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# MINIREVIEW

# Inorganic Polyphosphate: Toward Making a Forgotten Polymer Unforgettable

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Inorganic polyphosphate (poly P) is a linear polymer of many tens or hundreds of orthophosphate ($P_i$) residues linked by high-energy phosphoanhydride bonds (Fig. 1). Likely a prominent precursor in prebiotic evolution, poly P is now found in volcanic condensates, deep-oceanic steam vents, and every living thing—bacteria, fungi, protozoa, plants, and mammals (27). Yet, poly P has been ignored in textbooks and dismissed as a "molecular fossil." This minireview intends to make a case for poly P as a "molecule for many reasons" (24). Numerous and varied biological functions are performed by poly P, depending on the need and its location—species, cell, or subcellular compartment. Among these functions are acting as a reservoir of energy and phosphate, as a chelator of metals (e.g., $Mn^{2+}$ and $Ca^{2+}$), as a buffer against alkali, as a capsule of bacteria, in competence for bacterial transformation, in ecological disposal of pollutant phosphate, and, of great interest, in physiologic adjustments to growth, development, stress, and deprivation.

## METACHROMATIC GRANULES ARE INORGANIC POLYPHOSPHATE

Poly P was first seen as metachromatic granules in microorganisms in the form of particles stained pink by basic blue dyes and was called "volutin" early in this century (33). For some time poly P granules were mistaken for nucleic acids. With the advent of electron microscopy, these particles were seen to be highly refractive and appeared to volatilize while viewed under the electron beam; they were then identified as poly P (51). Like other polyanions, poly P shifts the absorption of a bound basic dye, such as toluidine blue, to a higher wavelength.

Historically, the poly P particle was recognized as a diagnostic feature of medically important bacteria, such as *Corynebacterium diphtheriae*. Decades later, poly P became of interest in biochemistry in connection with the major biochemical riddle of the 1940s, i.e., how $P_i$ is fixed by an anhydride bond to ADP in aerobic (oxidative) phosphorylation. Such studies led first to the source of inorganic $PP_i$ (23) and then to a curiosity about how the many more phosphoanhydride-linked residues in poly P were assembled. Although *Escherichia coli*, a major source of biochemical insights, lacks any visible content of poly P, it still proved to be a rich source of an enzyme which makes poly P (poly P kinase [PPK]) and catalyzes the more favored conversion of poly P to ATP (25, 26) as follows:

$$n\text{ATP} \leftrightarrow \text{poly P}_n + n\text{ADP}$$

## ASSAY OF POLY P

Despite the prominence of poly P in many organisms, such as in the vacuolar deposits in yeast cells that may represent 10 to 20% of cellular dry weight, this molecule has received relatively scant attention. Studies by Kulaev, Harold, Wood, and a few others (17, 29, 53) disclosed the ubiquity of poly P and identified a few related enzyme activities. Yet, poly P has remained a largely forgotten polymer. One of the reasons for this is the lack of evidence for any essential metabolic role. Another reason has been the inadequacy of methods to establish the authenticity and size of poly P and its abundance at very low concentrations.

In addition to the staining and appearance of the granular accumulations observed by light and electron microscopy, nuclear magnetic resonance (NMR) analysis has been used to identify poly P in intact cells. However, NMR detection requires high concentrations and fails to measure the poly P in aggregates and in metal complexes. Otherwise, identification of poly P rests on crude and cumbersome separations in cell extracts from the known phosphate-containing polymers followed by determination of the acid-lability characteristic of phosphoanhydride bonds (i.e., conversion to $P_i$ in 7 min at 100°C in 1 M HCl). These assay methods are not sufficiently quantitative to be conclusive, especially for low concentrations of poly P.

Enzymology offers an attractive route to analysis as well as to physiologic functions. Enzyme isolation has on many occasions revealed a novel mechanism or sometimes an insight into a metabolic or biosynthetic pathway. Now, the purified enzyme has also opened the route of reverse genetics; its peptide sequence leads to its gene and thereby to the means to knock out the gene or overexpress it. By manipulating expression of the gene and the cellular levels of its product, phenotypes are created which may provide clues to metabolic functions. More immediate and decisive, as in the studies of poly P, the enzyme can be a unique and invaluable reagent for analytic and preparative work. Toward this end, several enzymes have been purified and used for studies of poly P metabolism (1, 3, 54).

One enzyme, PPK, purified to homogeneity from *E. coli*, catalyzes the readily reversible conversion of the terminal ($\gamma$) phosphate of ATP to poly P (1) (see equation above). The enzyme, a tetramer of 80-kDa subunits bound to cell membranes, is responsible for the processive synthesis of long poly P chains (ca. 750 residues) in vivo; labeling with $^{32}$P in vitro provides such chains for use as substrates and standards. With ADP in excess, PPK converts near 90% of poly P to ATP, identified by the use of either [$^{14}$C]ADP or $^{32}$P-poly P as substrates. A second *E. coli* enzyme, exopolyphosphatase (PPX), encoded by a gene in the *ppk* operon (see below), hydrolyzes the terminal residues of poly P to $P_i$ processively and nearly completely with a strong preference for a long-chain substrate (3).

A third enzyme, an exopolyphosphatase (sc PPX1) isolated

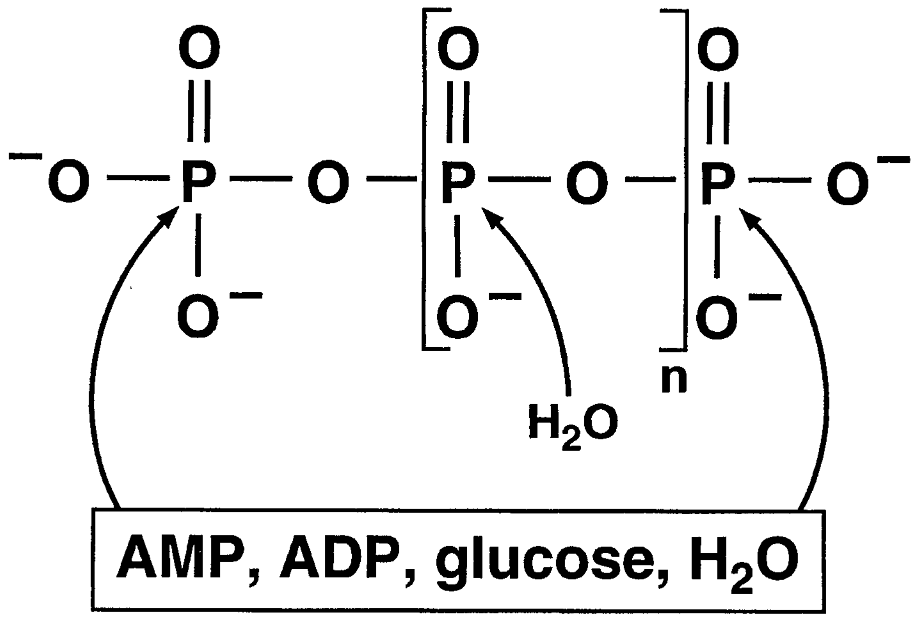


FIG. 1. Inorganic poly P as a substrate. Chains are attacked at their termini by AMP, ADP, glucose, or $H_2O$, catalyzed respectively by poly P-AMP phosphotransferase, poly P kinase, poly P glucokinase, and exopolyphosphatases and internally by endopolyphosphatases.

from *Saccharomyces cerevisiae*, is the most powerful of these analytic reagents, releasing 30,000 $P_i$ residues per min per enzyme molecule at 37°C (54). It acts with about 40 times the specific activity of the *E. coli* PPX, exhibits a far broader size range among poly P chains (i.e., 3 to 1000 residues), and enables poly P to be measured accurately at a level of 0.5 pmol when labeled with $^{32}P$. Cloning the gene for this polyphosphatase enabled the enzyme to be overproduced in *E. coli* (55). Application of this potent polyphosphatase to remove the poly P that contaminates DNA preparations from yeast and other poly P-rich organisms (42) may solve a problem that has bedeviled the action of restriction nucleases and the use of shuttle vectors for expression of fungal genes in *E. coli*.

Two more enzymes available as reagents for analysis of poly P are the glucokinase, which attacks the terminal residues of the poly P chain with glucose (19) (Fig. 1), and a phosphotransferase, which attacks the termini with AMP (6).

With respect to the sources of poly P, it is imperative, as it is with analysis of other cellular constituents, especially in eukaryotes, to distinguish subcellular compartments: nucleus, mitochondria, lysosomes (vacuoles), other vesicular entities, and the cytosol. A dramatic example is the yeast vacuole, which may contain more than 99% of the cellular poly P and mask the not insignificant remainder in the mitochondria and the nucleus.

## BIOSYNTHESIS OF POLY P

The only pathway for the synthesis of poly P that has been established is the polymerization of the terminal phosphate of ATP through the action of PPK in *E. coli* (1). The gene encoding the kinase is part of an operon in which the gene for PPX is located immediately downstream (2). Interruption of the operon produces mutants which, for lack of long-chain poly P, are defective in survival in the stationary phase (see below). How the operon is regulated to balance two counteracting enzymes that are roughly equal in activity has yet to be explained.

Although a PPK activity has been purified from other bacteria (41, 47) and reported in yeast (29), such an enzyme action has yet to be proven in animal systems. While PPK as the device to produce long chains of poly P (ca. 750 residues) has been validated in *E. coli* by genetic studies, mutants lacking this enzyme can still make short poly P chains, about 60 residues long, by an undefined pathway (9).

Several other plausible routes for the biosynthesis of poly P need to be considered. These are from ADP by reversal of an AMP phosphotransferase, from acetyl P, from 1,3-diphosphoglycerate, from dolichyl pyrophosphate (43), and, of special interest, by proton motive forces, known to fix $P_i$ in inorganic $PP_i$ (34) as well as in ATP.

## FUNCTIONS OF POLY P

**ATP substitute and energy source.** PPK converts poly P to ATP by catalyzing an ADP attack on the termini of the poly P chain (Fig. 1). An aggregate of poly P associated with this membrane-bound enzyme could generate large amounts of ATP at that very spot. Another source of ATP could come from an AMP attack on poly P (Fig. 1) by AMP-phosphotransferase to produce ADP, which is readily converted to ATP by coupling with PPK or with adenylate kinase:

$$\text{poly } P_n + \text{AMP} \rightarrow \text{poly } P_{n-1} + \text{ADP}$$

$$\text{poly } P_{n-1} + \text{ADP} \rightarrow \text{poly } P_{n-2} + \text{ATP}$$

$$2\,\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$$

AMP-phosphotransferase has been purified from an *Acinetobacter* strain (6) and has been identified in *E. coli* (21) and *Myxococcus xanthus* (44); adenylate kinase is a potent and ubiquitous enzyme.

Through the action of these enzymes, poly P is a potential phosphagen in cells where and when its levels far exceed those of ATP. Compared to the usual cellular ATP levels of 5 to 10 mM, the massive vacuolar deposits in yeast cells, expressed on the basis of total cell volume, can exceed 200 mM; in *Myxobacteria* cells in stationary phase the granular aggregates of poly P can reach 50 mM (44, 49).

In view of its energy equivalence to ATP, poly P qualifies as an ATP substitute in all of its kinase roles, involving a variety of acceptors. In addition to the observed transfers to AMP and ADP, poly P, as already noted, can replace ATP in the phosphorylation of glucose in many bacteria. All of these glucokinases use either ATP or poly P as donors; the more phylogenetically ancient species appear to show a preference for poly P over ATP (19). One might expect to find poly P kinases for other sugars, sugar derivatives (e.g., nucleosides and coenzyme precursors), proteins, and carboxylic acids. Indeed, phosphorylation of a 40-kDa protein in the ribosomal fraction of the archaebacterium *Sulfolobus acidocaldarius* was observed with poly P as the donor (46).

An energy recycling mechanism operating in the efflux of organic end products (e.g., lactate in enteric bacteria) in symport with protons can generate a proton motive force (48). Such a mechanism may function in the utilization of poly P. The efflux of a protonated metal chelate of $P_i$ released from poly P creates a proton motive force that may be coupled to the accumulation of amino acids from the medium or the synthesis of ATP.

**A reservoir for $P_i$.** A stable level of $P_i$, essential for metabolism and growth, can be insured by a reservoir in which poly P can be converted to $P_i$ by associated exopolyphosphatases. The polymer, as an aggregate complexed with multivalent counterions, enjoys a clear osmotic advantage over free $P_i$. Regulation of the *ppk* operon, which encodes both the poly P kinase and exopolyphosphatase in *E. coli* (3), appears to be responsive to the *pho* regulon that controls more than 20 genes related to phosphate metabolism (39). Multiple exopolyphosphatases in *E. coli* (3, 20) and in yeast (4, 54, 55) are potentially available to produce $P_i$ in various cellular locations.

**Chelator of metal ions.** As expected of a phosphate polyanion, poly P is a strong chelator of metal ions. *Lactobacillus plantarum*, unusual in lacking a superoxide dismutase, a metalloenzyme that catalyzes the removal of the damaging superoxide radical, has an inorganic catalyst instead—an extraordinarily high, 30-mM level of $Mn^{2+}$ chelated to 60 mM poly P (5). With regard to chelation of $Ca^{2+}$, the regulation of cellular $Ca^{2+}$ in yeast cells by vacuolar $Ca^{2+}$ depends on its binding to poly P; the poly P acts as a $Ca^{2+}$ sink within the vacuole lumen (12). Chelation of $Ca^{2+}$ and $Mg^{2+}$, structurally essential in the cell walls of gram-positive bacteria, is regarded as the basis for the antibacterial action of poly P (31). Chelation of other metals (e.g., Zn, Fe, Cu, and Cd) may either reduce their toxicity or affect their functions.

**Buffer against alkali ions.** Algae, like yeasts, accumulate poly P in their vacuoles. In the halotolerant green alga *Dunaliella salina*, deposits of poly P reach levels near 1 M in $P_i$ equivalents. When stressed at alkaline pH, amines enter the algal vacuoles and are neutralized by protons released by the enzymatic hydrolysis of poly P (36). The specific polyphosphatase, presumably activated by the amines, produces tri-poly P by mechanisms that have yet to be determined. Thus, poly P, as a result of its hydrolysis, can provide a high-capacity buffering system that sustains compartmentation of amines in vacuoles and protects the cytoplasmic pH. This alga, cultivated in large outdoor ponds, is an important commercial source of β-carotene for health foods and for food coloring.

**Channel for DNA entry.** Transforming competent *E. coli* with DNA for the cloning and expression of genes is currently one of the world's favorite indoor sports. Despite the widespread use of a $Ca^{2+}$ recipe to induce competence, there is little understanding of the mechanism whereby the highly charged DNA molecule penetrates the lipid bilayer membranes surrounding the cell. A significant advance was the discovery of polyhydroxybutyrate (PHB) complexed with $Ca^{2+}$ and poly P in the membranes of competent cells (40). In a proposed structure, $Ca^{2+}$ is bonded by ion dipoles to the carbonyl ester groups of PHB and by ionic interactions with poly P. This complex produces profound physical changes in the competent-cell membranes—increased rigidity at ambient temperatures and biphasic melting (9, 40). Whether and how these alterations facilitate DNA entry remains unclear.

In current studies, the PHB-$Ca^{2+}$-poly P complex has been reconstituted in large, unilamellar vesicles by adding PHB, $Ca^{2+}$, and poly P to phospholipids (9). The capacity of these vesicles for the uptake of small and large molecules, charged and neutral, needs to be extended. Although mutants lacking the long-chain poly P can attain competence, their membranes still contain a short poly P chain of about 60 residues, synthesized by a novel route during the development of competence (9).

**Regulator for stress and survival.** Regulatory roles for poly P, a phosphate polyanion with some resemblance to RNA and DNA, seem reasonable. Poly P readily interacts with basic proteins (e.g., histones) and with basic domains of proteins, as in polymerases, and has been observed in association with nonhistone nuclear proteins (35). Such roles could affect gene functions in positive or negative ways. Inasmuch as poly P is present in several sizes and complex forms, is located in the nucleus and other cell compartments, and fluctuates in response to nutritional and other parameters, it seems possible that poly P might function in the network of responses to stresses and the many signals that govern stages in the cell cycle and development.

We were surprised to discover that a novel exopolyphosphatase, identified in *E. coli* mutants that lacked the exopolyphosphatase gene (*ppx*) encoded in the *ppk* operon, was guanosine pentaphosphate (pppGpp) hydrolase (20), the enzyme that produces guanosine tetraphosphate (ppGpp; "magic spot"), the powerful effector in the bacterial stringent response (8). Upon deprivation of an amino acid, the RelA enzyme generates pppGpp, which upon conversion to ppGpp represses many genes and activates others. The significance of poly P and of the polyPase activity of pppGpp hydrolase need to be studied. Also unclear is the part that poly P may play in other stringencies, such as those of carbon, energy, or phosphate.

The possibility of poly P involvement in the stringent response suggested that it might be among the multiple metabolic adjustments in the stationary phase of the cell cycle. "Life after log" in *E. coli* (45) is a dynamic interval in which many genes are induced to cope with environmental stresses to ensure survival. Although the *ppk* mutant lacking long-chain poly P shows no phenotypic changes in the exponential phase of growth, striking deficiencies are evident when it is examined in the stationary phase (11, 38). The mutant survives less well, is less resistant to heat, oxidants, and osmotic challenge, and shifts to a small-colony phenotype, suggestive of an adaptive genotypic change (18). Thus, poly P may enter in the cascade of events that prepare cells for coping with "life in the slow lane."

**Regulator of development.** Developmental changes in microorganisms—fruiting body and spore formation in *Myxobacteria* species (e.g., *M. xanthus*), sporulation in bacteria (e.g., *Bacillus* species), and fungi, and heterocyst formation in cyanobacteria (e.g., *Anabaena* species)—occur in response to starvation of one or another nutrient. In view of the involvement of poly P in the stationary stage of *E. coli*, poly P may well participate in other instances of cellular adjustments to deprivation.

Upon the conclusion of vegetative growth in *M. xanthus*, the levels of poly P and of poly P-AMP phosphotransferase activity increase more than 10-fold (44). When present at concentrations as high as 50 mM (49), poly P may be an energy source for fruiting bodies and for deposition in spores. Inasmuch as an increase in ppGpp precedes poly P formation and mutants that fail to produce ppGpp also fail to increase their poly P levels (44), it seems that ppGpp has a regulatory role in poly P formation.

**Cell capsule.** Poly P is a component of the capsule (47) which is loosely attached to the surface of *Neisseria* species and represents about half of the cellular poly P. Whether the capsule contributes to the pathogenesis of infections, and if so, whether it is by combatting phagocytosis, by chelating metals needed in complement fixation, or by some other way has yet to be discovered.

## POLY P IN ANIMAL CELLS AND TISSUES

Although the presence of poly P in fungi and algae has been widely noted, the distribution and abundance of poly P in more complex eukaryotic forms has remained uncertain. The very low levels of poly P in animal cells (13) and subcellular compartments and the lack of definitive and sensitive methods have left its metabolic and functional roles entirely obscure.

Our exploratory studies with improved enzymatic assay methods have confirmed that poly P is present in a wide variety of cell cultures and animal tissues. The concentrations of poly P generally range from 10 to 100 $\mu$M (expressed in $P_i$ equivalents) and in sizes of 100 to near 1,000 residues. Among the subcellular organelles, poly P has been identified in lysosomes (37) and in mitochondria (32) and is relatively enriched in nuclei (30). In rat brain, poly P is present throughout the course of embryonic and postnatal development (30). Uptake of $P_i$ into poly P has been observed in cultured mammalian cells (10, 30), lysosomes (37), mitochondria (32), and broken-cell preparations (10). A strikingly rapid turnover of poly P was seen in a confluent culture of PC 12 cells, a neuron-like cell line derived from an adrenal pheochromocytoma. Although these cells have a generation time of 48 to 72 h, the turnover was nearly complete in 1 h (30). Studies of the dynamics of poly P formation and utilization in a variety of cells should reveal novel functions for the polymer in different stages of growth and metabolism.

## EVOLUTIONARY ROLE OF POLY P

RNA may have preceded DNA and proteins in prebiotic evolution, but it seems likely that poly P appeared on earth before any of these organic polymers. Poly P arises simply from the dehydration and condensation of $P_i$ at elevated temperatures and is evident in volcanic condensates (56) and oceanic steam vents. The anhydride-bond energy and the phosphate of poly P are plausible sources for nucleoside triphosphates, the activated building blocks of RNA and DNA (28, 50); mixed carboxylic-phosphate anhydrides provide a route to chemical polypeptide synthesis starting with amino acids and poly P (15).

Among the species of poly P, mention should be made of the very simplest, namely, inorganic pyrophosphate ($PP_i$). Once regarded solely as a metabolic product of biosynthetic reactions (22) and as being fated to be hydrolyzed by potent and ubiquitous inorganic pyrophosphatases to drive these pathways, $PP_i$ was revealed by later studies to be a substitute for ATP (52). A role for $PP_i$ as well as for poly P in prebiotic events leading to the evolution of ATP deserves attention. Inasmuch as the synthesis of one ATP by extant de novo pathways requires the input of at least eight ATPs, how was ATP made in the first place?

Poly P accumulations, also prominent in archaebacteria, may well be the substrates for enzymatic attack by nucleosides to produce nucleoside mono-, di-, or triphosphates. A systematic search among these ancient organisms might uncover enzymes that carry out such salvage reactions in the biosynthesis of nucleotides, coenzymes, and other factors.

## INDUSTRIAL APPLICATIONS OF POLY P

Aspects of the chemical and physical properties of poly P that emerge from its industrial uses are of general interest and in some instances may impinge directly on its place and functions in biological systems.

**Depollution of $P_i$ in the environment.** $P_i$ accumulation in waste waters containing runoff of fertilizers and discharges of industrial agents is a global problem that results in destructive algal blooms in bays, lakes and waterways. The use of lime, alum or ferric chloride to remove $P_i$ is expensive and inefficient. Currently, sanitary engineers employ a biological process in which aerobically activated bacteria take up the $P_i$ and convert it to poly P, which is then removed along with the bacteria as a sludge. Because the microbial process is slow and inadequate, genetically engineered improvements are needed in *Acinetobacter* strains and other gram-negative organisms that dominate the flora. A start has been made in this direction by showing that the *ppk* gene on a runaway plasmid introduced into a selected coliform species vastly increases the rate and extent of $P_i$ removal from the medium (16). When coupled with an improved *pst* uptake system for $P_i$, poly P accumulations have reached 38 to 48% of the dry weight of the cell.

**Antibacterial action.** Poly P is a safe additive to meats, enhancing water binding, emulsification, and color retention while retarding oxidative rancidity. In its use in virtually all processed meat, poultry, and fish products, poly P also serves as an antibacterial agent (31).

**Source of ATP.** The cost of ATP for use as an enzymatic phosphorylating agent on an industrial scale is prohibitive, as is the cost of agents, such as creatine phosphate and phosphoenolpyruvate, that might be used in an enzymatic ATP-regenerating system. In their place, poly P has been employed to regenerate ATP using PPK immobilized on a column (7). In this system, a commercial form of poly P costing 25¢/lb can provide ATP equivalents that would cost over $2,000/lb.

**Insulating fibers.** Phosphate fibers form bones and teeth. Polyphosphates are added to cheese, meats, toothpaste, and drinking water. A calcium polyphosphate fiber has been synthesized with all the properties of asbestos (14) and could be a safe substitute, but has been abandoned by its inventor, Monsanto Chemical Company, which cited its fear of litigation from lawyers trained to bring suits for injuries from mineral fibers. Unfortunately, the decision to abandon poly P fibers as an insulator also extends to its use for nonflammable infant sleepwear, hospital mattress covers, and fabric for aircraft interiors.

## SUMMARY

Pursuit of the enzymes that make and degrade poly P has provided analytic reagents which confirm the ubiquity of poly P in microbes and animals and provide reliable means for measuring very low concentrations. Many distinctive functions appear likely for poly P, depending on its abundance, chain

length, biologic source, and subcellular location. These include being an energy supply and ATP substitute, a reservoir for $P_i$, a chelator of metals, a buffer against alkali, a channel for DNA entry, a cell capsule and, of major interest, a regulator of responses to stresses and adjustments for survival in the stationary phase of culture growth and development. Whether microbe or human, we depend on adaptations in the stationary phase, which is really a dynamic phase of life. Much attention has been focused on the early and reproductive phases of organisms, which are rather brief intervals of rapid growth, but more concern needs to be given to the extensive period of maturity. Survival of microbial species depends on being able to manage in the stationary phase. In view of the universality and complexity of basic biochemical mechanisms, it would be surprising if some of the variety of poly P functions observed in microorganisms did not apply to aspects of human growth and development, such as aging and the aberrations of disease.

Of theoretical interest regarding poly P is its antiquity in prebiotic evolution, which along with its high energy and phosphate content make it a plausible precursor to RNA, DNA, and proteins. Practical interest in poly P includes many industrial applications, among which is its use in the microbial depollution of $P_i$ in marine environments.

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