	PolyP (μM)
Rat liver	26
Cytosol	12
Nucleus	89
Rat brain	54
Rat heart	58
Rat kidneys	34
Rat lungs	26
Mouse brain	95
Mouse heart	114

addition, exopolyphosphatase and endopolyphosphatase were found in animal cells (Kumble and Kornberg, 1996; Schröder *et al*., 1999, 2000).

It is rather difficult to compare the earlier and more recent data on the PolyP contents in animal tissues, because these have been calculated in different ways. However, animal tissues contain less PolyPs than microorganisms. Thus, here we present the data from the work of Kornberg and co-workers in Table 8.11 (Kumble and Kornberg, 1995; Kornberg, 1999) and from the work of Schröder and co-workers in Table 8.12 (Schröder *et al*., 1999, 2000). The latter table shows the concentrations measured for 'soluble' long-chain PolyPs (mainly polymers of 10–50 P_i residues) and 'insoluble' long-chain PolyPs (mainly polymers of $>$ 50 P_i residues) in different cells and extracellular fluids from animals. The concentrations of soluble long-chain PolyPs were determined to be higher than those of insoluble long-chain PolyPs. PolyPs was also present extracellularly in human blood plasma and serum. However, the concentrations of insoluble long-chain PolyPs in cell-free blood fractions is much lower those in human peripheral blood mononuclear cells (PBMCs) and erythrocytes. It is not yet known whether the plasma PolyPs are synthesized within this body fluid or appear as a result of the lysis of erythrocytes, as suggested by its smaller size. The highest amounts of PolyPs in humans were found in bone-forming osteoblasts (see Table 8.12).

Some interesting data on the changes in PolyP content and chain length were obtained during the development of lower (sponge) and higher (mammals) animals.

Dramatic changes in PolyP metabolism were revealed in the course of gemmule germination in the freshwater sponge *Ephydatia muelleri* (Imsiecke *et al*., 1996). In the process of germination, a rapid rise in the exopolyphosphatase activity and a strong decrease (by 94 % in 2 d) in the PolyP level were observed (Imsiecke *et al*., 1996). Since germination does not require exogenic energy sources, it was proposed that PolyP can serve as a phosphate and energy source for this process (Imsiecke *et al*., 1996; Schröder et al., 1999).

The interrelation of PolyP and RNA synthesis was observed in the course of embryonal development of the frog (Shiokawa and Yamana, 1965) and at early stages of rat liver regeneration (Mansurova *et al*., 1975a).

The PolyP content and exopolyphosphatase activity in rat tissues changed in the course of ageing and development (Lorenz *et al*., 1997a). The PolyP level in rat brain increased sixfold

Table 8.12 Concentrations of long-chain PolyPs in human cells and blood plasma (Schröder *et al*., 2000).[a]

Cells or blood fraction	PolyP fraction	PolyP content (μM P)	PolyP content (pmol (mg protein)$^{-1}$)
Osteoblasts[b]	Soluble long-chain	394.3 ± 30.6	4331 ± 336
	Insoluble long chain	133.8 ± 15.2	1469 ± 167
Gingival cells	Soluble long-chain	141.3 ± 15.3	1605 ± 174
	Insoluble long chain	14.9 ± 5.1	170 ± 58
PBMC[c]	Soluble long-chain	56.0 ± 2.2	622 ± 24
	Insoluble long chain	28.9 ± 7.0	321 ± 78
Erythrocytes	Soluble long-chain	71.2 ± 14.7	918 ± 190
	Insoluble long-chain	28.1 ± 4.1	362 ± 53
Plasma	Soluble long-chain	48.7 ± 7.0	641 ± 92
	Insoluble long-chain	2.5 ± 1.9	33 ± 25

[a] Extraction of the PolyPs was performed as described by Clark *et al*. (1986). The amounts of 'soluble' long-chain PolyPs (10–50 P_i residues) and of "insoluble" long-chain PolyPs (> 50 P_i residues) are shown.
[b] Unstimulated osteoblasts.
[c] Periferal blood mononuclear cells.

after birth. Mainly long-chain PolyPs caused this increase. The maximal level of PolyP in brain was found in 12 month old animals. In 'old' rat brain, the total PolyP content decreased to about 50 %. In rat liver, the age-dependent changes in PolyP content were lower. The highest activities of exopolyphosphatase (Lorenz *et al*., 1997b) and endopolyphosphatase (Kumble and Kornberg, 1996) were found when the PolyP level was low.

PolyP was shown to display characteristic changes in its chain length during apoptosis in human leukemic HL60 cells (Lorenz *et al*., 1997b). These cells contained a long-chain PolyP of ∼ 150 residues and a short-chain PolyP of 25–45 residues, which could be well distinguished by electophoresis. In apoptotic cells, the long-chain PolyP disappeared simultaneously with DNA fragmentation. This finding indicates that PolyP may be involved in the processes of apoptosis by affecting the stability of DNA–protein complexes or by regulation of nuclease activity (Schröder *et al*., 1999).

Schröder and co-workers (Schröder *et al*., 1999, 2000) studied PolyP metabolism in bone tissues and osteblast cultures. They revealed that PolyP metabolism in human osteoblasts was modulated by stimulators of osteoblast proliferation and differentiation (Leyhausen *et al*., 1998). A combined treatment of the cells with dexamethasone, β-glycerophosphate, epidermal growth factor (EGF), and ascorbic acid resulted in a dramatic decrease in PolyP content. This decrease is caused mainly by a decrease in the amount of soluble long-chain PolyPs. The amount of this PolyP fraction, but not the amount of insoluble long-chain PolyPs, further decreases after additional treatment of the cells with 1α, 25-dihydroxyvitamin D_3. The decrease in PolyP content during treatment with dexamethasone, β-glycerophosphate, EGF and ascorbic acid is accompanied by a decrease in exopolyphosphatase activity. However, additional treatment with 1α, 25-dihydroxyvitamin D_3 results in a significant increase of the enzyme activity. Therefore, it is reasonable to assume that PolyP

may act as an additional regulator of calcification and decalcification in bone tissue, in addition to PP_i. The treatment of cultures of human osteosarcoma cell line SaOS-2 with PolyP causes a strong suppression of mineralization induced by β-glycerophosphate (Schröder *et al*., 2000), thus indicating that this polymer may act as a mineralization inhibitor.

Exopolyphosphatase activity in osteoblasts is much higher than in other mammalian cells and tissues tested. Moreover, endopolyphosphatase activity is present in bone-forming osteoblasts (Schröder *et al*., 2000). All of these data suggest that, besides PP_i, PolyP is involved in the mineralization process in bone tissue.

Thus, the current data available, despite their 'fragmentariness', demonstrate the importance of further investigation of PolyP accumulation and utilization in animals, because PolyPs are probably an important additional regulatory factor in higher eukaryotes.

9

APPLIED ASPECTS OF POLYPHOSPHATE BIOCHEMISTRY

9.1 Bioremediation of the Environment

9.1.1 Enhanced Biological Phosphate Removal

The presence of considerable amounts of P_i in wastewaters due to the run-off of fertilizers, and industrial and household discharges, is a major problem, because destructive blue algae blooms may develop in natural waters, where under normal conditions the P_i concentration is most often the limiting factor for algae growth (Godd and Bell, 1985).

It is also important to understand how $PolyP_3$ contained in common detergents is hydrolysed in the environment and what ways may improve its removal. The major factor contributing to $PolyP_3$ degradation in wastewater was shown to be biological by nature, with the most likely mechanism being enzymatic hydrolysis (Halliwell *et al*., 2001).

The currently available methods for removing P_i from wastewater rely primarily on PolyP accumulation in sludge bacteria, which accumulate considerably more phosphate than is required for normal bacterial growth. This process is called 'enhanced biological phosphorus removal' (EBPR) (Toerien *et al*., 1990). The process of EBPR is now an accepted and low-cost strategy for controlling eutrophication. The excess of taken-up P_i is stored in the form of PolyP granules, and thus PolyPs play the key role in EBPR. In addition, some other extracellular polymers associated with cell clusters may act as a phosphorus reservoir (Cloete and Oosthuizen, 2001).

The literature data on EBPR are now very numerous. There are many detailed reviews, which summarize the data on technology, biochemistry, microbiology and molecular biology of this process (Kortstee *et al*., 1994; Van Loosdrecht *et al*., 1997; Mino *et al*., 1998; Bond and Rees, 1999; Kortstee and Van Veen, 1999; Ohtake *et al*., 1999; Kortstee *et al*.,

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2000; Ohtake and Kuroda, 2000; Mino, 2000; Keasling *et al*., 2000; Blackall *et al*., 2002; Seviour *et al*., 2003; McGrath and Quinn, 2003).

EBPR involves the cycling of microbial biomass and influent wastewater through anaerobic and aerobic zones to achieve selection of microorganisms with high capacities for PolyP accumulation in cells in the aerobic period. In the anaerobic zone of the treatment system, the cells are electron-acceptor deficient and carbon-rich. It has been proposed that PolyP is degraded to P_i, which is excreted from the cell to increase the transmembrane proton gradient. Carbon is then taken up via a proton-symport pump and stored inside the bacterial cell as polyhydroxyalkanoate (PHA). In the subsequent aerobic zone of the treatment system, the environment is electron-acceptor-rich but carbon-deficient. It has therefore been proposed that PHA is degraded and PolyP is synthesized from ATP generated from PHA metabolism. Since more P_i is taken up during the aerobic phase than is secreted during the anaerobic phase, there is a net removal of P_i from the wastewater.

Although the anaerobic–aerobic process for EBPR is an established process from the engineering point of view, it has not been clearly defined in microbiological terms. For example, the phylogenetic or taxonomic groups responsible for EBPR and general structures of the EBPR microbial community have not been described once and for all. Very few pure cultures have been isolated as candidates for playing the key role in EBPR processes. Studies of the metabolic aspects of EBPR have been based mainly on enriched mixed cultures but not on pure cultures. Polyphosphate kinase activity and the occurrence of *ppk* genes have been directly investigated in activated sludge performing enhanced biological phosphorus removal (Bolesch and Keasling, 2000b).

There are two groups of microorganisms involved in EBPR. These are the PolyP-accumulating organisms and their supposed 'carbon competitors', known as glycogen-accumulating organisms. *Acinetobacter* spp. was first proposed as the bacteria responsible for EBPR (Fuhs and Chen, 1975) and intensive studies of their physiology, genetics and PolyP-metabolizing enzymes were carried out (Deinema *et al*., 1980, 1985; Van Groenestijn *et al*., 1989; Bonting *et al*., 1991, 1993a,b; Van Veen *et al*., 1994; Geissdörfer *et al*., 1998). These data are summarized in a number of reviews (Kortstee and Van Veen, 1999; Kortstee *et al*., 2000). Many strains of *Acinetobacter* were isolated from activated sludge (Vasiliadis *et al*., 1990; Kim *et al*., 1997). However, *Acinetobacter* could produce only a small proportion of cells in some sludges, where other bacteria prevailed (Auling *et al*., 1991; Bond *et al*., 1999).

Nakamura *et al*. (1995) isolated a new PolyP-accumulating bacterium *Microlunatus phosphovorus* by a laboratory-scale EBPR process. *M. phosphovorus* accumulated large amount of PolyPs under aerobic conditions, which was then consumed along with the anaerobic uptake of carbon sources such as glucose. However, it lacks the key metabolic characteristics of EBPR; it neither takes up acetate nor accumulates PHA under anaerobic conditions. Using the 16Sr RNA-targeted probe, *M. phosphovorus* was found to be about 2.7 % of the total bacteria, while the percentage of PolyP-accumulating bacteria detected by the DAPI stain for PolyP was about 9 % of the total bacteria (Kawaharasaki *et al*., 1999).

Many authors believe that bacteria phylogenetically related to the *Rhodocyclus* group are responsible for EBPR in activated sludge communities (Hesselmann *et al*., 1999; Mino, 2000; Keasling *et al*., 2000; Jeon *et al*., 2003). Using fluorescent *in situ* hybridization techniques, the communities of *Rhodocyclus*-related organisms in two full-scale wastewater treatment plants were estimated to be between 13 and 18 % of the total bacterial population

(Zilles *et al*., 2002). Certain species within the *Rhodocuclus* group should be mainly responsible for EBPR, at least under certain circumstances.

Modern approaches to the identification and quantification of microorganisms in activated sludge include use of the 16S rRNA-targeted oligonucleotide probe (Wagner *et al*., 1994; Brdjanovic *et al*., 1999; Kawaharasaki *et al*., 1999; Onda and Takii, 2000; Liu *et al*., 2001; Serafim *et al*., 2002) and quinone profiling (Hiraishi *et al*., 1998). The results of 16S rDNA clone library and fluorescence *in situ* hybridization (FISH) with rRNA-targeted, group-specific oligonucleotide probes revealed many new bacterial species in activated sludge. Staining of PolyP and PHA granules confirmed that these bacteria accumulate PHA and PolyP just as predicted by the metabolic models for EBPR. For example, C*orynebacteria* (Bark *et al*., 1993), *Microthrix parvicella* (Erhardt *et al*., 1997), *Tetracoccus cechii* (Blackall *et al*., 1997), Gram-positive cocci belonging to a new genus, *Tetrasphaera* gen. nov., and two new species of *Tetrasphaera japonica*, i.e. *Tetrasphaera australiensis* sp. nov. (Maszenan *et al*., 2000) and *Tetrasphaera elongata* sp. nov. (Hanada *et al*., 2002), *Gemmatimonas aurantiaca* gen. nov., sp. nov. (Zhang *et al*., 2003) and *Accumulibacter phosphatis* (Hesselmann *et al*., 1999; Liu *et al*., 2001) were identified in activated sludges. Unexpectedly, one paper reported that the major PolyP-accumulating cells in the studied sludge were clustered spores of yeast (Melasniemi and Hernesmaa, 2000).

DAPI and PHA staining procedures could be combined with FISH to identify directly the PolyP- and PHA-accumulating traits of different phylogenetic groups. For example, *Accumulibacter phosphatis* (Hesselmann *et al*., 1999; Liu *et al*., 2001) and the representatives of a novel gamma-proteobacterial group were observed to accumulate both PolyPs and PHA. The representatives of another novel group, closely related to coccus-shaped *Tetrasphaera*, and one filamentous group resembling *Nostocoidia limicola*, were found to accumulate PolyPs but not PHA (Liu *et al*., 2001). An interesting example of PolyP accumulation was observed in the denitrifying bacterium *Paracoccus denitrificans* (Barak and Rijn, 2000). PolyP synthesis by this bacterium took place with either oxygen or nitrate as the electron acceptor and in the presence of an external carbon source. It was concluded that *P. denitrificans* is capable of combined P_i and nitrate removal with no need of alternating anaerobic–aerobic or anaerobic–anoxic switches. The observed diversified functional traits suggested that different substrate metabolisms were used by predominant phylogenetic groups in EBPR processes (Liu *et al*., 2001).

According to the modern data, EBPR is realized by complicate microbial communities (Bond and Rees, 1999; Mino, 2000; Keasling *et al*., 2000; Blackall *et al*., 2002; Hollender *et al*., 2002; Serafim *et al*., 2002; Seviour *et al*., 2003). Some bacteria of these communities are very difficult to isolate and cultivate in pure cultures. The microbial community structure of the EBPR process depends on waste composition, organic substrates and inorganic compounds. High microbial diversity of the EBPR sludge has been demonstrated by new techniques. It was suggested that EBPR sludges consist of several different chemotaxonomic groups. In other words, the EBPR sludge phylogenetically consisted of several different microbial groups, and their metabolic co-operation allows them to grow in special conditions such as wastewater treatment plants.

In EBPR processes, one of the problems is utilization of accumulated phosphate of the sludge. It has been discovered that nearly all PolyPs could be released from activated sludge simply by heating it to 70 ˚C for about 1 h (Kuroda *et al*., 2002). The chain lengths of the released PolyPs ranged from 100 to 200 P_i residues. The addition of $CaCl_2$ precipitated

approximately 75 % of the total phosphorus without pH adjustment. The formed precipitate contained more phosphorus and less calcium than typical natural phosphorite deposits. Hence, in combination with EBPR, the present method has a potential for development of a simple process for recovering phosphorus in reusable form from wastewaters (Kuroda *et al*., 2002).

There are many important problems remaining concerning the PolyP biochemistry of EBPR. First, a clear definition of the microbial community structure of EBPR processes and the mechanisms of ecological selection for such processes is needed. Since many microorganisms from EBPR plants seem to be non-cultivated in pure cultures, molecular methods are surely powerful tools for this purpose. A common EBPR metabolism seems to include phylogenetic diverse microbial populations. This suggests a possibility of the key genes of EBPR metabolism being common among different bacteria. It is important and interesting to determine such key genes and to find out how they are regulated.

9.1.2 Removal of Heavy Metals from Waste

PolyPs have been implicated as strong chelators of divalent cations, including cations of heavy metals. PolyP metabolism plays an important role in the bioremediation of phosphate contamination in municipal wastewaters and may play a key role in heavy metal tolerance and bioremediation (Boswell *et al*., 1999). Some genetic constructions for enhancing the tolerance of bacteria to heavy metals have been developed (Keasling and Hupf, 1996; Keasling *et al*., 2000). A plasmid was constructed for mercury bioaccumulation, using the fusion of the well-known *mer*-operon from *Pseudomonas* with the *ppk* gene from *Klebsiella aerogenes*. The *E. coli* strain with the plasmid accumulated 10-fold more Hg^{2+} and two-fold more phenylmercury from contaminated medium (Pan-Hou *et al*., 2002). A large amount of PolyP was identified in the mercury-induced bacterium but not in the cells without mercury induction. It was suggested that PolyP may play a direct role in mercury resistance, probably via chelate formation rather than precipitation of mercury, by releasing P_i from the PolyP (Pan-Hou *et al*., 2002). A strain of *Acinetobacter johnsonii* was capable of removing La^{3+} from solution via precipitation of cell-bound $LaPO_4$ (Boswell *et al*., 2001). The PolyP-mediated accumulation from waste could serve as a useful strategy for the direct remediation of organic and inorganic heavy-metal-containing pollutants.

9.2 Polyphosphates and Polyphosphate-Metabolizing Enzymes in Assay and Synthesis

The cheapness of PolyP, which may be easily obtained by chemical synthesis from P_i, is the reason for attempts to employ methods where its high-energy phosphoanhydryde bonds can be used in assay and synthesis processes. At the present time, many PolyP-depending enzymes are available in large quantities and with high degrees of purification, and this has facilitated the development of such methods.

Therefore, a method of glucose determination by immobilized polyphosphate glucokinase (EC 2.7.1.63, polyphosphate:glucose phosphotransferase) has been elaborated (Kowalczyk and Szymona, 1991). The enzyme was covalently coupled with collagen-coated silica gel beads and used to determine glucose in serum and other samples, as a packed-bed reactor. The method was based on spectrophotometric measurement of the NADPH produced by two consecutive reactions, similar to the hexokinase method. The immobilized-enzyme reactor showed good operational stability during a month of usage, losing about 12 % of its initial activity (Kowalczyk and Szymona, 1991). An endopolyphosphatase assay using the same enzyme has also been described (Kowalczyk and Phillips, 1993).

While PolyP is cheaper than AMP, ADP and ATP, it was proposed as a phosphodonor in enzymatic synthesis. Effective ATP regeneration systems based on polyphosphate kinase (EC 2.7.4.1, ATP:polyphosphate phosphotransferase) have been elaborated (Butler, 1977; Murata *et al*., 1988; Hoffman *et al*., 1988; Haesler *et al*., 1992). For example, polyphosphate kinase partially purified from *E. coli* and immobilized on glutaraldehyde-activated aminoethyl cellulose could carry out the synthesis of ATP from ADP, using long-chain inorganic PolyP as a phosphoryl donor. Immobilized polyphosphate kinase loses no activity when stored in an aqueous suspension for 2 months at 5 °C or for 1–2 weeks at 25 °C. It may be stored indefinitely as a lyophilized powder at −10 °C. Storage stability, purity and yield of its ATP product and low values of the Michaelis constants for its substrates make it a highly promising enzyme for ATP regeneration (Hoffman *et al*., 1988).

The overproduction of polyphosphate kinase achieved by recombinant DNA technology makes the practical use of polyphosphate kinase and PolyP more possible. This activity of pure polyphosphate kinase enables the practical synthesis of oligosaccharides and their derivatives (Noguchi and Shiba, 1998; Shiba *et al*., 2000; Ishige *et al*., 2001).

For example, galactose 1-phosphate (Gal-1-P) was synthesized with *E. coli* galactokinase and an ATP-regeneration system consisting of PolyP and polyphosphate kinase prepared from ppk-overproducing *E. coli* cells (Shiba *et al*., 2000). The phosphorylation efficiency of galactose with PolyP and polyphosphate kinase was shown to be almost the same as that with ATP. Via a combined action, polyphosphate kinase and adenylate kinase catalysed the formation of ADP from AMP, followed by ATP formation from ADP. The addition of adenylate kinase to an ATP-regeneration system consisting of PolyP and polyphosphate kinase is more promising, because cheap AMP can be substituted for expensive ATP or ADP as an essential compound initially added to the reaction system, and the supplement of adenylate kinase significantly enhances the efficiency of ATP-regeneration, possibly because ADK can efficiently catalyse ADP phosphorylation using another ADP as a phospho-donor yielding ATP and AMP. Eventually, with only 4 mM AMP, 28 mM Gal-1-P was synthesized under the action of polyphosphate kinase and adenylate kinase in the presence of PolyP (Shiba *et al*., 2000).

Polyphosphate kinase has been found able to phosphorylate nucleoside diphosphates to give nucleoside triphosphates, using PolyP as a phosphate donor. Therefore, the possibility of using PolyP and polyphosphate kinase instead of phosphoenol pyruvate and pyruvate kinase for enzymatic oligosaccharide synthesis was examined, because PolyP is quite cheap when compared with phosphoenol pyruvate (Noguchi and Shiba, 1998; Shiba *et al*., 2000). Attempts were made to synthesize *N*-acetyllactosamine (Gal (β1-4) GlcNAc) using the nucleoside diphosphate kinase-like activity of polyphosphate kinase, where UDP-Glc pyrophosphorylase and UDP-Glc 4-epimerase catalyse the synthesis of UDP-Glc from

glucose 1-phosphate and UTP, and the isomerization of UDP-Glc to UDP-Gal, respectively. The galactosyltransferase transfers galactose from UDP-Gal to *N*-acetylglucosamine, yielding *N*-acetyllactosamine. The UDP formed through the galactosyl transfer reaction should be regenerated to UTP under the action of PPK in the presence of PolyP. 14 mM *N*-acetyllactosamine was accumulated after 48 h of reaction, even if only 4 mM UTP was initially added, thus demonstrating that PPK and PolyP efficiently catalyse the regeneration of UTP from UDP. It is obvious that PolyP and PPK can replace PEP and pyruvate kinase in the regeneration of NTPs and are available for enzymatic cyclic synthesis of oligosaccharides (Shiba *et al*., 2000).

When CMP-*N*-acetyl neuraminic acid synthetase (EC 2.7.7.43) of *Haemophilus influenzae*, polyphosphate kinase and CMP kinase were added to the reaction mixture containing equimolar concentrations (15 mM) of CMP and *N*-acetyl neuraminic acid, and PolyP (150 mM in terms of phosphate), CMP-*N*-acetyl neuraminic acid was synthesized up to 67 % yield (Ishige *et al*., 2001).

It can be expected that the potential of PolyP as a phosphodonor in the enzymatic synthesis of biologically active compounds will not be depleted.

9.3 Polyphosphates in Medicine

9.3.1 Antiseptic and Antiviral Agents

PolyPs display antiseptic, cytoprotective and antiviral activities. At a concentration of 0.1 % or higher, PolyP had a bacteriocidal effect on log arithmic-phase *Bacillus cereus* cells (Maier *et al*., 1999). The growth inhibition effect of PolyP was observed with *Staphylococcus aureus* (Jen and Shelef, 1986; Matsuoka *et al*., 1995) and *Aeromonas hydrophila* (Palumbo *et al*., 1995).

PolyP with a chain length of more than four P_i residues inhibited human immunodeficiency virus type 1 (HIV-1) infection of cells *in vitro* at concentrations of $\geq 300\ \mu$M (in terms of P_i residues), whereas $PolyP_3$ was ineffective (Lorenz *et al*., 1997b). This long-chain PolyP also inhibited HIV-1-induced syncytium formation. The anti-HIV effect of PolyPs may be due to the binding of the compounds to both the host cell surface and the virus, thereby inhibiting adsorption of the virus (Lorenz *et al*., 1997b).

9.3.2 Polyphosphate Kinase as a Promising Antimicrobial Target

The *ppk1* and *ppk2* sequences encoding prokaryotic polyphosphate kinases have a high degree of conservation among diverse bacterial species, including some of pathogens of the major infectious diseases (Tzeng and Kornberg, 1998; Zhang *et al*., 2002). In view of the essentiality of polyphosphate kinase and PolyP for stationary phase responses and viability in *E. coli* (Rao and Kornberg, 1996), *Vibrio cholerae* (Ogawa *et al*., 2000b), *Pseudomonas aeruginosa* (Rashid *et al*., 2000a,b), *Helicobacter pylori* (Shirai *et al*., 2000), *Shigella and Salmonella* (Kim *et al*., 2002) and *Porphyromonas gingivalis* (Chen *et al*., 2002), their

similar roles seem plausible in the expression of virulence factors, which also appear in the stationary phase of some pathogens. In mutants lacking *ppk*, the phenotype changes related to virulence decrease were established. These are as follows: growth defects at the stationary phase, defective responses to stress and starvation, higher sensitivity to stress factors, including heat, antibiotics, antiserum, UV-light and other effectors, impairment in motility, and biofilm formation (Ogawa *et al*., 2000b; Rashid and Kornberg, 2000; Rashid *et al*., 2000a,b; Kim *et al*., 2002; Chen *et al*., 2002).

Some evidence for the enhanced virulence of pathogenic strains with increased polyphosphate kinase activity has been obtained. A deletion in the *ppk* gene of the *Helicobacter pylori* strain Hp141 led to the higher enzymatic activity of polyphosphate kinase, and the variant with such a deletion exhibited a better capacity for colonizing mice. Taking into account that the modified gene is known to be involved in adaptation to a new environment, it was suggested that PPK is an important virulence factor in *H. pylori* (Ayraud *et al*., 2003).

The genes with similarity to bacterial *ppk1* and *ppk2* were not found in higher eukaryotes (Kornberg *et al*., 1999; Zhang *et al*., 2002). Because polyphosphate kinase or/and PolyP were found necessary for virulence, polyphosphate kinase has become an attractive target for antimicrobial drugs (Kornberg, 1999; Kornberg *et al*., 1999). The absence of any similar enzyme in the higher eukaryotes makes toxicity less likely. Large-scale screening for inhibitors of *E. coli* and *P. aeruginosa* polyphosphate kinases has given candidates which are unique among the known kinases and active at low concentrations (Kim *et al*., 2002).

9.3.3 Polyphosphates as New Biomaterials

Calcium PolyP fibre has been synthesized (Griffith, 1992) and new high-performance calcium polyphosphate bioceramics has been proposed as a bone-substitute material (Nelson *et al*., 1993; Pilliar *et al*., 2001). The *in vivo* experiments, in which porous rods of calcium PolyP were implanted in the distal femur of rabbits, show that these rods can support bone ingrowth and give no adverse reaction (Grynpas *et al*., 2002).

A biodegradable PolyP matrix system was developed as a potential delivery vehicle for growth factors. Polyphosphate was synthesized using a triethylamine catalyst in an argon environment and characterized by using elemental analysis, gel permeation chromatography, and Fourier-transform infrared spectroscopy. It was concluded that this system might be an effective carrier for morphogens, growth factors or other classes of bioactive molecules (Renier and Kohn, 1997). Calcium PolyP fibres were used as scaffold materials for tendon tissue engineering *in vitro* (Sun and Zhao, 2002).

9.3.4 Polyphosphates in Bone Therapy and Stomathology

In view of the fact that PolyP is probably involved in the regulation of phosphate metabolism in bone tissues (Schröder *et al*., 1999; 2000), attempts were made to prove the potential therapeutic uses of PolyPs in the treatment of some bone diseases.

The dissolving action of sodium $PolyP_3$, cyclic trisodium phosphate and sodium PolyP on synthetic crystals of calcium pyrophosphate dihydrate, and on crystalline aggregates of menisci from patients with chondrocalcinosis, was determined (Cini *et al*., 2001). The

results of this study indicated that PolyP is effective for dissolving both synthetic and *ex vivo* crystal aggregates. This suggests possible use of these molecules in the treatment for chondrocalcinosis (Cini *et al*., 2001).

Based on such PolyP properties as complexing ability, buffer capacity and antiseptic properties, new dentifrices with sodium hexametaphosphate (high-polymeric PolyP) have been elaborated. These dentifrices produced stronger and more lasting effects on surface film chemistry than pyrophosphate or other polymeric-based dentifrice systems (Busscher *et al*., 2002). The *in vitro* studies confirmed the anticaries potential and hard tissue safety of a novel sodium hexametaphosphate dentifrice technology, which provides dual-action tooth whitening (i.e. stain prevention, as well as stain removal), while simultaneously providing improved anticalculus action (Pfarrer *et al*., 2001). The chewing gum, containing 1 % of pyrophosphate and 1 % of $PolyP_3$, reduced calculus formation during a clinical study by 37.6 %, when compared with 'no-gum' treatment (Porciani *et al*., 2003).

9.4 Polyphosphates in Agriculture

Ammonium polyphosphate is one of the most often used phosphoric fertilizers (Corbridge, 1980). It was found to be equally effective when compared with single superphosphate and diammonium phosphate for increasing the yields of wheat and maize and for increasing the available phosphorus content in soil during field experiments (Sharma and Singh, 1998).

However, the excess of phosphates and PolyPs in soil due to technogenic pollution and the use of fertilizers has a great influence on the transformation of mineral and organic compounds in soil, the composition of soil microbial communities, and finally agriculture productivity (Kudeyarova, 1993).

9.5 Polyphosphates in the Food Industry

In the food industry, PolyPs are used for different purposes, for instance, water holding in the product, emulsion stabilization, etc. Their high buffering capacities, polyanion and sequestering properties, dry improvements, adhesion reduction abilities and antibacterial effects are the main technological properties which make these effective and multifunctional ingredients of food, such as ham, bacon, meat poultry, fish and shellfish. The main function of PolyPs in food is maintenance of the optimal pH level. This helps prolong the product lifetime, and avoids undesirable changes of the product colour and fat decomposition during storage. PolyP and pyrophosphate also help stabilize dispersions, emulsions and suspensions, favouring water-binding capacity and gel formation.

Some allowed food additives, for example, E-451 and E-452, contain the following PolyPs: triphosphates – pentasodium triphosphate and pentapotassium triphosphate; polyphosphates – sodium polyphosphate, potassium polyphosphates and calcium polyphosphate.

There are many commercial phosphate-containing products for meat and fish treatment. For example, Puron CC, a fine granular powder consisting of sodium polyphosphates, is used in amounts of 0.2–0.5 % of the raw products as a component of brine for the manufacture of

smoked products, poultry and fish products. Puromix 80, consisting of sodium di-, tri- and polyphosphates, can be effectively used with both normal raw meat and meat with defects (pale, soft, watery, etc.) (http://tharnika.ru/; http://www.meat.ru).

The improvement of methods of food treatment with PolyPs (Young *et al*., 1999) and of PolyP assays in food (Cozzani *et al*., 1996; Sekiguchi *et al*., 2000) is continuing.

It should be taken into account that the abundance of PolyPs in food may have some unstudied effect on health. For example, if rats were fed with a high-phosphorus diet, they developed nephrocalcinosis. This was more severe in rats fed on $PolyP_3$ than in those fed on pyrophosphate (Matsuzaki *et al*., 2001). Thus, the influence of PolyPs in food on human health needs further investigations, especially in view of those diseases associated with phosphate metabolism.

In conclusion, it should be noted that we live in an environment with a permanent phosphorus excess. This factor, resulting from the wide use of phosphate-containing detergents, fertilizers and food additives may have some negative effects on the environment and human health. The biochemistry of PolyPs may offer new ways for overcoming unfavourable factors caused by phosphate contamination of the environment and for controlling infections and some other diseases.

10

INORGANIC POLYPHOSPHATES IN CHEMICAL AND BIOLOGICAL EVOLUTION

The achievements of contemporary science have enabled biochemical investigations to be extended beyond the biochemistry of contemporary organisms, and it is now possible to pose and to answer many questions concerning evolutionary and comparative biochemistry. The present stage of development of biochemistry and molecular biology is characterized by a steadily increasing interest in a great variety of evolutionary problems. Modern genomics and proteomics offer many new possibilities for understanding the evolution of protein families, biochemical pathways and other functions of living cells. A wealth of experimental material has been provided, from which far-reaching conclusions may be drawn concerning the origin and development of life on Earth. These investigations have been mainly concerned with those aspects of chemical evolution which must have preceded the appearance of life on Earth (Miller, 1953, 1955; 1986; Belozersky, 1959a–c; Griffith *et al*., 1973; Beck and Orgel, 1965; Lohmann and Orgel, 1968; Ponnamperuma *et al*., 1963; Rabinowitz *et al*., 1968; Schwartz and Ponnamperuma, 1968; Oro *et al*., 1990). It worth noting that the experimental data so far obtained are a great triumph for the theory of the origin of life on Earth, as previously put forward by A. I. Oparin (Oparin, 1924, 1938, 1957, 1965, 1976).

However, despite the outstanding achievements of evolutionary biochemistry, many problems still await solution. Among these unsolved and relatively little-investigated problems of evolutionary biochemistry, there are the role of phosphorus compounds in chemical evolution, which preceded the appearance of life on Earth, and the evolution of phosphorus metabolism from primitive organisms to contemporary living creatures.

The Biochemistry of Inorganic Polyphosphates I. S. Kulaev, V. M. Vagabov and T. V. Kulakovskaya
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10.1 Abiogenic Synthesis of Polyphosphates and Pyrophosphate

Phosphorus, being one of constituent elements of living cells, must without doubt have played an important role at the earliest stages of emergence and evolution of life. It was considered that even when the Earth possessed a reducing atmosphere, phosphorus was present as phosphate rather than in a reduced form such as phosphite (Miller and Parris, 1964). However, model experiments showed that electrical discharges in water-saturated N_2 containing 1–10 % CH_4 reduce phosphate to phosphite. This mechanism was suggested as a possible source of water-soluble phosphorus-containing compounds in volcanic environments on the prebiotic Earth. By introducing small amounts of H_2 and CO into gas mixtures, in which CO_2 and N_2 are the main components, surprisingly high conversions to phosphite were obtained and several percent reduction of apatite occurred (De Graaf and Schwartz, 2000). Phosphites are known to be highly unstable compounds (Miller and Parris, 1964; Schwartz, 1971), but their occurrence from insoluble apatite might provide a possibility for engaging the insoluble forms of phosphate in different chemical, and later biochemical, processes. It appears that, even at the very earliest stages of life on Earth, phosphorus was taken up by primitive living organisms from the environment in the form of phosphate or its derivatives.

Condensed inorganic phosphates could arise on the primitive Earth through a wide variety of abiogenic processes. They could be formed by condensation of inorganic phosphates at high temperatures (Schramm *et al*., 1962, 1967), in the reaction between calcium phosphate and cyanide (Miller and Parris, 1964), and under the action of heat on mixtures of ammonium phosphate and urea (Ostenberg and Orgel, 1972). They could therefore easily be present at the time when life first appeared on the Earth. It was shown both in the experiments that simulate magmatic conditions and in the analysis of volatile condensates in volcanic gas, that volcanic activity can produce water-soluble PolyPs (Yamagata *et al*., 1991). Some authors, however, doubt that PolyP synthesis by heating phosphate minerals under geological conditions on the primitive Earth may be an effective process, but they do not exclude an undiscovered robust prebiotic synthesis of PolyP or mechanisms for concentrating it (Keefe and Miller, 1996). PolyP production as a result of heating the mineral apatite in the presence of other minerals has been reported (De Graaf and Schwartz, 2000).

On the other hand, pyrophosphate (the lowest member of this homologous series of compounds) could also be formed on the primitive Earth. It could arise from orthophosphate, either by inorganic redox reactions or following preliminary activation of the phosphate by cyanogen, cyanate or dicyandiamide. Miller and Parris (1964), Degani and Halmann (1971) and Steinman *et al*. (1964) have demonstrated this in model experiments. These activating agents could apparently have existed on the primeval Earth. Furthermore, from the work of Orgel and co-workers (Beck and Orgel, 1965; Lohmann and Orgel, 1968), the Miller–Parris reaction (i.e. the conversion of hydroxyapatite into calcium pyrophosphate in the presence of cyanates) can take place under aqueous conditions.

The pyrophosphate formed in this or other ways could be, according to Lipmann (Lipmann, 1965, 1971), ‘the simplest compound present on the primeval Earth to be involved in the accumulation and transfer of energy-rich bonds’. Pyrophosphate is an energy-rich

compound and the hydrolysis of its phosphoric anhydride bond liberated 4.5–5 kCal of energy per mole (Flodgaad and Fleron, 1974).

In model experiments, the conditions were found for non-enzymatic synthesis of pyrophosphate, by the phosphorylation of orthophosphate in the presence of certain cations, and by means of ATP and $PolyP_3$, respectively (Lowenstein, 1958; Tetas and Lowenstein, 1963; Le Port *et al*., 1971).

It was shown experimentally that this reaction proceeded much more rapidly with PolyP, as follows (Kulaev and Skryabin, 1971, 1974):

$$PolyP_n + [^{32}P]\ \text{orthophosphate} \longrightarrow [^{32}P]\ \text{pyrophosphate} + PolyP_{n-1} \qquad (10.1)$$

It was observed that radioactive pyrophosphate was formed non-enzymatically in substantial amounts when [^{32}P] orthophosphate was phosphorylated by incubation with $PolyP_{40}$ in an aqueous solution at 37 °C for 15 h at pH 9 in the presence of certain divalent cations. The 16–30 % of initial high-molecular-weight polyphosphate was utilized in phosphorylation, and among the cations tested, the greatest amount of incorporation was achieved with Ba^{2+} (33 %) and the least with Mg^{2+} (16 %):

Cation	Mg^{2+}	Ca^{2+}	Mn^{2+}	Cd^{2+}	Ba^{2+}	None
PP_i/ PolyP (× 100 %)	16	22	23	26	33	0

It is interesting that, when organic and inorganic tripolyphosphates were employed under the same conditions, only 0.2–0.6 % of the phosphate donor was utilized (Lowenstein, 1958, 1960; Tetas and Lowenstein, 1963; Le Port *et al*., 1971). Our experimental findings thus lead to the conclusion that, as the Earth cooled and a hydrosphere was formed on its surface, a variety of transphosphorylation reactions became possible in the primeval ocean, in particular, the phosphorylation of P_i by PolyP to give pyrophosphate. It should be noted that the non-enzymatic synthesis of PolyP and pyrophosphate on the primitive Earth could take place not only in solutions, but also on the surface of some minerals with anion-exchange properties (Arrhenius *et al*., 1993, 1997).

10.2 Phosphorus Compounds in Chemical Evolution

Phosphate ion is a unique link between living organisms and the inorganic world. In the opinion of some investigators (Arrhenius *et al*., 1993; De Graaf *et al*., 1998; Arrhenius *et al*., 1997), among anionic species oligophosphate ions and charged phosphate esters could have been of great importance in the proposed 'RNA world'. Phosphorylation is shown to result in selective concentration by surface sorption of compounds, otherwise too dilute to support condensation reactions. It provides protection against rapid hydrolysis of sugars and induces oligomerization of aldehydes by selective concentration. As a manifestation of life arisen, phosphate already appears in the organic context in the oldest preserved sedimentary record (Arrhenius *et al*., 1997).

Some experiments have shown efficient condensation of simple aldehyde phosphates in the hydroxide mineral (such as hydrotalcite, ($[Mg_2Al(OH)_6][nH_2O]$) interlayer to form

hexose sugar phosphates, which may be considered as a model for precursor components of RNA (Arrhenius *et al*., 1993; Pitsch *et al*., 1995; De Graaf *et al*., 1998). Other minerals, e.g. montmorillonites, catalyse self-condensation of 5′-phosphorimidazolide of nucleosides in pH 8 aqueous electrolyte solutions at ambient temperatures leading to the formation of RNA oligomers (Ferris and Ertem, 1993; Ertem and Ferris, 1997, 1998). These model experiments support the postulate that the origin of the 'RNA world' was initiated by RNA oligomers produced by polymerization of activated monomers formed in the course of prebiotic processes (Ferris and Ertem, 1993; Ertem and Ferris, 1997, 1998). It is not improbable that P_i, oligophosphates and PolyP as active anions might have possibilities for modulating the adsorbtion and catalytic properties of the above minerals and thereby affect the synthetic processes at the earliest stages of chemical evolution.

Phosphate minerals might have taken an important place at the earlier stages of chemical evolution and in the model experiments reconstituting the biomolecular stage of evolution on the Earth. For example, non-enzymatic formation of 5′-ADP, starting from phosphorylation of 5′-AMP in the presence of either calcium phosphate or calcium pyrophosphate precipitates, has been reported. This reaction was taken as a model example for the study of heterogeneous catalysis of transphosphorylation in prebiotic conditions (Tessis *et al*., 1995). Depending on the precipitation times of the samples and medium composition, the structural analysis of these precipitates by electron and X-ray diffraction showed changes in their 'grade' of crystallinity. It was proposed that these changes are responsible for modulation of the quantity of adsorbed nucleotides to the surface of solid matrices, as well as the catalytic activity of the precipitates (Tessis *et al*., 1995).

Many model experiments (Fox and Harada, 1958, 1960; Ponnamperuma *et al*., 1963; Schramm *et al*., 1962, 1967; Rabinowitz *et al*., 1968; Schwartz and Ponnamperuma, 1968; Gabel and Ponnamperuma, 1972; Schoffstall, 1976; Oro, 1983) have shown that high-molecular-weight PolyPs, in contrast to pyrophosphate, could have functioned on the primeval Earth as condensing agents in reactions leading to the formation of nucleosides, nucleotides (including adenosine triphosphate), simple polynucleotides, polypeptides and even primitive protein-like materials. It should be noted that divalent cations, especially Mg^{2+}, were often needed for effective realization of these processes. For example, condensation of glycylglycine to oligoglycine with cyclotriphosphate in an aqueous solution containing Mg^{2+} have been observed (Yamagata and Inomata, 1997). Magnesium ion was found to have a remarkable catalytic effect on the phosphorylation of adenosine by cyclotriphosphate in an aqueous solution under mild conditions at pH 7.0 and 41 °C. The product was primarily 2′,3′-cyclic AMP, together with lesser amounts of ATP (Yamagata *et al*., 1995).

Some observations proposed that PolyP may be a catalyst in the abiotic synthesis of peptides (Rabinowitz *et al*., 1969; Rabinowitz and Hampai, 1984; Chetkauskaite *et al*., 1988).

It was observed that condensation reactions, in which high-molecular-weight PolyPs functioned as activating agents, could be carried out either at high temperatures in non-aqueous media or at room temperature in an aqueous solution. This gives grounds to suppose that these reactions could be involved in the synthesis of macromolecules, which were subsequently incorporated into living cells, both before and after the appearance of the hydrosphere on Earth (Kulaev, 1971, 1973).

Prebiological energy conversion at the prenucleotide level was suggested to involve a 'thioester world' (De Duve, 1987), an 'iron–sulfur world', in which pyrite (FeS_2) is

mandatory (Walker and Brimblecomble, 1985; Wachtershauser, 1992) and a 'pyrophosphate world' (Baltscheffsky, 1997). Taking into account the ability of PolyPs for transphosphorylation reactions and for the catalysis of some condensation reactions, it may be proposed that these polymers also participated in the earlier stages of chemical evolution. Some investigators have proposed that geothermal PolyP might be used by primitive membrane-anchored kinases for ancient energy-transduction processes (Cavalier-Smith, 2001).

In recent years, interest in the 'RNA-world' (Gilbert, 1986; Dworkin *et al*., 2003) has increased greatly. This is connected with discoveries of the catalytic properties of RNA and of the ability of RNA for replication without the involvement of any other biopolymers. The conception of this possible stage of evolution is described in detail by Spirin (2001). According to this hypothesis, RNA was synthesized by abiogenic processes and then the ancient RNA world arose, in which self-replicated RNA functioned as pre-genetic material and a catalyst (Spirin, 2001). Taking into account the ability of PolyPs to form complexes with RNA, it may be proposed that in such an ancient world PolyPs could yet participate in the regulation of different RNA activities. Figure 10.1 shows a hypothetical scheme of the origin of life according to the idea of the primarility of RNA in biochemical evolution (Spirin, 2001), in which the possible functions of PolyPs are inserted. It would be enticing to add to this scheme PolyP–polyhydroxybytyrate complexes as the simplest channels. Such

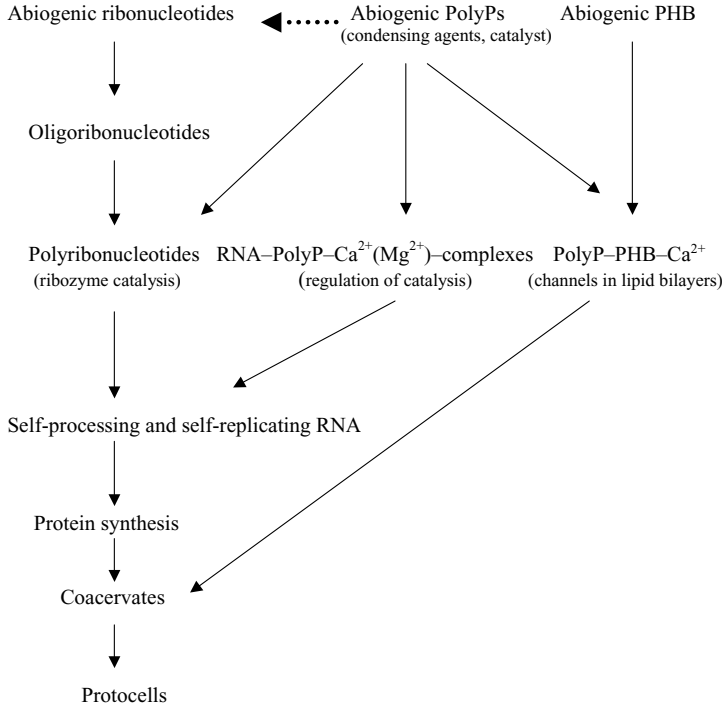

Figure 10.1 Schematic conception of the origin of life according to the idea of primarility of RNA (Spirin, 2001) with addition of the probable role of PolyPs and PolyP–polyhydroxybutyrate (PHB) complexes in the early stages of evolution.

complexes might be present in the coacervates and provide an exchange of micro- and macromolecules between these proto-cells. The investigations of Reusch (Reusch, 1999a; 2000) showed that these channels exist in the membranes of nearly all classes of organisms and probably were ancient membrane channels. Earlier, Gabel (1965, 1971) proposed the involvement of PolyPs in formation of the first cell membranes.

10.3 Polyphosphates and Pyrophosphates: Fossil Biochemical Reactions and the Course of Bioenergetic Evolution

Model experiments, however, have not yet provided any reliable information concerning the functions of high-molecular-weight PolyPs and pyrophosphate in the earliest living creatures, although some conclusions as to the role of these primitive high-energy compounds in the metabolism of protobionis may be drawn from comparative biochemistry. By studying the metabolism of more ancient, comparatively primitive forms of contemporary organisms, there may be discerned, as Lipmann (1971) has said, 'antediluvian' metabolic features and fossil biochemical reactions, which have been preserved since ancient times.

Investigations in this field of biochemistry, which could be termed as 'biochemical palaeontology', could lead, and have indeed led, to the detection of archaic metabolic features, which in all probability derive from primitive life forms.

Thus, Baltscheffsky and co-workers (Baltscheffsky, 1967a,b; Baltscheffsky *et al*., 1966) and Keister *et al*. (Keister and Yike, 1967a,b; Keister and Minton, 1971, 1972) have shown that in the phylogenetically ancient and primitive photosynthesizing bacterium *Rhodospirillum rubrum* photosynthetic phosphorylation results in the production of high-energy phosphate much more in the form of pyrophosphate than in the form of ATP. The synthesis of pyrophosphate can proceed in the chromatophores of this bacterium even when the formation of ATP is totally suppressed. Later, it was shown that pyrophosphate in *Rhodospirillum rubrum* is accumulated only in light (Keister and Minton, 1971, 1972; Kulaev *et al*., 1974a). The energy stored in the pyrophosphate molecule could be utilized both for reversed electron transport and for the active transport of ions through the chromatophore membranes in this bacterium (Baltscheffsky, 1967a,b; Baltscheffsky *et al*., 1966).

The light-dependent synthesis of pyrophosphate was also observed in the chloroplasts of higher plants (Rubtsov *et al*., 1977). The results obtained by Libermann and Skulachev (1970) supposed that the energy of pyrophosphate in chromatophores of *Rhodospirillum rubrum* is utilized via electrochemical proton potential ($\Delta\mu H^+$). The gene of proton-pumping PP_i synthetase from *Rhodospirillum rubrum* was cloned (Baltscheffsky *et al*., 1998) and appeared to have a homology with plant vacuolar H^+PPases (Baltscheffsky *et al*., 1999). In the vacuolar membranes of plants (Davies *et al*., 1997) and yeast (Lichko and Okorokov, 1991), H^+ PPases generate $\Delta\mu H^+$ by using the energy of the PP_i phosphoanhydride bond. The vacuolar membranes of some archae and protozoa also possess such H^+ PPase (Drozdowicz *et al*., 1999; Docampo and Moreno, 2001).

Mansurova and co-workers (Mansurova *et al*., 1973a,b, 1975b, 1976; Mansurova, 1989) have shown that the same process occurs in animal and yeast mitochondria. Pyrophosphate is synthesized in rat liver mitochondria together with ATP (Figure 10.2). However, in rat liver

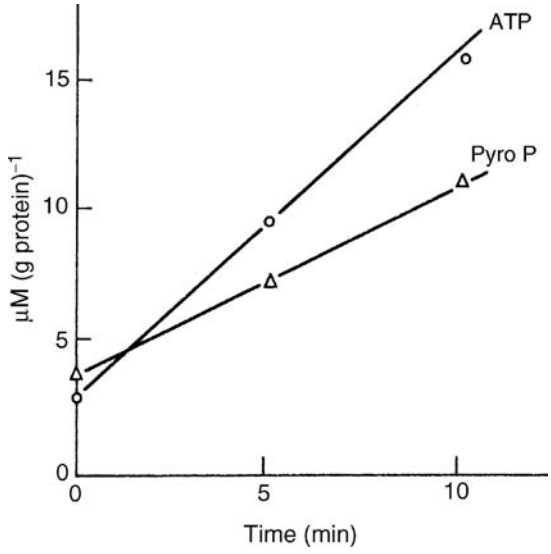

Figure 10.2 Synthesis of pyrophosphate and ATP in rat liver mitochondria (Mansurova *et al.*, 1973b).

mitochondria, pyrophosphate was synthesized at about one tenth the rate of the synthesis of ATP and ADP (AMP was present in the incubation medium). The inhibitors of the respiratory chain, rotenone (2 μg per mg of protein), antimycin (1 μg per mg of protein), and cyanide (1 mM), together with the uncoupler 2,4-DNP (0.4 mM), completely suppressed the biosynthesis of pyrophosphate in rat liver mitochondria. This was shown both in intact mitochondria and in fragments of the internal mitochondrial membrane. It is possible that pyrophosphate is formed in the mitochondria as a secondary product, by the cleavage of some part of ATP to AMP and pyrophosphate. However, experiments on the effect of oligomycin (2 μg per mg of protein), which inhibits the formation of ATP in mitochondria, showed that under these conditions the production of pyrophosphate increased substantially. These findings suggest that pyrophosphate is synthesized in animal mitochondria during the functioning of the respiratory chain independent of ATP, and to a certain extent in opposition to it. Similar results were obtained with yeast mitochondria (Mansurova *et al.*, 1975b).

The lack of dependence of pyrophosphate synthesis in animal and yeast mitochondria *on* ATP was even more established in experiments with mitochondria, which had been depleted in ADP and ATP by pre-incubation with glucose (40 mM), hexokinase (0.1 mg ml^{-1}) and oligomycin (1 μg per mg of protein). In these experiments, the synthesis of pyrophosphate in yeast mitochondria proceeds in the total absence of ADP and ATP (Figure 10.3). The addition of P_i enhanced the above synthesis. Much more pyrophosphate than ATP is synthesized in the chromatophores of *Rh. rubrum*, whereas substantially more ATP is formed in animal mitochondria. In addition, the quantity of pyrophosphate synthesized in yeast mitochondria may approximate to their ATP content under certain conditions of incubation.

Comparative biochemistry thus suggests that, since ancient times, and perhaps since the appearance of the earliest organisms, pyrophosphate has been involved in energetic

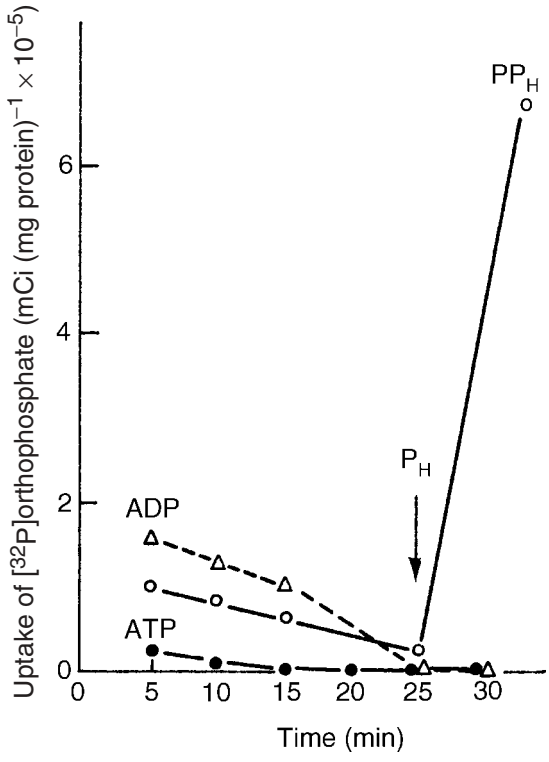

Figure 10.3 Synthesis of pyrophosphate by yeast mitochondria depleted in ADP and ATP (Mansurova *et al*., 1975b).

processes taking place in the membranes, and above all in photosynthetic phosphorylation and phosphorylation in the respiratory chain.

In the opinion of some investigators, the PP-dependent H^+-pumps are more ancient than the H^+-ATPases (Baltscheffsky, 1997; Baltscheffsky *et al*., 1999). However, it should be noted that all contemporary microorganisms, including the most ancient archae, possess in their membranes H^+-ATPases of different types (Nelson, 1992).

It cannot be excluded therefore that the ATP- and pyrophosphate-based energetics were developed in the course of evolution in parallel and their joint existence in some organisms is one of the ways for the best adaptation to the changing environment. In some cases, the pyrophosphate-dependent enzymes might arise from ATP-dependent ones as a later adaptation. For example, the single mutation in the pyrophosphate-dependent phosphofructokinase of *Entamoeba histolytica* changed the preference of the enzyme from PP_i to ATP (Chi and Kemp, 2000). This result suggested the presence of a latent ATP-binding site in this enzyme and it was proposed that the ancestral pyrophosphate-dependent phosphofructokinase was ATP-dependent.

In the opinion of Oparin (1924, 1938), the earliest process to provide energy for the first living organisms on the Earth, even before the appearance of oxygen, was anaerobic

fermentation of hexoses to lactic acid and ethanol. This suggestion was based first of all on a very reasonable assumption that when life originated on Earth the atmosphere did not contain oxygen but possessed reducing properties, and that a variety of organic substances were present in abundance on the Earth's surface. Secondly, all retained and most essential energy-providing mechanisms encountered in living organisms today (the cleavage of hexoses during respiration and the pentose phosphate and photosynthetic cycles) involve anaerobic fermentation reactions. From these considerations, it may be deduced with a reasonable degree of certainty that in primitive living organisms the principal, and perhaps the only, energy-providing process was anaerobic fermentation of hexoses, which seemed to be already present in the 'primeval soup'.

On the basis of results (Uryson and Kulaev, 1968; 1970; Kulaev *et al*., 1971; Szymona *et al*., 1962), Kulaev (1971) has suggested that the energy-providing processes involved in glycolysis were mediated in the earliest organisms by high-molecular-weight PolyPs rather than by ATP and pyrophosphate.

In certain contemporary organisms, for instance, bacteria and fungi, 3-phospho-D-glyceroyl-phosphate:polyphosphate phosphotransferase activity was found (Kulaev and Bobyk, 1971; Kulaev *et al*., 1971). The phosphate was transferred from 1,3-diphosphoglyceric acid, not to ADP to form ATP, as one could expect from the Meyerhof–Embden–Parnas scheme, but directly to PolyP. This fossil reaction was most expressed in an adenine deficient yeast mutant under cell adaptation to ATP depletion.

The second 'fossil' reaction is phosphorylation of glucose, not by ATP but by PolyP. The polyphosphate hexokinase activity was detected only in the phylogenetically ancient organisms, which are closely related to each other (Table 10.1). It can be seen from the latter that in the more ancient representatives of this group of microorganisms such as the Micrococci, Tetracocci, Mycococci, and the propionic bacteria, polyphosphate hexokinase activity exceeded that of ATP hexokinase, whereas in phylogenetically younger representatives ATP hexokinase activity was substantially higher than that of polyphosphate hexokinase.

As was shown by Phillips and co-workers (Phillips *et al*., 1993, 1999), PolyP and ATP glucokinase activities are catalysed by a single enzyme. The data obtained by the investigation of kinetic parameters of purified enzyme from some bacteria suggest a hypothesis of gradual transition from PolyP to ATP as a phosphoryl donor in the course of evolution. According to 16s RNA sequence analysis (Stackelbrandt and Woese, 1981), *Propionibacteria* are phylogenetically older than *Mycobacteria*. The purified enzymes of *Propionibacterium shermanii, Mycobacterium tuberculosis* and *Propionibacterium arabinosum* differ in their preference for PolyP. When the substrate specificity constant k_{cat}/K_m ratios, for the utilization of PolyP and ATP were compared, it was found that the ratios decreased progressively with the enzymes from older to younger organisms (Phillips *et al*., 1999). These results show that utilization of PolyP as a donor of active phosphate in the phosphorylation of glucose is apparently more ancient from the evolutionary point of view than utilization of ATP.

The above experimental findings support the view of Belozersky (1958) who suggested that the high-molecular-weight PolyPs in the earliest organisms functioned in the same way as ATP in the contemporary organisms. Lipmann (1965) and Oparin (1965) also confirmed this suggestion. They indicate that the high-molecular-weight PolyPs may be primarily involved in protobionts in the coupling of glycolysis with the phosphorylation of sugars, for

Table 10.1 Activity of polyphosphate glucokinase and ATP glucokinase in different bacteria. Results are given in mE per mg of protein (Uryson and Kulaev, 1968; Uryson *et al*., 1973; Szymona *et al*., 1962; Szymona *et al*., 1967).

Organism	PolyP glucokinase	ATP glucokinase	PolyP glucokinase/ ATP glucokinase
Micrococcus lysodeikticus	34	7	4.9
Micrococcus citreus	43	15	2.9
Micrococcus aurantiacus	23	9	2.6
Micrococcus sulfureus	12	4	3.0
Micrococcus sp. strain 11 5N	97	23	4.2
133N	146	35	4.2
220N	124	39	3.2
Mycococcus sp. strain 36F	125	54	2.3
61N	14	8	1.8
196N	94	50	1.9
357N	199	97	2.0
12N	125	45	2.8
114N	66	19	3.5
338N	54	19	2.9
Sarcina lutea	34	8	4.2
Staphylococcus aureus	0	63	—
Streptococcus faecalis	0	97	—
Propionibacterium shermanii	100	10	10
Propionibacterium friedenreichii	37	Trace	1
Lactobacterium lindneri	0	110	—
Lactobacterium cereale	0	157	—
Mycobacterium tuberculosis	87	10	8.7
Mycobacterium scotochromogenus	237	118	2
Mycohacterium phlei	169	130	1.3
Mycobacterium smegmatis	263	233	1.2
Mycobacterium friburgensis	177	155	1.1
Arthrobacterium (Mycobacterium) citreus	173	97	1.8
Arthrobacterium (Mycobacterium) globiformis	23	11	2.1
Corynebacterium xerosis	19S	62	2.0
Proactinomyces ruber strain 45 1	247	26	9.5
Nocardia (Proactinomyces) madurea	150	22	7.0
Nocardia (Proactinomyces) turbatus	43	15	2.9
Micropolyspora viridis	233	107	2.2
Micropolyspora mesophylica	92	51	1.8
Nocardia (Proactinomyces) gardneri	67	57	1.2
Nocardia (Proactinomyces) minima	172	158	1.1
Nocardia (Proactinomyces) brasiliensis	83	110	0.8
Nocardia (Proactinomyces) asteroides	78	187	0.4
Proactinomyces ruber strain 408	30	273	0.1

Table 10.1 (*Continued*)

Organism	PolyP glucokinase	ATP glucokinase	PolyP glucokinase/ ATP glucokinase
Nocardia (Proactinomyces) paragvensis	0	112	—
Proactinomyces pelletieri	Trace	71	—
Streptomyces (Actinomyces) globisporus	80	122	0.6
Streptomyces (Actinomyces) olivaceus	60	127	0.5
Streptomyces (Actinomyces) fradiae	30	267	0.1
Streptomyces (Actinomyces) aureofadens	38	252	0.1
Streptomyces (Actinomyces) griseus	38	312	0.1
Dermatophylus congolensis	3	23	0.1
Streptomyces (Actinomyces) somaliensis	9	174	0.05
Thermoactinomyces (Micromonospora) vulgaris	Trace	12	—
Micromonospora fusca	Trace	95	—
Stretosporangium roseum	Trace	252	—
Actinoplanus armeniacus	0	154	—

instance, in a sequence of reactions such as those shown in Figure 10.4. High-molecular-weight polyphosphates are able to phosphorylate glucose to glucose-6-phosphate, which is converted into 1,3-diphosphoglyceric acid. The latter may give rise to the synthesis of PolyP.

Thus, at the earliest stages of evolution of the energy systems in living organisms, the function of linking exoenergetic and endoenergetic processes, which is normally accomplished in contemporary organisms by ATP, could apparently be carried out to some extent by the more primitively structured high-energy compounds, inorganic PolyPs.

Discussing the role of PolyP in bioenergetics, it should be taken in account that the synthesis of PolyP may be related to $\Delta\mu H^+$. The possibility of PolyP synthesis from PP_i was demonstrated in chromatophores of *Rh. rubrum* (Oh and Lee, 1987). In addition, the accumulation of PolyP in yeast is inhibited by ionophores, which destroy the $\Delta\mu H^+$ on different cellular membranes (Beauvoit *et al*., 1991; Trilisenko *et al*., 2003).

To summarize, the participation of PolyP in energy-liberating and energy-requiring processes in living cells, including the most ancient pathways, is shown in Figure 10.4. The interaction of PolyP and $\Delta\mu H^+$ was probably developed later than the processes, in which PolyP participated directly in glycolysis. These reactions have been preserved to the utmost in the evolutionary older microorganisms, whereas the $\Delta\mu H^+$-dependent accumulation of PolyP is most supported in lower eukaryotes.

The experimental data so far obtained, especially those derived from our own work, make it possible even now to envisage a role for high-molecular-weight PolyPs in chemical

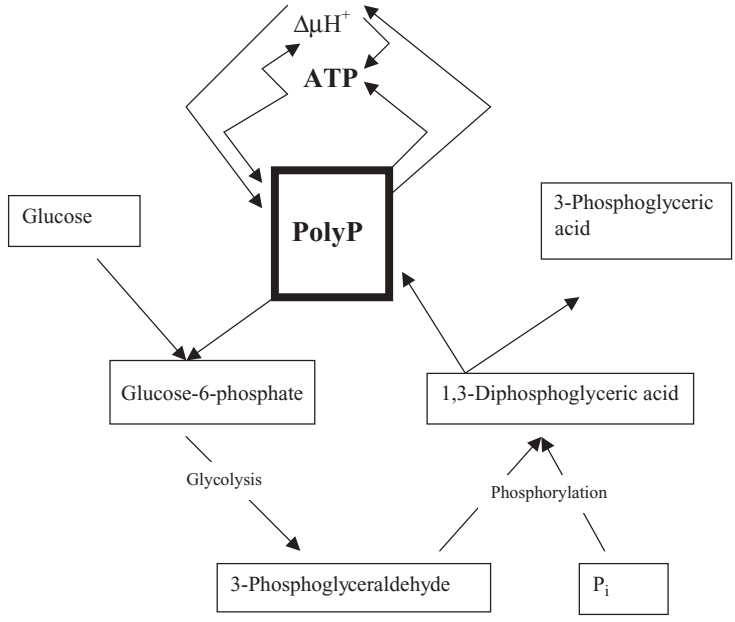

Figure 10.4 Hypothetical scheme for the participation of PolyP in the coupling of energy-liberating and energy-requiring processes.

and biochemical evolution (Figure 10.5). When the Earth was still very hot, phosphate occurred largely in the form of PolyP. As the temperature fell and a hydrosphere was formed, the polyphosphates were able to participate in abiogenic transphosphorylation reactions, with the formation of pyrophosphate. At any moment, ATP could have been formed in the primeval ocean. During the development of the earliest organisms, the functions, which were initially largely carried out by pyrophosphate, for example, participation in redox reactions taking place in membranes, and by high-molecular-weight PolyPs in reactions occurring in solution, were gradually transferred to ATP. In the metabolism of contemporary organisms, however, neither pyrophosphate nor PolyPs has been totally superseded. Alongside ATP, they are biopolymers with many functions, especially gene-activity control, energy reserve, participation in membrane transport, phosphate reservation, cation chelation and enzyme-activity regulation.

10.4 Changes in the Role of Polyphosphates in Organisms at Different Evolutionary Stages

Quite a number of facts mentioned above support the idea that PolyPs are of very ancient evolutionary origin. Taking into account the hypothesis of the primarility of RNA in the origin of life, the regulatory and energetic functions of PolyPs may be primary in evolution (Figures 10.1 and 10.5). It is probable that this function of PolyPs, when life first appeared

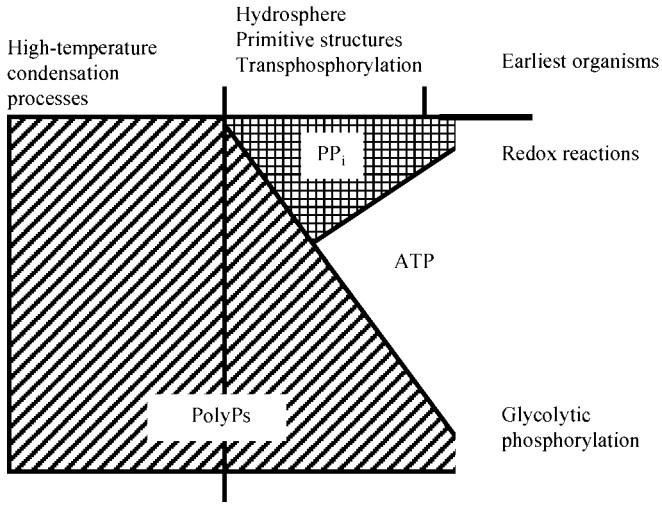

Figure 10.5 Hypothetical scheme for the formation and reactions of high-molecular-weight PolyPs, pyrophosphate and ATP at different stages of appearance of life on Earth (Kulaev and Skryabin, 1974).

on the Earth, was their main, although by no means, their only function. In general, it is assumed that the polyfunctionality of compounds was an important criterion in their selection as components of living cells.

It is possible, for instance, that in protobionts PolyP was not merely a coupling compound, but also provided a comparatively long-term depot for phosphorus and energy, which enabled organisms to become independent of their environment to some extent. PolyP was able to detoxify free orthophosphoric acid and its salts, which could accumulate in large amounts in cells. Being an excellent ion exchanger, PolyP could also be a regulator of cation metabolism in the earliest organisms, since it could regulate the functions of many enzymes through binding one cation and liberating another. At some stage of evolution, however, the high-molecular-weight PolyPs ceased to fully satisfy the requirements of an organism. More specific compounds were needed, which would have had structures capable of an even greater variety of functions, as well as more precise and specific interactions with other cell components.

In general, it is very likely that the ability to interact with other cell components has also been an important factor in the evolution of metabolism of living organisms. Thus, PolyP, which possesses a monotonic macromolecular, essentially linear, structure without any special features, could have become a somewhat unsatisfactory compound at a certain stage of cell development. The limited capacity of PolyP for precise and very specific interactions with other cellular metabolites resulted in an inconsistency with its function of coupling exo- and endoenergetic processes. Hence, ATP was selected for the above functions, because it has a much more specific, and therefore more readily recognized, structure. Moreover, ATP was capable of many other functions, which could not be performed by PolyP.

Numerous investigations (Ponnamperuma *et al*., 1963; Rabinowitz *et al*., 1968; West and Ponnamperuma, 1970; Oro, 1983) have demonstrated a relative easiness of formation of

adenine as compared with other nitrogenous bases, under conditions similar to those which probably occurred in the pre-biological stage of the Earth's development. It was also shown that adenine is much more resistant to various types of radiation, which undoubtedly played an important part in the processes taking place on the primeval Earth. A series of brilliant investigations carried out by Ponnamperurna and co-workers (Ponnamperuma *et al*., 1963; Rabinowitz *et al*., 1968; Schwartz and Ponnamperuma, 1968) demonstrated that adenosine and adenosine polyphosphates could have been formed under conditions similar to those which existed on the Earth at the time when life first appeared. It is very important to note that in these investigations the abiogenic formation of ATP took place only in the presence of ethyl polyphosphates or labile inorganic PolyPs. This makes even more plausible the hypothesis that inorganic PolyPs, rather than ATP, were the first to appear and to function as acceptors and donors of phosphate in primitive organisms.

At the following stages of evolution, three domains of extant life (Bacteria, Archaea, Eucarya) have emerged from a multiphenotypical population of primarily chemolithoauthotrophic pre-cells by cellularization and further evolution (Woese *et al*., 1990; Kandler, 1993). In modern prokaryotes, in addition to the functions of energy and phosphate reserve, the function of gene-activity control has become very significant. This function might arise already in the RNA world, because of the great ability of PolyPs to form complexes with RNA via divalent cations. It is not improbable that such complexes affected the catalytic and replication properties of RNA molecules at this early stage of evolution.

The contemporary organisms with a low degree of organization are still heavily dependent on the environment, and for this reason they need to possess a mechanism for conserving large amounts of active phosphate. One such mechanism for the storage of active phosphate in contemporary microorganisms, both prokaryotes and eukaryotes, remains the accumulation of PolyPs, which renders them independent of the changes in their external environment to a substantial extent. Furthermore, an important factor leading to the retention in contemporary organisms of the ability to accumulate high-molecular-weight PolyPs appears to be the lack of balance of their metabolism. The accumulation of PolyPs in these organisms appears to be an efficient means of detoxifying free P_i and storing it in an active form as PolyPs, so that they can be utilized for rapid growth once favourable conditions for their development appear. In this case, the phosphorus and energy contained in the PolyPs must greatly facilitate the rapid and simultaneous synthesis of large amounts of the nucleic acids required at an early stage of rapid growth and cell division. If a microorganism cannot accumulate PolyPs, the excess of P_i in the medium becomes an unfavourable factor. For example, the halophilic archae *Halobacterium salinarium* has no ability to synthesize PolyPs in great amounts (Smirnov *et al*., 2002a,b). Thus, it accumulates P_i in the form of magnesium phosphate under P_i excess in the medium. This leads to certain changes in cell morphology and death of some part of the population.

The cells of the higher, multicellular organisms are to a lesser extent dependent on the environment, and as a result the ability to accumulate large amounts of inorganic polyphosphates could have been lost during the evolutionary process. In certain highly specialized organs in animals, in particular, the muscles, the requirement for a long-term depot of activated phosphate again arose in the course of evolution because of the rhythmic nature of muscular contraction. In this case, still more specialized forms of phosphorus and energy storage appeared, namely, arginine phosphate and creatine phosphate. However, the active phosphate stored in these phosphagens is both accumulated and utilized in muscle tissue

only via ATP. In this respect, the phosphagens differ essentially from inorganic PolyPs, which can also be utilized and synthesized, as we have seen, by other routes.

It is therefore reasonable to suppose that the phosphagens, as distinct from inorganic PolyPs, arose in the course of evolution subsequent to ATP. The function of detoxification of orthophosphate has in most cases, however, evidently ceased to be necessary as a result of evolutionary development in the higher organisms of a very delicately balanced metabolism and of mechanisms for its precise control. In many cases, the need of elimination of P_i surplus in a form, which is non-toxic to the cell, has again appeared in certain tissues of the higher organisms.

Such a case is apparently the accumulation in the seeds of some plants of significant amounts of phytin, calcium and magnesium salts of inositol hexaphosphoric acid. A number of investigators, in particular, Sobolev (1962), have in fact observed that phytin is formed in large amounts during the ripening of the seeds of higher plants, in parallel with the accumulation of reserve substances such as starch and fats. The accumulation of phytin under these conditions, in the light of our hypothesis, can be regarded as a means of detoxifying orthophosphate, which is liberated during the synthesis of starch and fats and cannot be removed from the ripening seeds by any other means. This method of P_i detoxification, i.e. deposition as phytin, may be regarded as much more advanced from the evolutionary point of view. This follows from the work carried out by Kulaev and co-workers (Kulaev *et al*., 1964c) on the metabolism of phosphorus during germination of cotton seeds. It was shown, in particular, that one of the products of phytin cleavage during germination was 3-phosphoglycerie acid. From the evolutionary point of view, this is obviously a greatly improved process, as a result of which large amounts of 3-phosphoglyceric acid accumulate during seed germination. This compound is known to occupy the central position in the Calvin photosynthetic cycle and other energetic and plastic processes in the higher plants. However, high-molecular-weight PolyPs are also present at the sites where large amounts of phytin accumulate in the higher plants (e.g. the seeds) (Asamov and Valikhanov, 1972), which is highly significant. For example, high-molecular-weight polyphosphates occur in the brains of mammals, i.e. at a site where usually a typical phosphagen, creatine phosphate, is found as well.

In the course of evolution from prokaryotes to eukaryotes, the energetic role of PolyP decreased. However, other functions came to the fore, such as phosphate storage, cation chelation, regulation of enzyme activities, gene expression and membrane transport (Figure 10.6). The significance of the regulatory functions of PolyP increased in eukaryotes.

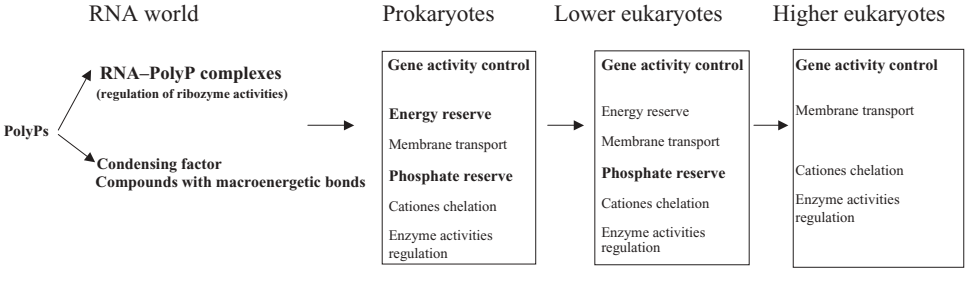

Figure 10.6 Changes in the functions of PolyPs during evolution.

These functions are predominant in animal cells, where PolyP participates mainly in the transport across the membranes and in the regulation of gene expression.

Therefore, future comparative investigations of PolyP metabolism in archae, bacteria and eukaryotes will provide better understanding of the evolution of the functions of PolyPs. In microbial cells, inorganic PolyP plays a significant role increasing cell resistance to unfavourable environmental conditions and regulating different biochemical processes; whereas in animal cells, which possess a hymoral and neuorous regulatory mechanisms, the PolyP functions become narrow but do not disappear. The ability to synthesize high-molecular-weight PolyPs is apparently of great importance even in the higher animals and plants, since their cells are thereby rendered less dependent on external factors.

The PolyP metabolism in eukaryotic cells has specific peculiarities in each cellular compartment. For example, a large amount of evidence has been obtained for yeast, suggesting that the synthesis and degradation of PolyP in each specialized organelle and compartment of the cells is mediated by different sets of enzymes. This is consistent with the endosymbiotic hypothesis of eukaryotic cell origin.

According to this hypothesis (Margulis, 1993), the eukaryotic cell is a result of symbiosis of different prokaryotic cells, where mitochondria originated from eubacteria, and chloroplasts – from cyanobacteria, and vacuoles – from archae.

The main argument in favour of this hypothesis is the presence in chloroplasts and mitochondria of DNA, which is different from the nuclear DNA and similar to the DNA of prokaryotes, as well as the similarity of chloroplast, mitochondrial and bacterial ribosomes and their significant difference from cytoplasmic ribosomes of eukaryotic cells. The chloroplasts and mitochondria were found to be close to bacterial cells in additional other biochemical features: the presence in their membranes of phospholipid cardiolipin, which is absent in the plasma membrane of eukaryotes, and ATPases of one and the same type F_1F_0.

The homology of V-ATPases and pyrophosphatases of the vacuoles and plasma membranes of archae indicates a possibility of endosymbiotic descent of vacuoles from ancient representatives of this domain (Nelson, 1992).

PolyP metabolism in mitochondria and chloroplasts has been little studied as yet. The question of retention in these organelles of some peculiarities of PolyP metabolism characteristic of bacterial cells is still open. In particular, computer analysis of the genomes of chloroplasts and mitochondria in some plants and yeast has not revealed any sequences similar to the genes of bacterial polyphosphate kinase *ppk1* and exopolyphosphatase *ppx* (Kulakovsky, unpublished results). These sequences are highly conserved in bacteria (Reizer *et al*., 1993; Kornberg *et al*., 1999), and their absence points to a possible loss of the corresponding genes in the course of evolution.

It should be mentioned that the ribosomes of chloroplasts and mitochondria synthesize only a comparatively small part of the proteins required for the formation and function of these organelles. This may be due to the fact that the process of symbiosis occurred in a very distant epoch and many genes have shifted from autonomous genomes into nuclei since that time.

For example, the genes encoding putative guanosine 3′,5′-bispyrophosphate (ppGpp) synthase–degradase, which is a member of the RelA-SpoT family of bacterial proteins, were identified in the nuclear genomes of the unicellular photosynthetic eucaryote *Chlamydomonas reinhardtii* (Kasai *et al*., 2002) in the halophyte *Suaeda japonica* (Yamada *et al*.,

2003). The encoded protein of *Chlamydomonas reinhardtii* (Kasai *et al*., 2002) possesses a putative chloroplast-targeting signal at its NH_2-terminus and can be translocated into chloroplasts. The presence of ppGpp synthase–degradase activities in eukaryotic organisms suggests that the eubacterial stringent control mediated by ppGpp and tightly bound with PolyP has been conserved during the evolution of chloroplasts from photosynthetic bacterial symbiont.

It is not improbable that further investigation of PolyP metabolism in mitochondria and chloroplasts would reveal novel features of similarity with eubacteria in favour of the endosymbiotic theory of the origin of eukaryotes.

In general, investigations of the metabolism of polyphosphates in living organisms at different stages of evolution are of great importance for progress in evolutionary biochemistry.

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