ACCUMULATION AND DEGRADATION OF POLYPHOSPHATE IN ACINETOBACTER SP.

Promotor:	**dr A.J.B. Zehnder,** **hoogleraar in de microbiologie**
Co-promotor:	**dr M.H. Deinema,** **universitair docent**

J.W. van Groenestijn

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr C.C. Oosterlee,
in het openbaar te verdedigen
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des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

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STELLINGEN

1. De biochemische modellen van het mechanisme van de biologische defosfatering van afvalwater, zoals gepresenteerd op het internationale congres over dit proces, gehouden van 28 t/m 30 september 1987 te Rome, kunnen niet verklaren waarom het Phostrip proces zo goed werkt.
   - R. Ramadori (ed.) Biological phosphate removal from waste waters. Proceedings of an IAWPRC specialized conference, Rome, 28-30 Sept. 1987. Adv. Wat. Poll. Control. Pergamon Press.

2. In tegenstelling tot de bewering van Kulaev hoeft de productie van ATP uit extracellulair adenosine en intracellulair polyfosfaat door afstervende cellen van Corynebacterium sp. niet te duiden op een polyfosfaat:AMP fosfotransfersase activiteit.
   - V.D. Butukhanov, M.A. Bobyk, V.Zh. Tsyrenov and I.S. Kulaev (1979) Possible role of high-molecular-weight polyphosphate in ATP synthesis from exogenous adenine by a culture of Corynebacterium species, strain VSTI-301. Biokhimiya 44:1321-1327.
   - I.S. Kulaev and V.M. Vagabov (1983) Polyphosphate metabolism in microorganisms. Adv. Microbiol. Physiol. 24:83-171.

3. De conclusie van Neijssel en Tempest dat glucose gelimiteerde groei van Klebsiella aerogenes NCTC 418 koolstof gelimiteerd moet zijn spreekt de hypothese van Linton en Stephenson tegen.
   - J.D. Linton and R.J. Stephenson (1978) A preliminary study on growth yields in relation to the carbon and energy content of various organic growth substrates. FEMS microbiol. Lett. 3:95-98.
   - O.M. Neijssel and D.W. Tempest (1976) The role of energy-spilling reactions in the growth of Klebsiella aerogenes NCTC 418 in aerobic chemostat culture. Arch. Microbiol. 110:305-311.

4. Het homogeniseren van aktief slib met een vortex, dat in sommige publicaties wordt beschreven als enige voorbehandeling ten behoeve van celtellingen op platen, kan tot foutieve conclusies leiden omtrent de aantallen cellen in dit slib.

5. Zolang grote hoeveelheden fosfaat aanwezig blijven in de effluenten van afvalwater zuiveringsinstallaties, is de verwijdering van de laatste ppm's NH_4^+ en organische koolstofverbindingen uit afvalwater vaak een zinloze bezigheid. De introductie van fosfaat in de heffingsformule voor de lozing van effluent is het gewenste politieke instrument dat deze vaak toegepaste handelswijze kan voorkomen.

6. Door het introduceren van fosfaatvrije wasmiddelen zal biologische defosfatering van afvalwater steeds gunstiger worden t.o.v. chemische defosfatering.

7. De houding van regeringen inzake de emissie van afgassen is een typische 'na-mij-de-zonvloed-politiek'.

8. De tijdelijke volksverontwaardiging tijdens de introductie van de eerste treinen in Europa, wordt vaak ten onrechte aangehaald als er weer een controversiële technologische innovatie geïntroduceerd moet worden.

9. Natuurtalenten zijn vaak slechte docenten omdat zij niet inzien waarom een leerling de stof niet begrijpt.

10. Voor een wetenschapper met een achternaam van vier lettergrepen waarin 'oe' en 'ij' voorkomen zal internationale bekendheid niet snel zijn weggelegd.

Stellingen behorende bij het proefschrift "Accumulation and degradation of polyphosphate in Acinetobacter sp." van J.W. van Groenestijn.

Wageningen, 17 juni 1988

VOORWOORD

Voordat u in dit proefschrift gaat lezen moet u beseffen dat de verschillende activiteiten die hebben geleid tot de totstandkoming van dit boek niet het werk is geweest van een enkeling, de auteur, maar in grote mate ondersteund zijn geweest door anderen. Deze mensen wil ik hier bedanken:
Prof. Zehnder en Mia Deinema voor het mogelijk maken van dit onderzoek, voor hun wetenschappelijke begeleiding, het kritisch doorlezen van het manuscript en hun stimulerende invloed.
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En vele andere medewerkers van de vakgroepen Microbiologie en Waterzuivering die met raad en daad hebben bijgedragen aan de totstandkoming van dit proefschrift. Hun gezelschap en belangstelling vormden een hartverwarmende stimulans en een belangrijke steunpilaar onder het soms zo zware promotie-werk.
Ook mijn ouders komt alle dank toe: zij hebben mij gemotiveerd en gestimuleerd om te gaan studeren.

CONTENTS

CHAPTER 1

INTRODUCTION

PREAMBLE

A study on polyphosphate accumulation and degradation by Acinetobacter sp. is important for the following two reasons:

(1) Polyphosphate accumulating strains of Acinetobacter play an important role in the biological phosphate removal from waste water, a process that is performed in a number of wastewater treatment plants and results in activated sludge enriched with polyphosphate accumulating bacteria.

(2) Acinetobacter sp. are able to accumulate exceptionally high amounts of phosphorus (up to 100 mg per g dry biomass), mainly in the form of polyphosphate, and this property makes this bacterium very suitable for research on the reserve functions of polyphosphate.

The role of inorganic polyphosphate in biology has not yet been elucidated very well, especially its role as an energy reserve in vivo, which still remains to be a controversial subject. In this chapter the available information about both the application of polyphosphate accumulating bacteria in wastewater treatment and the physiological role of polyphosphate in microorganisms are summarized.

THE ROLE OF PHOSPHATE IN EUTROPHICATION OF SURFACE WATERS

In the early ninetheenseventies the awareness for water pollution grew exponentially and wastewater treatment plants were introduced on a large scale in the industrialized countries. These treatment plants were designed to reduce the biological oxygen consuming potential of the waste waters, i.e. organic carbon and reduced nitrogen. The effluents of these plants still may contain large amounts of phosphate and nitrate, which are both the major compounds responsible for the eutrophication of surface waters. Eutrophication, an overload of surface waters with nutrients, has become a growing problem, and many negative effects have been reported. Examples are: algal blooms, decrease of ecological quality and organism diversity in surface waters, and livestock deaths due to ingestion of toxic algae. The treatment of eutrophied water in conventional potable water treatment plants also presents problems. They include penetration of fil-

ters by algae, filter clogging, taste, odour, colour, turbidity and the formation of trihalomethanes from organic compounds released by algae as a result of chlorination. Death of algae can lead to anaerobic conditions in the water causing fish kill and odour nuisance. Excretion of proteins by algae in the sea can cause foam which may interfer with fishery. The flow of irrigation water in canals can be interferred by the abundant development of filamentous algae (Wiechers and Best 1985). In a majority of surface waters, phosphorus is the limiting nutrient. Its addition to this waters will cause eutrophication (Vollenweider 1985). According to Van Dieren et al. (1985) the eutrophication policy in The Netherlands should consist of four tracks: (1) phosphate ban from detergents, (2) water treatment (phosphate removal from waste water, dredging of sediments containing phosphate), (3) restriction of phosphate emission by agriculture (e.g. runoff) and (4) reduction of foreign influx of phosphate (by rivers). Only an integral approach will reduce algal growth. Much attention is given to the removal of phosphate from municipal waste water, which is currently in the Netherlands the most important source of phosphate as a pollutant (Olsthoorn 1986).

CHEMICAL PHOSPHATE REMOVAL FROM WASTEWATER

The conventional and most applied method for phosphate removal is its precipitation with chemicals, like alum ($Al_2(SO_4)_3.18\ H_2O$), ferric chloride, lime and AVR (Swedish abbreviation for wastewater treatment) which is a mixture of Al^{3+} and Fe^{2+} compounds. In addition to these salts also polymers can be used to improve flocculation or chemicals to correct the pH. Depending on the stages of the wastewater treatment process in which the precipitation process takes place, three categories can be distinguished:

(1) Pre-precipitation: the chemicals are added to the raw waste water and the precipitate is removed in the primary settling tank.

(2) Simultaneous precipitation: the chemicals are added to the tank in which biodegradation takes place and the precipitate is removed in the secundary settling tank.

(3) Post-precipitation: the chemicals are added to the secundary effluent and the precipitate is removed in a separate plant.

The application of the precipitation technique is not wide spread in wastewater treatment in The Netherlands, because of the following disadvantages of the process: high costs, pro-

duction of larger quantities of chemical sludge, formation of worthless by-products like hydroxyapatite and similar highly insoluble phosphates, introduction of extra anions such as Cl^- and SO_4^{2-} into the effluent, and the necessity of an advanced process control. New low-cost processes in which phosphate can be recovered in an useful form have been found in post-precipitation technology. Examples are: the precipitation of phosphate in secundary effluents by means of lime on grains of sand in an upflow reactor (Van Dijk and Braakensiek 1984), or the recovery as $MgNH_4PO_4$ by means of a combined anion exchange/precipitation process (Liberti et al. 1985).

BIOLOGICAL PHOSPHATE REMOVAL FROM WASTEWATER

An alternative for chemical phosphate removal is biological phosphate removal from waste water, which is based on the enrichment of activated sludge with polyphosphate accumulating bacteria. This sludge is able to remove phosphate almost completely from the waste water. The process does not have the above described drawback of chemical phosphate removal. The process of biological phosphate removal has already proved its practical applicability. However, the failures which still occur occasionally, are mostly difficult to understand, due to a lack of experience and a lack of understanding of the fundamental principles of the process.

The process

Treatment plants that spontaneously removed more phosphate than expected have been reported already in 1959 (Srinath et al.) and 1961 (Alarcon). The first investigations concerning this process started in the mid-sixties. From studies with full scale plants it appeared that biological phosphate removal took place in treatment plants with plug flow aeration reactors in which the return sludge and influent were added at the beginning of the reactor (Vacker et al. 1967, Milburg et al. 1971, Garber 1972, Yall et al. 1972). Barnard (1976) discovered that treatment plants designed for nitrogen elimination, with nitrification in aerobic zones and denitrification in anoxic zones, were able to remove large amounts of phosphate as well. A phosphate removal of 97% was possible. The presence of an anaerobic zone in the reactors was compulsory (Davelaar et al. 1978). Biological phosphate removal appeared in reactors with two zones namely an anaerobic zone at the beginning of the reactor, to which

influent and return sludge were added, and an aerobic zone at the end. In the anaerobic zone the sludge released large amounts of phosphate, while in the aerobic zone phosphate was taken up almost completely (Fig. 1).

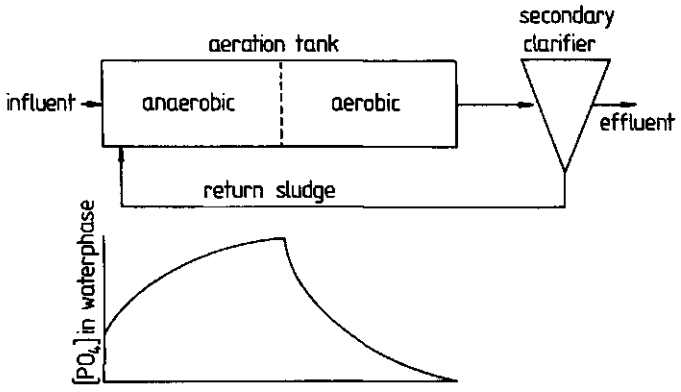

Fig. 1. Flow sheet of an activated sludge plant with biological phosphate removal; and the ortho-phosphate concentration in the aeration tank.

The above described process has not always been recognized as a biological process. Twenty years ago a chemical and a biological model were developped. According to the chemical model the improved phosphate removal was due to chemical precipitation reactions, and the temporary phosphate release was assigned to be due to a pH decrease (Menar and Jenkins 1970). However, evidence for a biologically catalyzed reaction was growing as Levin and Shapiro (1965) indicated that the excess phosphate removal was a biological process: the addition of 2,4-dinitrophenol to the sludge stopped the uptake of phosphate completely. The pH optimum for phosphate removal appeared to be 7.8. At pH 9.0, in comparison, less phosphate was removed which could not be explained by the chemical precipitation model of Menar and Jenkins. Arvin (1983) proposed that phosphate removal in wastewater treatment plants operating without chemicals was actually a combination of a biological and chemical process. He hypothesized that phosphate release and uptake were of biological nature, but because of the high phosphate concentration occurring under anaerobic conditions precipitation of calcium phosphates could take place at a pH higher than 7.5. In 1975 Fuhs and Chen isolated several Acinetobacter strains from phosphate removing activated sludge able to store phosphate intracellularly as polyphosphate in form of granules. Pure cultures accumulated phosphorus up to 50 mg per gram dry biomass in

continuous aerated growth media, without adaptation. Acinetobacter appeared to be the dominant genus in wastewater treatment plants where phosphate is removed biologically (Nicholls and Osborn 1979). Buchan (1983) reported that Acinetobacter could account for 66% of the number of cells in this type of activated sludge. In an acetate fed pilot plant with alternating aerobic and anaerobic conditions the main part of the organisms cultured aerobically were acinetobacters (Wentzel et al. 1987). These microbiological findings were important indications that the observed phosphate removal was indeed of biological nature.

Based on recent advancements in wastewater engineering and applied research two new proceedings have been developped for biological phosphate removal. They are the Phostrip process (Levin and Sala 1987) and the Renpho process (Rensink, in press). In the Phostrip process (Fig. 2) the influent is added in a completely and continuous aerated reactor. A part of the return sludge is made anaerobic to strip the phosphate from the sludge. The sludge is subsequently separated from the orthophosphate by settling and returned to the aerobic reactor.

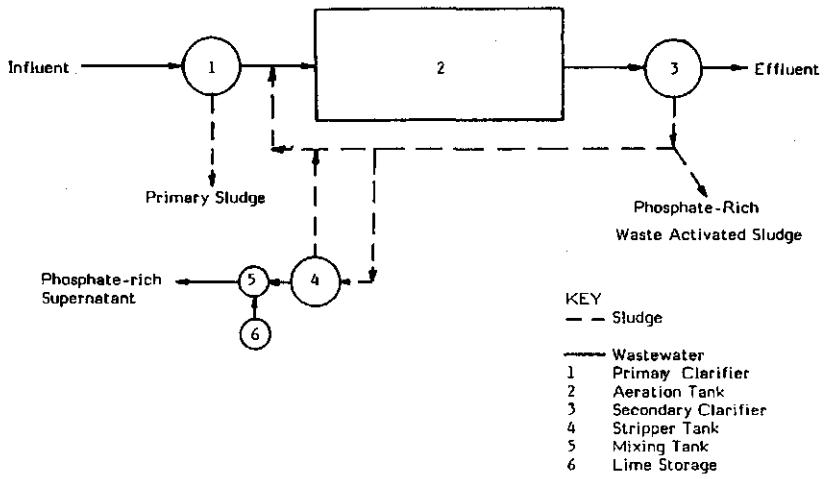

Fig. 2. Flow sheet of a standard Phostrip process.

The Renpho process (Fig. 3) is able to remove to a great extend both phosphorus and nitrogen from waste water. Influent and return sludge are added into an anaerobic zone in which phosphate is released, the mixed liquor is subsequently transported to an aerated zone in which phosphate is taken up and nitrification takes place. In a third, anoxic zone denitrification occurs. A part of the influent is directly added to the denitri-

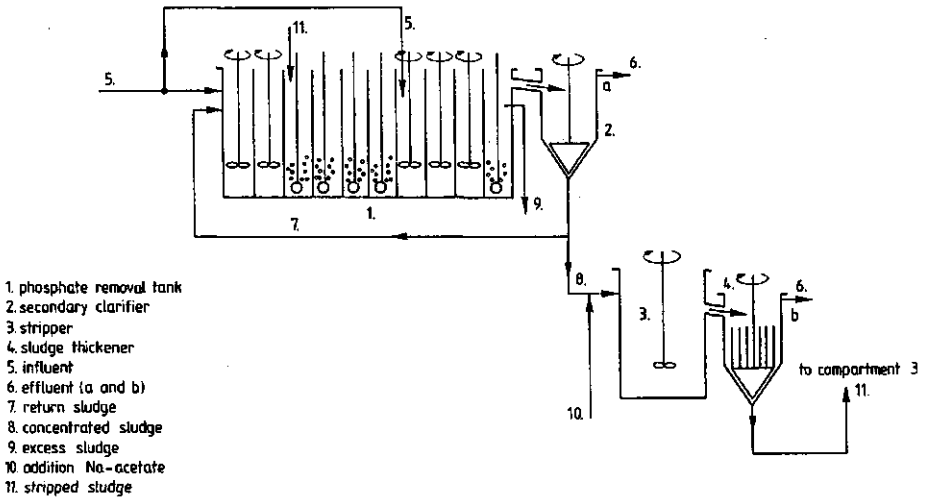

Fig. 3. Flow sheet of the Renpho process.

fication zone, because here the original influent is already devoid of degradable organic carbon compounds and the fresh waste water adds the necessary electron donors for denitrification. Phosphate is not released in this stage, since denitrifying conditions seem to inhibit phosphate release. At the end of the reactor an additional aerobic zone is inserted to remove the last traces of biodegradable organic pollutants. After settling, the sludge is devided into two parts: one part is returned directly to the beginning of the plant, and from the second part phosphate is stripped before it is returned to the first aerated zone. This stripping process is necessary if biological phosphate removal has to be applied in low loaded wastewater treatment processes; because at low sludge loadings the amount of sludge produced is too low to take up all phosphorus from the waste water.

Biochemical models

After the discovery of the biological nature of biological phosphate removal, research on the biochemical mechanisms was started to develop a biochemical model for this process. The necessity of an anaerobic zone was most intriguing and attracted the attention of many scientists. Since most acinetobacters cannot use glucose as substrate but grow well on fatty acids and alcohols, Fuhs and Chen (1975) stated that the anaerobic stage playes an important role in the production of short chain fatty acids by facultative anaerobic bacteria. Rensink (1981) demonstrated a lower fatty acid production from sewage in the anaero-

bic stage. The free fatty acid concentration decreased in the anaerobic zone within a few weeks simultaneously with the development of enhanced biological phosphate removal in a pilot plant. Fuhs and Chen (1975) suggested that the lower fatty acids were consumed anaerobically by polyphosphate accumulating bacteria, and stored in form of PHB (poly-β-hydroxybutyrate). PHB could be detected in pure cultures of Acinetobacter (Fuhs and Chen 1975, Nicholls and Osborn 1979), and it was hypothesized that this compound was used as an energy and carbon source for growth and polyphosphate accumulation, as in the aerobic zone no lower fatty acids were detectable. PHB accumulated in the sludge in the anaerobic stage of the biological phosphate removal process, and was broken down in the aerobic period (Fukase et al. 1982, Comeau et al. 1987, Mino et al. 1987). Lower fatty acids were taken up in the anaerobic period and this uptake correlated with the accumulation of PHB and poly-β-hydroxyvalerate (Comeau et al. 1987). Bordacs and Chiesa (1987) did experiments with ^{14}C labeled acetate which was added to the sludge at the beginning of the anaerobic period. Seventy percent of the ^{14}C was incorporated into PHB after anaerobiosis. The mass of anaerobically released phosphate appeared to be proportional to the mass of acetate added (Rensink 1981, Wentzel et al. 1985), ethanol added (De Vries et al. 1985) or COD added (Hascoet and Florentz 1985).

Concomitently with the above described observations biochemical models were designed. In an early model Nicholls and Osborn (1979) suggested that both PHB and polyphosphate were accumulated in the same organism, probably Acinetobacter, and that both were important in the survival and competition of this organism in activated sludge. In their model PHB was accumulated under anaerobic conditions as an electron sink of anaerobic fermentation products, and polyphosphate acted as a phosphorus reserve. A more sophisticated model was suggested by Rensink (1981) and Marais et al. (1983). In their model polyphosphate acts as an energy reserve, a hypothesis which has been put forward by many microbiologists (Harold 1966, Dawes and Senior 1973, Kulaev 1979) and was demonstrated in vitro in Escherichia coli (Kornberg 1957). With the energy generated by the degradation of polyphosphate lower fatty acids were taken up when the system was anaerobic and were transformed into PHB which was stored in the cells, just as has been suggested by Fuhs and Chen (1975). The model of Rensink (1981) and Marais et al. (1983) explains the PHB accumulation, the phosphate release and its response to lower fatty acid addition in the anaerobic stage and

the subsequent phosphate uptake in the aerobic period. Moreover, it explains why polyphosphate accumulating bacteria like Acinetobacter become dominant in a treatment plant with alternating anaerobic and aerobic conditions. According to the model of Rensink and Marais et al. Acinetobacter would be overgrown by more competitive aerobes like Pseudomonas and Flavobacterium in continuously aerated plants. The presence of an anaerobic zone gives a distinct advantage for Acinetobacter since it can there, in contrast to most other strictly aerobic bacteria, take up fermentation products. They can immediately be utilized for energy production when the sludge enters the aerobic zone where free substrates are scarce. In their model Comeau et al. (1986) hypothesize that the uptake of lower fatty acids dissipates the transmembrane protongradient and that as a consequence orthophosphate has to be transported across the membrane to restore the protongradient. The model of Wentzel et al. (1987) combines the models of Comeau, Rensink and Marais. However, one has to keep in mind that all models are hypothetical and mainly based on assumptions. In most models the utilization of polyphosphate as an energy reserve under anaerobic conditions plays an important role. Many authors are of opinion that this energy is generated by the action of polyphosphatekinase which catalyses: $(poly\text{-}P)_n + ADP \longrightarrow (poly\text{-}P)_{n-1} + ATP$. This enzyme has been chosen for the models because it was detected in many microorganisms other than Acinetobacter (Kornberg 1957, Kulaev and Vagabov 1983). Despite all these models and hypotheses ATP production from polyphosphate has never been demonstrated in Acinetobacter or in activated sludge and the microbial utilization of polyphosphate as an energy reserve in vivo has also not yet been proved.

A second not yet proved assumption in all these models says that the PHB accumulation and polyphosphate degradation in the anaerobic stage take place in the same organisms, namely in acinetobacters. Although polyphosphate degradation by pure cultures of Acinetobacter sp. has been demonstrated under anaerobic conditions (Adamse et al. 1984, Ohtake et al. 1985), the accumulation of PHB has only been shown to occur under aerobic conditions. Acinetobacter strains have been isolated from activated sludge in many research laboratories, but the anaerobic uptake of lower fatty acids and accumulation of PHB by pure cultures of this genus has never been reported. The causal relation between anaerobic polyphosphate degradation and PHB synthesis was doubted by Fukase et al. (1985), who incubated two types of activated sludge anaerobically, namely sludge from the

biological phosphate removal process with a dry weight containing 10% phosphorus, mainly poly-phosphorus, and conventional activated sludge with only 1.9% phosphorus. Both sludges took up the same amount of total organic carbon (TOC) under anaerobic conditions and ± 34% of this TOC was accumulated in form of PHB. Nevertheless these authors observed the same effect as reported by others: the phosphate rich sludge released phosphate proportional to the amount of TOC incorporated into the biomass. The phosphate poor sludge accumulated equal quantities of TOC but released only small amounts of phosphate. Most research and model developments were based on the process illustrated in Fig. 1. However, the Phostrip process works at the crucial steps completely different and is nevertheless able to remove phosphate succesfully primarily by biological means (Fig. 2, Levin and Sala 1987). Biological phosphate removal in the Phostrip process cannot be coupled to the anaerobic accumulation of PHB by polyphosphate containing bacteria, since the influent is added first to the aerobic part, leaving so little organic substrates for fermentation in the anaerobic step.

It can be concluded that the fundamental principles of the process of biological phosphate removal from waste water are still unknown and that its working mechanism is mainly based on emperical experience.

POLYPHOSPHATE IN MICROORGANISMS

Chemical structure of inorganic condensed phosphates

Inorganic condensed phosphates, the general term applied to all pentavalent phosphorus compounds in which various numbers of tetrahedral PO_4 groups are linked together by oxygen bridges, fall into three classes (Harold 1966):

(1) Cyclic condensed phosphates, or metaphosphates: Only tri- and tetrametaphosphate are known. They are present in some microorganisms (Kulaev 1979).

(2) Linear condensed phosphates: Unbranched structures with the elementary composition $M_{n+2}P_nO_{3n+1}$ (M = cation, generally metals) are called polyphosphates. The number of phosphate residues in these compounds from living organisms, may vary noticeably: from two in pyrophosphate, the simplest type, to several hundreds and thousands in high molecular-weight polyphosphates (Kulaev and Vagabov 1983). Linear polyphosphates are, based on their ability to dissolve in cold 5% TCA, divided into two classes: acid soluble polyphosphates ($n<20$) and acid insol-

uble polyphosphates (with a higher molecular weight) (Kulaev 1979).
(3) Cross-linked condensed phosphates, or ultraphosphates: Their characteristic feature is the presence of branching points, phosphate groups in which three oxygen atoms are shared with neighboring phosphate groups. Branching points are readily hydrolyzed in water and therefore the occurrence of ultraphosphates in living cells would not be expected.

Accumulation of polyphosphate by microorganisms

In 1888 Liebermann discovered polyphosphates in yeast. Up to now this biopolymer has been found in many taxonomical orders, from prokaryotes to higher plants and animals. The amount of polyphosphate present in the cells of microorganisms can vary considerably. In yeasts the polyphosphate content can account for 20% of the biomass dry weight (Liss and Langen 1960) and strains of Acinetobacter are able to accumulate this compound up to 24% of their dry weight (Deinema et al. 1985). In several fungi 0.2 to 1% polyphosphate-phosphorus has been found. More than 140 species of microorganisms containing polyphosphate are currently known (Kulaev 1979). The form in which high molecular weight polyphosphates are present in the cell is not yet very clear. Chain lengths of 500 to 600 phosphate-groups have been found in Aerobacter aerogenes (Harold 1963), and in the flagellate Crithidia fasciculata the polymer consists of 850 monomers (Janakidevi et al. 1965). The acid soluble form can be present in a free state (Mac Farlane 1939), while the acid insoluble form will be precipitated, possibly in combination with RNA, linked by Mg^{2+} and Ca^{2+}, or linked to proteins, acidic polysaccharides or phospholipids (Kulaev 1979). These precipitates form metachromatic granules or volutin granules (Wiame 1949). Polyphosphate granules usually do not contain membrane structures, but in mycobacteria, cultivated on oleic acid, the polyphosphate granules are surrounded by a lipid layer (Schaefer et al. 1965). Polyphosphate can be localized at several places in or outside the cytoplasm: on the cell surface outside the cytoplasmic membrane of yeasts (Van Steveninck and Booij 1964), in the nuclei of yeasts and fungi (Kulaev 1979), in vesicles of the endoplasmic reticulum in yeasts (Kulaev and Vagabov 1983) and in the chloroplasts of algae (Rubtsov et al. 1977). Two types of polyphosphate granules are found in yeasts, a vacuolar and cytoplasmic form (Indge 1968). In prokaryotes granules are found in the region of the nucleoplasm or near the thylakoids of cyanobacteria (Jensen and Sicko 1974), in the periplasmic region

of Mycobacterium smegmatis (Ostrovsky et al. 1980) or in association with glycogen inclusions in Myxococcus xanthus (Voelz et al. 1966).

The accumulation of polyphosphate by microorganisms depends on the availability of phosphorus, oxidizable substrates, K^+ or other metal cations and the activity of energy-providing processes such as respiration, fermentation, and photo-phosphorylation. The specific conditions for polyphosphate accumulation can be divided into three categories:

(1) The overplus phenomenon (Harold 1966), also called Ueberkompensation (Liss and Langen 1962) or polyphosphate supersynthesis (Kulaev 1979) which occurs after phosphate is added to phosphorus starved cells. Under phosphorus limitation the cells synthesize larger amounts of enzymes that play a role in the polyphosphate metabolism, as soon as phosphate is introduced into the medium unusual large amounts of polyphosphate are temporarily accumulated and broken down again. This effect was first observed by Liss and Langen (1962) in yeast, and by Harold (1966) in Aerobacter aerogenes, in which an increased biosynthesis of polyphosphatekinase and polyphosphatase was demonstrated. This mechanism has also been found in Hydrogenomonas (Kaltwasser 1962), Micrococcus denitrificans (Kaltwasser et al. 1962) and in vacuoles of Saccharomyces carlsbergensis (Lichko et al. 1982).

(2) Luxury uptake of phosphate under conditions unfavourable for growth: This reaction is performed by microbial cells that accumulate large amounts of polyphosphate in stationary phases or other conditions when growth declines, provided that sufficient energy, phosphorus and cations are available. These organisms don't accumulate polyphosphate under normal growth conditions. Luxury uptake has been demonstrated in many bacteria, mostly under conditions of nutrient imbalance (e.g. N or S starvation). Examples are: Corynebacterium xerosis, mycobacteria (Kulaev 1979), Aerobacter aerogenes (Wilkinson and Duguid 1960), Bacillus cereus and B. megaterium, Agrobacter radiobacter (Szymona et al. 1962) and Chlorobium thiosulfatophillium (Shaposhnikov and Fedorov 1960).

(3) Luxury uptake of phosphate under growth conditions: in a few species polyphosphate is accumulated in the growth phase. For example in Acinetobacter sp. in the logarithmic growth phase (Fuhs and Chen 1975, Deinema et al. 1980) or in Actinomyces aureofaciens with a maximum accumulation during the second half of the logarithmic and the beginning of the stationary growth phases (Kulaev et al. 1976). In Chlorella a specific polyphos-

phate fraction was accumulated only under conditions of photosynthesis. This fraction, called poly-P "C", was insoluble either in cold PCA (5%) or in cold weak alkali (pH 8-10) but it was extractable with 2 N KOH at 37°C and co-precipitable with $KClO_4$ which is formed upon neutralization of the KOH-extract with $HClO_4$ (Miyachi et al. 1964).

More examples of polyphosphate accumulation have been found in other bacteria. Polyphosphate can be accumulated by Escherichia coli at the initial stages of logarithmic growth (Nesmeyanova et al. 1973) or during the latent period which precedes the phase of rapid growth as found in Corynebacterium xerosis (Kulaev 1979). On entering the logarithmic growth phase both organisms utilize their polyphosphates. In synchronous cultures of Corynebacterium diphteria (Sall et al. 1958) and Azotobacter sp. (Kulaev 1979) it was demonstrated that polyphosphates play a specific role during cell division. Polyphosphate accumulated at the stage preceding cell division and was consumed very rapidly during the division process. Polyphosphate accumulation can also be dependent on the carbon substrate used. A formation of a polyphosphate storage occurred in Propionibacterium shermanii grown with lactate but not in the cells grown with glucose. When lactate-grown cells were suspended in a solution of glucose, the content of polyphosphate in the cells decreased rapidly. It was proposed that cells grown on glucose as the substrate, do not accumulate polyphosphate since this polymer is utilized as rapidly as it is formed by the phosphorylation of the glucose that is being metabolized (Wood 1985). Recently a Thiobacillus strain has been found that accumulates polyphosphate up to 50% of its dry weight. This occurs in continuous growing cultures under carbon and energy limitation, but only at high ortho-phosphate concentrations in the medium (6.8 mM). At low concentrations (0.68 mM) no luxury uptake can be found (Gommers and Kuenen, in press).

The role of cations in polyphosphate accumulation

Cations may be necessary in polyphosphate accumulation as a counterion of the polyanion or as a prerequisite for other processes that are necessary for the synthesis of polyphosphate. According to Kulaev (1979) the importance of K, Be, Mg, Mn, Zn, Fe and Ca has been reported by several authors. Mg^{2+} as a polyphosphate counterion has been found in Micrococcus lysodeikticus (Friedberg and Avigad 1968) and in Desulfovibrio gigas in which large amounts of Mg-tripolyphosphate were present (Jones and

Chambers 1975). In vacuoles of Neurospora crassa considerable quantities of polyphosphate, Mg^{2+} and Ca^{2+} have been found (Cramer and Davis 1984). The presence of K^+ as a polyphosphate counterion in Chlorella sp. has been reported by Cembella et al. (1984). According to Baxter and Jensen (1980) K^+ is present in polyphosphate granules of Plectonema boryanum, but dependent on their concentrations in the environment also divalent cations could be found in the granules. The importance of K^+ in the medium as a crucial factor for polyphosphate accumulation in Corynebacterium diphteriae was found by Sall et al. (1956). Both K^+ and Mg^{2+} in the medium were essential for polyphosphate accumulation in Aerobacter aerogenes (Wilkinson and Duguid 1960).

The functions of polyphosphate in microorganisms

According to Kulaev (1979) the physiological functions of polyphosphates remain unknown. Because of their different molecular weights, diverse responses to the physiological state of the cell, and various localization within the cell, it appears to be very likely that polyphosphate may perform more than one function in the vital processes of those organisms in which they are present. Polyphosphates can act as a phosphorus reserve which enable the microorganisms to increase their biomass many fold during growth in a phosphorus-free medium (Harold 1966, Van Groenestijn and Deinema 1985). Moreover, they represent a valuable pool of activated phosphate which can be utilized in various metabolic processes, primarily in those connected to different stages of carbohydrate and nucleic acid metabolism (Kulaev and Vagalov 1983). It has been suggested that the earliest organisms in the evolution of life used polyphosphate or pyrophosphate as the energy carrier instead of ATP (Harold 1966). Polyphosphates are regulators of the intracellular concentration of important metabolites including ATP, ADP, other nucleoside polyphosphates, pyro- and ortho-phosphate. There is substancial evidence for the participation of polyphosphates in the transport of glucose through the plasmamembrane of yeasts. The polyphosphates on the outside of the membrane are the ultimate phosphoryl donors in transport-associated phosphorylation (Van Steveninck et al. 1986). The practical significance of polyphosphate as a biological ion-exchanger is unknown and only hypothesized (Baxter and Jensen 1980, Kulaev and Vagabov 1983). Polyphosphate is probably important for cell division of Corynebacterium diphtheria. Sall et al. (1958) hypothesized that during this division polyphosphate is utilized as a phosphagen

as a reaction to the increased cellular energy demand. Many authors have speculated about a function of inorganic polyphosphate as an energy source or reserve in vivo (Dawes and Senior 1973), but there are only a few experimental observations that may support this hypothesis. The growth of some anaerobic bacteria has been reported to be stimulated by pyro- and polyphosphates in the medium (Varma and Peck 1983, Cruden et al. 1983). Streptomyces levoris used inorganic polyphosphates and not ATP as an energy source during the synthesis of the antibiotic levorin (Zyuzina et al. 1981). However, up to now there is still a lack of convincing evidence for a role of polyphosphate as an energy reserve.

Polyphosphate in Acinetobacter sp.

The genus Acinetobacter is defined as a nonmotile short rod which is unable to denitrify. Cells are Gram negative, cytochrome oxydase negative, heterotrophic, and obligate aerobic. They grow well on many substrates such as alcohols, hydrocarbons, aliphatic acids, aromatic compounds, amino acids and amines, but mostly not on glucose as the sole carbon source (Baumann et al. 1968, Juni 1978). In 1975 Fuhs and Chen isolated different Acinetobacter strains from a wastewater treatment plant with biological phosphate removal, and demonstrated that these organisms were able to accumulate large amounts of polyphosphate. After that report polyphosphate accumulating acinetobacters have been isolated in a number of laboratories. Their ability to take up large amounts of phosphate in the logarithmic phase was demonstrated by Deinema et al. (1980). The bacteria contained granules which were electrondense (Fig. 4), and coloured violet-blue with Neisser staining (Gurr 1965). By means of energy dispersive microanalyses with X-rays (EDAX) the high phosphorus content of these granules was demonstrated (Buchan 1983, Van Groenestijn and Deinema 1985). The presence of polyphosphate in Acinetobacter sp. was proved qualitatively by NMR analyses (Florentz 1983, Ohtake et al. 1985) and quantitatively by chemical fractionation (Fuhs and Chen 1975, Ohtake et al. 1985). The polyphosphate was mainly present in the acid insoluble form. The phosphorus content of different strains diverged strongly. Fuhs and Chen (1975) did not find more than 50 mg phosphorus per g dry biomass, Ohtake et al. (1985) found around 30 mg phosphorus, Deinema et al. (1985) reported 54-120 mg phosphorus and Hao and Chang (1987) investigated a strain that contained 48 mg phosphorus per g dry biomass. Under anaerobic

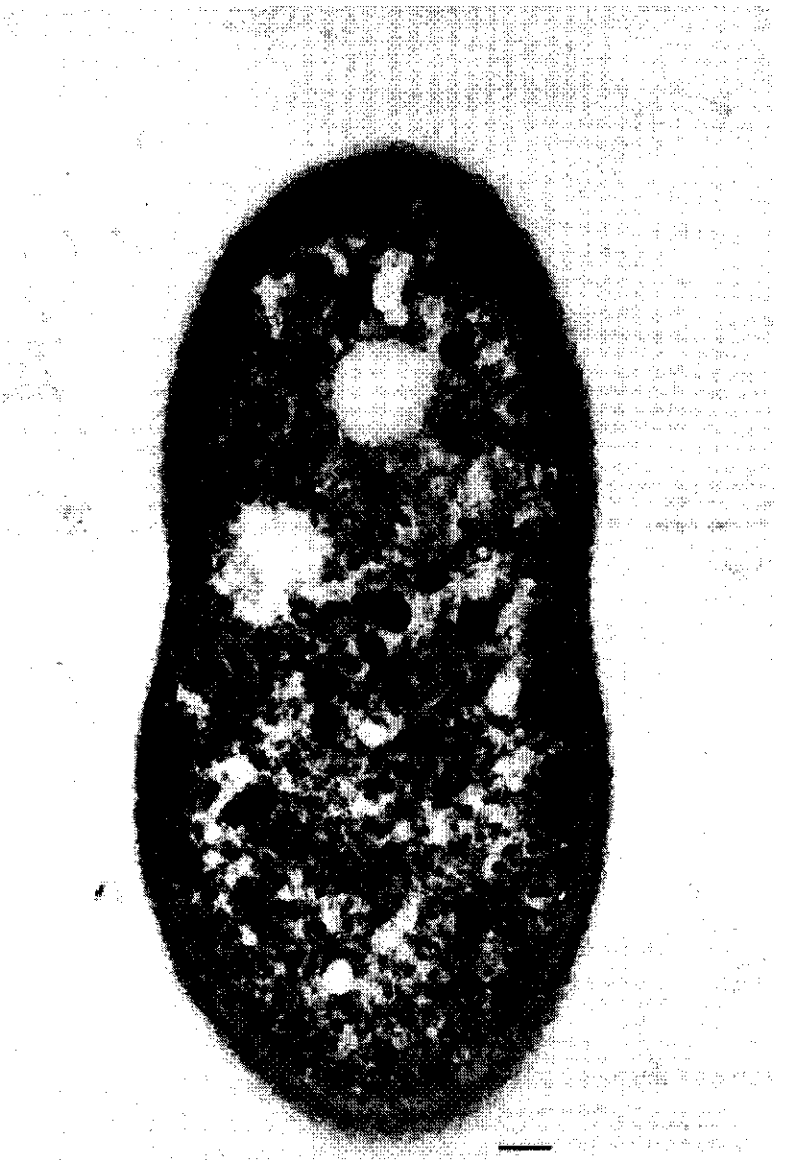

Fig. 4. Electron micrograph of Acinetobacter strain 210A. The black polyphosphate granules are distributed throughout the cell. The bar indicates 0.1 µm. The micrograph was made with the EM Philips-400T of the Technical and Physical Engineering Research Service in Wageningen.

conditions ortho-phosphate is released by the cells (Adamse et al. 1984). The biochemical pathway of the polyphosphate degradation is still unknown. Up to now only a polyphosphatase activity has been found (Ohtake et al. 1985). For the synthesis of polyphosphate polyphosphatekinase has been detected in some Acinetobacter strains (T'Seyen et al. 1985), however, in very low activities of around 1 $nmol.min^{-1}.mg^{-1}$ protein. The best investigated strain so far is Acinetobacter strain 210A (Van Groenestijn and Deinema 1985). This strain grows well on fermentationproducts, like acetate, lactate and ethanol. NH_4^+, NO_3^- and ureum can serve as nitrogen sources, and SO_4^{2-}, $S_2O_3^{2-}$, methionine and cystine as sulphur sources. The strain does not require growth factors. The K_m for dissolved oxygen was measured to be 0.08 $mg.l^{-1}$.

ENZYMES OF THE POLYPHOSPHATE METABOLISM

Biosynthesis of polyphosphates

The enzyme polyphosphatekinase catalysing the reaction

$$ATP + (poly\text{-}P)_n \rightleftarrows (poly\text{-}P)_{n+1} + ADP.$$

was first demonstrated by Yoshida in 1953 who was able to synthesize polyphosphates from ATP with the aid of cell-free extracts from yeast (Kulaev 1979). Kornberg et al. (1956) isolated the same enzyme from Escherichia coli and purified it hundred fold. It was shown that the enzyme was requiring Mg^{2+} and was strongly inhibited by ADP. In 1957 Kornberg reported that the reverse reaction was also possible. Muhammed (1961), however, was not able to demonstrate such a reverse reaction with a purified polyphosphatekinase from Corynebacterium xerosis. By 1979 polyphosphatekinases have been detected in 16 species of bacteria and in yeasts and fungi, in some cases the reaction was reversible, in other cases only the synthesis reaction was catalyzed (Kulaev et al. 1971, Kulaev 1979). Two isoforms of polyphosphatekinase have been found in yeasts. The main isoform is localized in the cytoplasm and appeares to perform only the degradation of high molecular weight polyphosphates. The isoform in the vacuolar fraction, however, catalyzed both synthesis and the reverse reaction (Shabalin et al. 1977). According to Nesmeyanova (1973) the direction in which polyphosphatekinase operates may change at different phases of growth in Escherichia coli.

Besides polyphosphatekinase also 1,3-diphosphoglycerate:polyphosphate phosphotransferase is able to synthesize polyphosphate:

$$1,3\text{-diphosphoglycerate} + (\text{poly-P})_n \rightleftarrows 3\text{-phosphoglycerate} + (\text{poly-P})_{n+1}$$

This reaction was first detected by Kulaev et al. (1968) in Neurospora crassa. The enzyme associates polyphosphate synthesis with glycolytic phosphorylation reactions.

A number of microorganisms contain only polyphosphatekinase, others only 1,3-diphosphoglycerate:polyphosphate phosphotransferase, and in some both enzymes are present (Kulaev 1979). There are at least some organisms in which polyphosphatekinase is wholy or largely responsible for polyphosphate biosynthesis, for example in Aerobacter aerogenes. Polyphosphatekinase negative mutants of this bacterium were unable to synthesize any polyphosphate (Harold and Harold 1963).

Biodegradation of polyphosphates

In addition to polyphosphatekinase and 1,3-diphosphoglycerate:polyphosphate phosphotransferase, which have been described above, other enzymes are able to degrade polyphosphate:
(1) Polyphosphate:AMP phosphotransferase:

$$(\text{poly-P})_n + \text{AMP} \longrightarrow (\text{poly-P})_{n-1} + \text{ADP}$$

The first indications for the existence of polyphosphate:AMP phosphotransferase in mycobacteria has been reported by Winder and Denneny (1957). Dirheimer and Ebel (1965) isolated the enzyme from Corynebacterium xerosis and purified it partly. The enzyme needed Mg^{2+} and was specific for AMP and high molecular weight polyphosphates. Because the K_m for AMP was 2.10^{-2} M, the physiological significance was doubted. This enzyme has been found only in mycobacteria and corynebacteria.
(2) Polyphosphatase:

$$(\text{poly-P})_n + H_2O \longrightarrow (\text{poly-P})_{n-1} + P_i$$

Polyphosphatase has been isolated from many organisms (Kulaev and Vagabov 1983). Polyphosphatases with a high affinity for high molecular weight polyphosphates have been reported (Muhammed 1959) as well as seperate enzymes for the hydrolyses of

tetra-, tri- and pyrophosphate (Kulaev 1979). The activity is mostly dependent on the Mg^{2+} concentration, and can be stimulated by K^{+} (Kulaev 1979). The enzyme can be localized intracellularly or on the outer side of the plasma membrane (Kulaev and Vagabov 1983). In E. coli this enzyme is derepressed under phosphorus starvation, and its synthesis is regulated by the same genes which also control the formation of alkaline phosphatase (Kulaev and Vagabov 1983).
Other polyphosphate degrading enzymes detected in microorganisms are polyphosphate depolymerase, polyphosphate dependent NAD-kinase, polyphosphate glucokinase, polyphosphate fructokinase, polyphosphate mannokinase and polyphosphate gluconatokinase (Kulaev 1979, Kulaev and Vagabov 1983).

OUTLINE OF THE PRESENT RESEARCH

To fully control the biological phosphate removal from waste water, the fundamental microbiological and biochemical principles of this process have to be elucidated. Knowledge on the microbiological fundamentals of phosphate removal will allow to taylor the treatment process in such a way that the specific needs of the microorganisms involved can best be satisfied. Only the thorough understanding of the microbiology of phosphate removal will enable the engineers to optimize this process and to increase the safety of operation for the most divergent waste waters in large scale plants. Since organisms of the genus Acinetobacter seem to be responsible for the removal of phosphate in activated sludge, it was appropriate to investigate the physiology of these organisms in more details.

The aim of this thesis was therefore to study the environmental and regulatory factors responsible for the accumulation and degradation of polyphosphate in Acinetobacter sp.. Special attention was given to the possibility of polyphosphate acting as an energy reserve.

The accumulation of polyphosphate in Acinetobacter sp. and the influence of environmental factors on this process is described in chapter 2. Accumulation and release of phosphate are considered to be dependent on cations because of the expected role of these ions in the neutralization of the negative charges of polyphosphate and ortho-phosphate. Chapter 3 deals with the role and behaviour of cations in phosphate accumulation and release. Emphasis is given to the elucidation of the polyphosphate degradation pathway to obtain information on the functions of this polymer in the cell. The production of ATP from poly-

phosphate by the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase in cell-free extracts of Acinetobacter is reported in chapter 4. Chapter 5 deals with the conditions under which polyphosphate is degraded in vivo and ortho-phosphate is released from the cell. The property of this compound to act as an energy reserve has been demonstrated. Other polyphosphate degrading enzymes that reflect other functions of the polymer are reported in chapter 6. In addition, the involvement of the most important enzymes in polyphosphate breakdown have been measured in activated sludge to show the validity of pure culture studies for the application.

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CHAPTER 2

REGULATION OF POLYPHOSPHATE ACCUMULATION IN *ACINETOBACTER* SP.

Johan W. van Groenestijn, Martina Zuidema, Jacques J.M. van de Worp, Maria H. Deinema & Alexander J.B. Zehnder.

ABSTRACT

The regulation of and the optimum conditions for polyphosphate accumulation in *Acinetobacter* sp. were determined. *Acinetobacter* strain 210A accumulated polyphosphate in the presence of an intra- or extracellular energy source. The accumulation of polyphosphate during endogenous respiration was stimulated by streptomycin and inhibited by KCN. The highest amount of polyphosphate was found in cells in which energy supply was not limited, namely at low growth rates under sulphur limitation, and in the stationary phase of growth when either the nitrogen or the sulphur source was depleted. The phosphorus accumulation was not affected by the pH between 6.5 and 9. There was a pronounced effect of the temperature on phosphorus accumulation but it varied from strain to strain. *Acinetobacter* strain 210A accumulated more phosphate at low temperatures, strain B8 showed an optimum accumulation at 27.5^{o}C, while strain P accumulated phosphorus independently of the temperature. The optimum temperature for growth of *Acinetobacter* strains tested ranged from 25 to 33^{o}C, and the optimum pH was between 6 and 9.

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INTRODUCTION

In 1975 Fuhs and Chen discovered that some Acinetobacter strains were able to accumulate large amounts of phosphate. These strains had been isolated from activated sludge-type sewage treatment plants designed for biological phosphate removal. Essential in this process is the circulation of sludge through anaerobic and aerobic zones. During aerobiosis phosphate is taken up by the sludge and released again under anaerobic conditions (Barnard 1976, Rensink et al. 1981). Enhanced biological phosphate removal depends on the enrichment of the sludge with Acinetobacter (Buchan 1983).

Microorganisms mostly accumulate polyphosphate under certain growth conditions and at certain stages of their development. In general the accumulation depends on the availability of phosphorus, oxidizable substrates, K^+ or other metal cations, and the function of energy providing-processes, such as respiration, fermentation and photophosphorylation. There is a considerable variation of specific conditions under which microorganisms synthesize polyphosphate (Kulaev 1979). The physiological functions of inorganic polyphosphates remain obscure and many speculations have been made about their role as an energy storage or reserve material (Dawes & Senior 1983). The main criterion for a compound to act as an energy storing material is the fact that it is accumulated under conditions when the supply of energy in the cell is in excess of that required for growth and related processes at that particular moment of time (Wilkinson 1959). The quantity of the excess energy will firstly be dependent on availability and depletion of different elements, and secondly on growth rate, and physical-chemical limitations such as temperature and pH.

The current investigation was therefore undertaken to determine and quantify the effect of temperature, pH, and various nutrient limitations at different growth rates on the synthesis of polyphosphate in three strains of Acinetobacter isolated from three different activated sludge plants.

MATERIALS AND METHODS

Organisms

Three Acinetobacter strains were isolated from different activated sludge plants according to the method of Deinema et al. (1980). Strain 210A originated from sludge in Renkum, strain P

from Bennekom and strain B8 from Bunnik (all three in The Netherlands). The strains were maintained on yeast extract agar slants (5 g glucose, 2.5 g yeast extract and 12 g agar per litre tapwater, pH = 7), subcultured every two months and stored at 4°C.

Media

The **basic medium**, used for the temperature experiments contained per 1000 ml demineralized water: 5,67 g Na-acetate.$3H_2O$, 1 g NH_4Cl, 0,6 g $MgSO_4.7H_2O$, 0,4 g KH_2PO_4, 0.1 g $Na_2S_2O_3.5H_2O$, 0,07 g $CaCl_2.2H_2O$ and 2 ml of a trace mineral solution (containing 50 g EDTA, 5 g $FeSO_4.7H_2O$, 1,6 g $CuSO_4.5H_2O$, 5 g $MnCl_2.4H_2O$, 1,1 g $(NH_4)_6$ $Mo_7O_{24}.4H_2O$, 2,2 g $ZnSO_4.7H_2O$, 50 mg H_3BO_3, 10 mg KI and 50 mg $CoCl_2.6H_2O$ per litre demineralized water). The pH was brought to 7.0 with 6 N HCl. In the experiments in which the effect of the pH was measured NH_4Cl was replaced by 1.6 $g.l^{-1}$ $NaNO_3$ (to avoid the precipitation of NH_4MgPO_4 at high pH values). Where indicated acetate has been replaced by lactate and ethanol both also at a concentration of 1 g carbon per litre. Continuous cultivation under different limitations was carried out with the following media:
Substrate limitation: Basic medium.
Sulphur limitation: Basic medium but $MgSO_4.7H_2O$ was replaced by equimolar amounts of $MgCl_2.6H_2O$, and a sulphate poor trace element solution was used as well (Van Groenestijn et al. 1987). 5 mg $Na_2S_2O_3.5H_2O$ per litre were added as sulphur source. To assure sufficient carbon source the acetate concentration was increased 1.7 times to guarantee an excess of energy source for 28 h in the experiments in which substrate addition was stopped after continuous cultivation.
Nitrogen limitation: Basic medium with only 0.25 g NH_4Cl per litre.
Phosphorus limitation (for cultivation of phosphorus starved cells): Basic medium with only 35 mg KH_2PO_4 and an additional 0.1 M Tris-HCl per litre for the buffering of the medium.
The **phosphate uptake medium** contained 66 mg KH_2PO_4, 600 mg $MgCl_2.6H_2O$ and 4 g Tris per litre demineralized water. The pH was adjusted to 7 with 6 N HCl.

Analytical methods

Dry weight of bacteria was measured after centrifugation of a 50 ml sample, washing it with demineralized water and drying at 100°C overnight. After treating the supernatant with amberlite

IR-120H ion exchanger, it was analysed for acetate with a gas-chromatograph (Varian 2400 Aerograph, Adamse 1980). NH_4^+ and ortho-phosphate (ascorbic acid method) were quantified according to Standard Methods (1976), and thiosulphate was measured spectrophotometrically (Sörbo 1957). The phosphorus and nitrogen content of the biomass was calculated from the increase of the biomass dry weight and the decrease of the ortho-phosphate and NH_4^+ concentrations in the bulk liquid. A quick estimation of the biomass dry weight was made by measuring the optical density at 660 nm and using a calibration curve (OD_{660} as function of dry weight). Polyphosphate granules were stained following the method of Neisser (Gurr 1965).

Experimental procedures

For the phosphate uptake experiment cells were precultivated in a phosphorus limited medium in Erlenmeyer flasks in a shaker at 15°C until growth stopped. The cells were harvested and washed with demineralized water, a part was used for dry weight estimation, the other part was resuspended in the phosphate-uptake medium and incubated in Erlenmeyer flasks at 25°C. At time intervals samples were taken to measure phosphate concentration and dry weight. Parallel experiments were done by adding KCN (1mmol per litre phosphate uptake medium) or streptomycin sulphate (200 $mg.l^{-1}$).

The maximum specific growth rate was determined in a batch culture in a 2 l bioreactor (Applikon, Schiedam, The Netherlands) with pH and temperature control. At time intervals the dry weight was estimated by measuring the OD_{660}. The maximum specific growth rate was calculated by plotting the natural logarithm of the dry weight against time. The phosphorus content of the biomass from the growth phase was calculated from the ortho-phosphate concentration in the supernatant and in the initial medium, and the biomass dry weight.

For continuous cultivation a 2 l bioreactor was used. All measurements were done when steady state was reached.

Growth and phosphorus accumulation as a function of physical-chemical parameters

The effect of the temperature on growth and polyphosphate accumulation for three strains of Acinetobacter. The maximum specific growth rate on acetate in batch cultures for the three strains investigated was 0.69 h^{-1} (Fig. 1a). The optimum growth temperature ranged from 25 to 33°C. Temperature had an important effect on the phosphate accumulation (Fig. 1b), but this effect was different for every strain. Strain 210A accumulated high amounts of phosphorus (100 $mg.g^{-1}$ dry weight) at low temperatures and phosphorus accumulation decreased with increasing temperatures. This behaviour of strain 210A was not an effect of

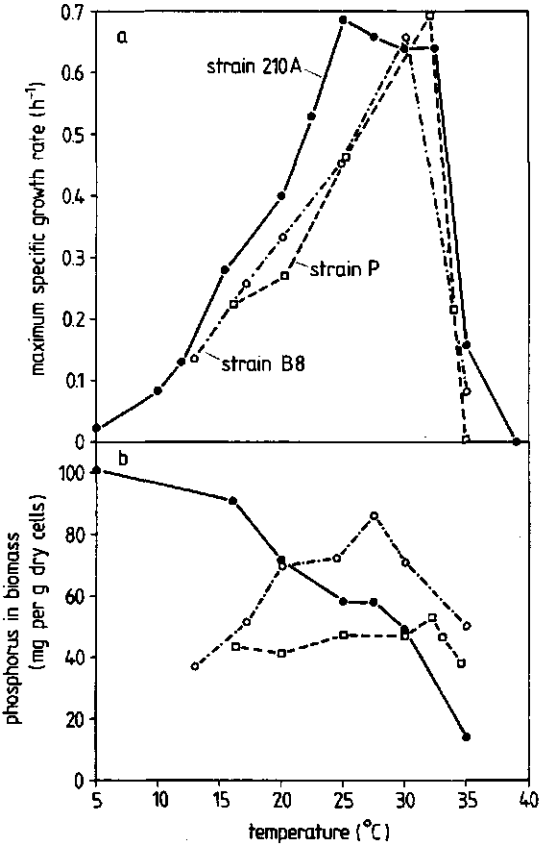

Fig. 1. The effect of the temperature on the maximum specific growth rate (a) and phosphorus accumulation (b) during growth in acetate medium for three different Acinetobacter strains: 210A (●), P (□) and B8 (○).

the growth rate (Fig. 4) since at 16°C a higher phosphorus content was found as compared to 25°C at several fixed growth rates. In contrast to strain 210A, strain B8 accumulated increasing amounts of phosphorus with increasing temperatures with an optimum at 27.5°C. For strain P temperature had hardly any effect on phosphate incorporation.

The effect of the pH on growth and polyphosphate accumulation for Acinetobacter strain 210A. Acinetobacter strain 210A was able to grow well between a pH of 6 and 9, some growth was still observed at a pH of 4.7 or at a pH of 9.5 (Fig. 2a). The optimum pH for growth was dependent on the nature of the carbon source. The carbon sources tested were acetate, ethanol and lactate. All three substrates yielded high growth rates of Acinetobacter strain 210A (Van Groenestijn & Deinema 1985). If acetate was used as substrate, an optimum for growth was found at pH 9 but no growth occurred below a pH of 6.0, while with the other substrates, ethanol and lactate, the optimum pH range for growth was very broad. The phosphorus accumulation by Acinetobacter

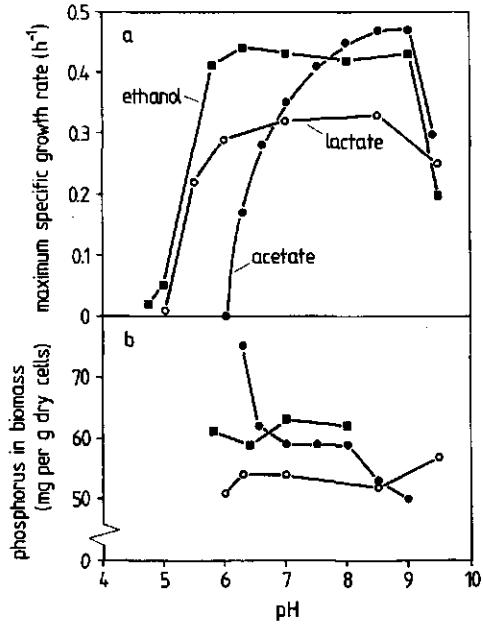

Fig. 2. The effect of the pH on the maximum specific growth rate (a) and phosphorus accumulation (b) during growth of Acinetobacter strain 210A on different substrates at 25°C: acetate (●), ethanol (□), lactate (○); every point in 2b represents the mean value of several measurements with an average standard deviation of ± 3.5 mg phosphorus per g dry biomass.

strain 210A was pH independent above a pH of 6.5 (Fig. 2b), at lower pH-values a clear pH effect could be observed. Growth on acetate at a pH of 6.3 resulted in a significantly higher amount of phosphorus in the biomass compared to its content at higher pH's. In these experiments the μ_{max} of 0.69 h^{-1} was not reached probably because NH_4Cl was replaced by $NaNO_3$. Thermodynamic considerations indicate that more energy is needed for cell synthesis when nitrate is used as nitrogen source instead of ammonium (McCarty 1965).

Polyphosphate accumulation during unlimited growth

In batch at pH 7.0 and 25°C Acinetobacter strain 210A was growing exponentially for several hours (Fig. 3). During this experiment, the phosphorus content of the biomass was constantly 58 mg per g biomass dry weight and did not increase after growth had stopped. Continuous cultivation at μ_{max} (0.69 h^{-1}), pH 7.0 and 25°C resulted also in a phosphorus content of 58 mg per g biomass dry weight (Fig. 4).

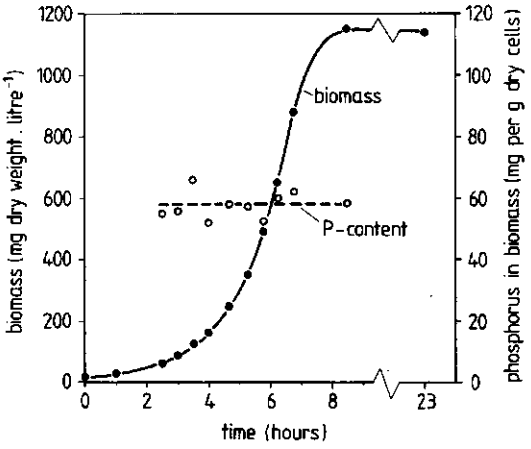

Fig. 3. Growth and accumulation of phosphorus by Acinetobacter strain 210A in batch culture in basic medium at 25°C and pH 7.0. The presented data are from one experiment that represents 5 other experiments with growth rate deviations of 4%.

The effect of substrate limitation on polyphosphate accumulation by Acinetobacter strain 210A

The effect of the growth rate. The accumulation of polyphosphate in Acinetobacter strain 210A at 25°C depended on the growth rate (Fig. 4). At low growth rates of around 0.1 h^{-1} the substrate in the chemostat was no longer detectable and the phosphorus

accumulation increased, reaching a maximum of 83 mg phosphorus per g biomass dry weight at a growth rate of 0.11 h^{-1}.

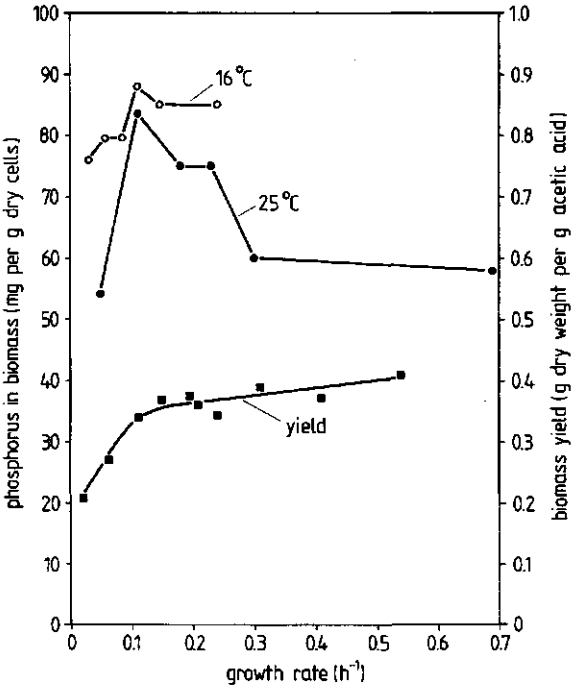

Fig. 4. Phosphorus accumulation by continuously growing Acinetobacter strain 210A at various dilution rates under substrate limitation at pH 7.0 and 25°C and 16°C. The biomass yield is given for growth at 25°C. Every phosphorus content represents the mean value of several measurements with an average standard deviation of 4 mg per g dry biomass.

Polyphosphate accumulation in absence of an extracellular substrate. A batch of Acinetobacter strain 210A was precultivated under phosphorus limitation until growth stopped. The cells were washed and subsequently suspended in the phosphate uptake medium. One g of these cells containing only 13 mg phosphorus was able to take up an additional 27 mg phosphorus from the medium within 9 hours, the largest part during the first 35 minutes (Fig. 5). Initially no polyphosphate granules were detectable with Neisser staining but after 1 hour granules were clearly visible. KCN almost entirely inhibited phosphate uptake indicating that phosphate accumulation is somehow depending on energy from the respiration process (endogenous respiration). As the medium did not contain any energy source, electrons for respiration had to be derived from intracellular storage products, e.g. lipids or other polymers. Interestingly strepto-

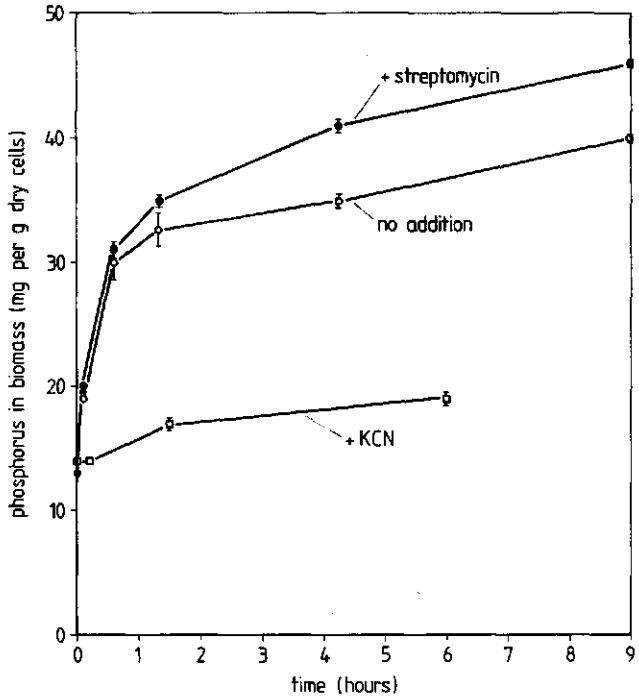

Fig. 5. Effect of streptomycinsulphate (200 $mg.l^{-1}$) and 1 mM KCN on the phosphorus accumulation in cells of Acinetobacter strain 210A in a mineral medium in absence of an external energy substrate (mean values of two independent experiments).

mycin stimulated phosphate uptake (Fig. 5). Since protein synthesis is an energy consuming process its inhibition by streptomycin might increase the energy available for phosphate uptake and polyphosphate synthesis. Both the KCN and the streptomycin experiment indicate that energy, probably from respiration processes, is used for polyphosphate synthesis.

The effect of nutrient limitation on polyphosphate accumulation by Acinetobacter strain 210A

The effect of the growth rate during nitrogen limitation. Acinetobacter strain 210A was continuously cultivated under nitrogen limitation. During this cultivation no NH_4^+ was detectable in the supernatant after harvesting the cells and acetate was still present in considerable amounts, while the biomass concentration was significantly lower than found under substrate limitation. The phosphorus accumulation was independent of the growth rate (Fig.6). The nitrogen content of the biomass of the nitrogen limited cultures remained constant at 120 $\pm$ 10 mg nitrogen per g

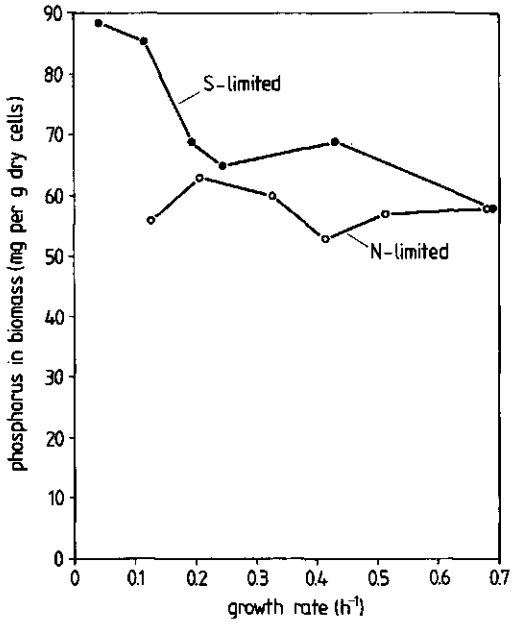

Fig. 6. Phosphorus accumulation by continuous cultivated Acinetobacter strain 210A under sulphur limitation and nitrogen limitation, both at pH 7.0 and 25°C.

dry biomass over the whole range of growth rates, indicating that no carbon reserve material was formed. The experiment was repeated 2 additional times at a growth rate of 0.11 h^{-1}, the cells contained 56 and 54 mg phosphorus per g dry biomass, thus similar amounts as in the first experiment.

Polyphosphate accumulation during a nitrogen limited stationary phase. An extra phosphorus accumulation was found in stationary cultures of Acinetobacter strain 210A during nitrogen limitation (Table 1). This stationary situation was reached after stopping the substrate addition (time = 0 h) to nitrogen limited cultures growing at a dilution rate of 0.31 h^{-1}.

The effect of the growth rate during sulphur limitation. During the continuous cultivation of Acinetobacter strain 210A under sulphur limitation no thiosulphate was detectable in the supernatant after harvesting the cells, acetate was still present in high amounts and the biomass concentration was lower than found under substrate limited continuous cultivation. The phosphorus accumulation in the biomass depended on the growth rate. It was highest at low growth rates (88 mg phosphorus per g dry biomass

at a growth rate of 0.04 h^{-1}) decreasing to 58 mg phosphorus per g dry biomass around the maximum growth rate (Fig. 6). To check the reproducability 5 independent experiments were carried out at 0.12 h^{-1}. The cells contained 96, 88, 88, 81 and 74 mg phosphorus per g dry biomass.

Table 1. Accumulation of phosphorus by stationary cultures of Acinetobacter strain 210A precultivated in a chemostat under nitrogen limitation at D = 0.31 h^{-1}. Medium and substrate addition were stopped at time = 0 h. The data presented are from one experiment which was representative of 3 similar experiments.

Time (h)	acetate in the medium (mM)	Biomass dry weight (mg l^{-1})	phosphorus content biomass (mg g^{-1} dry wt.)
0	17	480 ± 10	53 ± 2
0.75	15	490	65
1.75	11	500	65
2.75	6.3	507	74
4.50	0.5	525	71
5.75	0[a]	525	70
6.75	0	530	68

a: detection limit for acetate was 0.05 mM

Table 2. Accumulation of phosphorus by cultures of Acinetobacter strain 210A precultivated in a chemostat under sulphur limitation at D = 0.20 h^{-1}. Medium and substrate addition were stopped at time = 0 h. The presented data are from one experiment which was representative of 3 similar experiments.

Time (h)	acetate in the medium (mM)	Biomass dry weight (mg l^{-1})	P-content biomass (mg g^{-1} dry wt.)
0	42	655 ± 10	64 ± 2
4	31	810	71
8	22	880	76
13	15	965	77
24	4.5	1060	79
28	1.5	1070	80
32	0[a]	1080	80
80	0	1010	80

a: detection limit for acetate was 0.05 mM

Polyphosphate accumulation during prolonged sulphur starvation. After continuous cultivation of Acinetobacter strain 210A under sulphur limitation at a growth rate of 0.20 h^{-1} the substrate addition was stopped. At that time acetate was still present in the medium. The culture was still able to grow slowly, and the cellular phosphorus content increased to a high level (Table 2). Growth was measured by means of OD_{660} and by determining the dry weight.

DISCUSSION

Growth of Acinetobacter sp.

The temperature optima for growth of the Acinetobacter strains investigated ranged from 25 to 33°C (Fig. 1a). This agrees with temperature optima for Acinetobacter sp. strains isolated from soil, sediments and activated sludge reported by other authors (Abbot et al. 1973, Fuhs and Chen 1975, Du Preez 1978, Hao & Chang 1987). Growth is possible between a pH of 4.8 and 9.5, but growth on acetate is completely inhibited at a pH lower than 6 (Fig. 2a). According to Pirt (1975) the toxicity of weak acids such as acetic acid is often pH dependent. The free acid is more lipid soluble than the ionized form, hence reduction in pH will favour free diffusion of the more hydrophobic acid into the cell affecting so the pH gradient across the membrane. This effect is stronger for acetic acid than for lactic acid, because acetic acid is a weaker acid and more hydrophobic than lactic acid (log P_{oct} of lactic acid = -0.62 and log P_{oct} of acetic acid = -0.17; P_{oct} is the quotient of distribution of a compound between octanol and water; Verschueren 1983). Higher hydrophobicity facilitates diffusion through the cellmembrane.

Polyphosphate accumulation by Acinetobacter sp.

The amount of polyphosphate accumulated in Acinetobacter sp. depends on temperature, pH, nature of the carbon and nitrogen source, growth rate, growth phase, limitations of different elements, and on the selected Acinetobacter sp. strains. It is possible to understand most of the above described observations if the assumption is made that polyphosphate accumulation is the result of the production of excess energy in the cell, or the inhibition of the synthesis of other polymers. As polyphosphate in Acinetobacter sp. can act as an energy reserve in vivo (Van Groenestijn et al. 1988, submitted), its accumulation may obey

the criterion stated by Wilkinson (1959) that an energy reserve is accumulated if the energy supply is in excess of that required by the cell for growth and related processes. An energy storage product like glycogen for example is accumulated if excess energy and carbon is available in the cell (Dawes & Senior 1973). It can be expected that the criteria for polyphosphate storage are surplus of energy and phosphorus in the cell.

Polyphosphate in Acinetobacter sp. can be accumulated on the expence of an extra- or intracellular energy source (Figs. 3 and 5). In absence of an extracellular substrate intracellular compounds are respired to supply energy for maintenance and polyphosphate accumulation. In the presence of streptomycin the need for energy for maintenance and biosynthesis will be lower, and the higher amounts of accumulated polyphosphate under these conditions can be explained by an availability of a greater surplus energy.

Cultures of Acinetobacter strain 210A growing without nutritional limitation at their maximum rate on acetate at pH 7.0 and 25°C contain 58 mg phosphorus per g biomass dry weight (Figs. 3, 4 and 6), but under certain conditions more phosphorus can be accumulated, at the same temperature and pH, using the same substrate. Dependent on environmental conditions growing cells may generate more energy than is necessary for biosynthesis and maintenance. The surplus energy can be wasted in the form of heat by means of slip-reactions (energy spilling reactions). This was found by Neijssel & Tempest (1976) in substrate (glucose) limited continuous growing cultures of Klebsiella aerogenes which were carbon limited but not energy limited. Instead of wasting this surplus energy by means of slip-reactions the formation of an energy reserve such as polyphosphate may be used by Acinetobacter strain 210A as an energy sink.

Excess energy can be obtained when cell synthesis is limited by the lack of certain essential nutrients; e.g. sulphur limitation (Fig. 6) or nitrogen and sulphur depletion (Table 1 and 2). Cultures of Acinetobacter strain 210A growing continuously under sulphur limitation will restrict their biosynthesis at low dilutionrates in the bioreactor due to sulphur starvation. At lower growth rates the energy generated from the extracellular energy source will form a larger excess, giving rise to the formation of larger amounts of polyphosphate as an energy sink product, as can be observed in Fig. 6. A similar observation has been reported by Wilkinson & Munro (1967). Bacillus megaterium only accumulated polyphosphate at low dilution rates (0.11 h^{-1}) during sulphur limitation. This effect may be comparable with the accumulation of increasing amounts of glycogen by nitrogen

limited microorganisms at decreasing growth rates (Dawes & Senior 1973). The observation that nitrogen limited cultures of Acinetobacter strain 210A don't accumulate the same large amounts of polyphosphate at low growth rates as sulphur limited cultures may be due to different responses of the regulatory mechanisms of assimilation and dissimilation when nitrogen instead of sulphur is deficient (Fig. 6).

In a stationary phase after nitrogen or sulphur depletion, a large excess of cellular energy can be expected, while biosynthesis will be strongly reduced by the complete absence of the assimilable form of the structural elements nitrogen and sulphur in the medium. Under these conditions Acinetobacter strain 210A is able to take up an additional amount of phosphorus above the amount that is already present in the biomass (Tables 1 and 2). A similar effect has been found in Enterobacter (formerly Aerobacter aerogenes which accumulated polyphosphate under nitrogen or sulphur starvation in the presence of excess carbon source. These conditions reduced nucleic acid synthesis and favoured polyphosphate accumulation (Harold 1963). Contrary to Acinetobacter sp. this organism was not able to accumulate polyphosphate under substrate limitation.

Interestingly, Acinetobacter strain 210A shows a maximum of polyphosphate accumulation under substrate limitation around a dilutionrate of 0.11 h^{-1} (Fig. 4). According to Linton and Stephenson (1978) growth on limited amounts of acetate means energy limitation and not carbon limitation, which makes polyphosphate accumulation at these energy restricted conditions difficult to understand. Specific regulatory mechanisms as part of a survival strategy may cause a shift in the balance of synthesis of different biopolymers in favour of polyphosphate production. In natural environments energy limitation can be a precursor of energy depletion. In the absence of an external energy source polyphosphate will be needed as an energy reserve. A similar effect has been found by Wilkinson and Munro (1967) for the accumulation of poly- -hydroxybutyrate in Bacillus megaterium growing under substrate (glucose) limitation. A maximum accumulation was found at a growth rate of 0.4 h^{-1}, at 0.8 h^{-1} two times less poly- -hydroxybutyrate was present in the cells. The decrease of the phosphorus content in Acinetobacter strain 210A at growth rates lower than 0.1 h^{-1} at which also a decrease of the yield occurs, may be caused by the regulatory effects of alarmones. The concentration of these regulatory nucleotides increases when the yield becomes lower than 75% of its maximum (Van Verseveld et al. 1984). These compounds mobilise carbon and energy reserves (Rickenberg 1974) and can inhibit some energy

consuming processes like RNA synthesis (Gallant 1979) and maybe also the production of polyphosphate.

Polyphosphate accumulation by Acinetobacter sp. is strongly dependent on temperature and to a less extent on pH (Figs. 1b and 2b). If it is assumed that the amount of accumulated polyphosphate reflects the imbalance between energy generation and biosynthesis, then the overall effect of temperature and pH can be based on effects on energy generation, synthesis of structural biopolymers and synthesis of polyphosphate. These effects can be different for every strain, e.g. the polyphosphate accumulation in Acinetobacter strains 210A, B8 and P is influenced by temperature in different ways (Figs. 1b and 2b). Probably the effect of the growth rate on polyphosphate accumulation shows the same strain specificity, since Hao & Chang (1987) found an increase of polyphosphate accumulation with increasing growth rates of their Acinetobacter sp., which is contrary to the effect found in Acinetobacter strain 210A. From a fundamental point of view the differences between the strains are confusing, but from a practical point of view this means that biological phosphate removal from wastewater may be possible under divergent conditions, and is always based on Acinetobacter strains that are best adapted to the prevailing conditions. For example excellent biological phosphate removal from wastewater has been found at 5°C (Krichten et al. 1985) as well as at 20°C (Meganck et al. 1985).

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CHAPTER 3

THE ROLE OF CATIONS IN THE ACCUMULATION AND RELEASE OF PHOSPHATE BY ACINETOBACTER STRAIN 210A

Johan W. van Groenestijn, Gerard J.F.M. Vlekke, Désirée M.E. Anink, Maria H. Deinema and Alexander J.B. Zehnder

ABSTRACT

Cells of the strictly aerobic Acinetobacter strain 210A, containing aerobically large amounts of polyphosphate (100 mg phosphorus per g dry biomass), released in absence of oxygen 1.49 mmol inorganic phosphate, 0.77 mEq Mg^{2+}, 0.48 mEq K^+, 0.02 mEq Ca^{2+} and 0.14 mEq NH_4^+ per g dry biomass. The pH drop during this anaerobic phase was caused by the extrusion of 1.8 protons per PO_4^{3-} molecule. Cells of Acinetobacter strain 132 which did not accumulate polyphosphate aerobically, released per g dry biomass only 0.33 mmol ortho-phosphate and 0.13 mEq Mg^{2+}, but amounts of K^+ comparable to strain 210A. Stationary cultures of Acinetobacter strain 210A in which polyphosphate could not be detected with Neisser staining were taking up aerobically phosphate simultaneously with Mg^{2+}, the most important counterion in polyphosphate. In absence of dissolved phosphate in the medium no Mg^{2+} was taken up. Cells containing polyphosphate granules were able to grow in a Mg-free medium, while cells without these granules were not. Mg^{2+} was not essential as counterion, because it could be replaced by Ca^{2+}. The presence of small amounts of K^+ was essential for polyphosphate formation in cells of strain 210A. In batch experiments with media without K^+, Acinetobacter strain 210A took up 59% less phosphate, in spite of a higher cellular ATP content during this K^+ depletion. During continuous cultivation under K^+-limitation, cells of Acinetobacter strain 210A contained only 14 mg phosphorus per g dry biomass, while this element was accumulated in amounts of 59 mg per g dry biomass under substrate limitation and 41 mg per g dry biomass under Mg^{2+}-limitation. For phosphate uptake in activated sludge the presence of K^+ seems to be crucial.

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INTRODUCTION

Enhanced biological phosphate removal from waste water, a biological alternative to chemical phosphate precipitation, is based on the enrichment of activated sludge with polyphosphate accumulating bacteria (12). Acinetobacter is the most important genus in this process (4) and some studies on its polyphosphate accumulation and degradation have been carried out recently in pure cultures (8, 12, 14, 21). Just like activated sludge in which biological phosphate removal was observed, Acinetobacter takes up phosphate under aerobic conditions and releases it anaerobically (9). Polyphosphate in Acinetobacter is present in granules (12) and the negative charge of this polyanion is neutralized by cations (4, 13). As a result simultaneously with phosphate an uptake and release of cations can be expected. In fact in wastewater treatment plants with biological phosphate removal this effect has already been observed. In the anaerobic zones of these plants phosphate is released together with Mg^{2+} and K^{+} (3, 6, 19, 26), and during aerobic conditions the phosphate is taken up again simultaneously with Mg^{2+} and K^{+} and small amounts of Ca^{2+} (6, 26). Kainrath et al. (17) analysed the dry matter of the sludge from the biological phosphate removal process and found a correlation of the total P-content with Mg^{2+} and K^{+} but not with Ca^{2+}, Fe^{3+} and Al^{3+}. From these observations it was concluded that the enhanced biological phosphorus removal from waste water was indeed mainly a biological process, with chemical precipitation playing a minor role. However, it could be possible that with another composition of the influent, e.g. water with high calcium concentrations, chemical precipitation may be more important. An example of a combined chemical and biological phosphorus removal has been reported by Arvin and Kristensen (3). They found that during anaerobic conditions the phosphate release from the sludge was always accompanied by a release of K^{+} and Mg^{2+}, and a decrease of the Ca^{2+} concentration. In the two plants investigated, 50-60% of the phosphate in the sludge was precipitated and the dominating counterions in the precipitate were Ca^{2+} (high amounts) and Mg^{2+} (lower amounts). From these observations it might be concluded that only Mg^{2+} and K^{+} are important counterions in biological phosphate removal, however this is at variance with the findings of Buchan (4). By means of energy dispersive analyses with X-rays (EDAX) of the electrondense granules in sludge, this author proved that calcium plays a predominant role in the stabilisation of polyphosphate-ions and that a calcium requirement for enhanced phosphorus uptake does not necessarily point to chemi-

cal precipitation.

To distinguish between biological and chemical removal of phosphate and cations a study was undertaken with two pure cultures of the Acinetobacter, namely strain 210A and 132, both isolated from activated sludge (strain 210A did accumulate phosphate in form of polyphosphate whereas 132 did not). This paper reports the results of the experiments on the effect of K^+, Mg^{2+} and Ca^{2+} on polyphosphate formation and degradation as well as on the role of polyphosphate as possible cation (Mg^{2+}) reserve.

MATERIALS AND METHODS

Organisms. Acinetobacter strain 210A and strain 132 were isolated from activated sludge by the method described by Deinema et al. (8). Strain 210A is able to accumulate large amounts of polyphosphate, while strain 132 is not. The organisms were maintained on yeast extract agar slants (5 g glucose, 2.5 g yeast extract and 12 g agar per litre tapwater, pH = 7), subcultured every two months and stored at 4°C.

Media. - Medium for batch cultivation containing butyrate as carbon and energy source was prepared according to Van Groenestijn et al. (14). Medium for batch cultivation containing acetate as carbon and energy source was the same as the medium with butyrate except that Na-butyrate was replaced by 5.67 g Na-acetate.$3H_2O$. For optimal growth and polyphosphate accumulation, the acetate and butyrate medium were used, respectively (13).

- Medium for cultivation under phosphorus limitation contained per litre demineralized water: 7 g Na-acetate.$3H_2O$, 0.1 g $Na_2S_2O_3.2H_2O$, 1 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 40 mg KH_2PO_4, 0.06 g $CaCl_2.2H_2O$, 2 ml trace elements (14) and 12 g Tris. The pH was adjusted to 7.0 with 6 N HCl.
- Phosphate release medium contained per litre demineralized water: 2.5 g acetic acid and 4 g Tris. The pH was adjusted to 7.5 with 6 N HCl.
- Unbuffered phosphate release medium (used in the experiments in which the pH decrease caused by phosphate efflux was studied) contained per litre demineralized water: 20 mmol butanol and 6 g NaCl. The pH was adjusted to 7.5 with 0.1 N NaOH. Butanol, also a substrate of Acinetobacter strain 210A, was chosen because it does not buffer the medium as acetate or butyrate would do.
- Phosphate uptake medium for Acinetobacter contained per litre demineralized water: 136 mg KH_2PO_4, 11 mg NH_4Cl, 44 mg $CaCl_2.2H_2O$, 61 mg $MgCl_2.6H_2O$, 12 mg NaCl, 300 mg acetic acid, 6 g Tris and 200 mg streptomycinsulphate. The pH was adjusted to

7.0 with 6 N HCl. Experiments with various concentrations of K^+ were carried out replacing KH_2PO_4 by equimolar amounts of $NaH_2PO_4.2H_2O$. K^+ was added as KCl.
- Phosphate uptake medium for activated sludge contained per litre demineralized water: 0.6 g $MgSO_4.7H_2O$, 0.25 g $NaH_2PO_4.2H_2O$, 1 g NH_4Cl, 0.07 g $CaCl_2.2H_2O$, 1.5 g Tris and various amounts of KCl. The pH was adjusted to 7.0 with 6 N HCl.
- Medium without Mg^{2+} (used in the Mg-reserve batch experiments) contained per litre demineralized water: 5.67 g Na-acetate.$3H_2O$, 1 g NH_4Cl, 0.44 g KH_2PO_4, 0.1 g $Na_2S_2O_3.5H_2O$, 0.07 g $CaCl_2$ and 2 ml of a trace mineral solution (14). The pH was brought to 7.0 with 6 N HCl.

Continuous cultivation under substrate and cation limitation was carried out with the following media:
- Substrate limitation: Basic medium contained per litre demineralized water: 5.67 g Na-acetate.$3H_2O$, 1 g NH_4Cl, 0.6 g $MgSO_4.7H_2O$, 0.44 g KH_2PO_4, 0.1 g $Na_2S_2O_3.5H_2O$, 0.07 g $CaCl_2$ and 2 ml of a trace mineral solution (containing 50 g EDTA, 5 g $FeSO_4.7H_2O$, 1.6 g $CuSO_4.5H_2O$, 5 g $MnCl_2.4H_2O$, 1.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$, 2.2 g $ZnSO_4.7H_2O$, 50 mg H_3BO_3, 10 mg KI and 50 mg $CoCl_2.6H_2O$ per litre demineralized water). The pH was brought to 7.0 with 6 N HCl. The trace mineral solution used in this experiment differed from that used in the batch experiments, because of practical reasons: no precipitation was formed after extended sterilization of the complete medium in 10 l carboys.
- Mg^{2+}-limitation: Basic medium with only 80 mg $MgSO_4.7H_2O$ per litre.
- K^+-limitation: Basic medium, however KH_2PO_4 was replaced by equimolar amounts of $NaH_2PO_4.2H_2O$. 15 mg KCl was added.

Phosphate release experiments. Acinetobacter strain 210A and strain 132 were grown at 15°C in shaken Erlenmeyer flasks in a medium containing butyrate as carbon and energy source. Cultures from the log-phase of strain 210A containing 100 mg phosphorus per g biomass dry weight and of strain 132 containing only 26 mg phosphorus per g biomass dry weight were centrifuged and washed with demineralized water. The pellets were resuspended in serumvials containing a phosphate release medium. The vials were made free of oxygen by flushing them with a gas-mixture of N_2:CO_2 (99%:1%, v/v). Before incubation samples were taken with a syringe for the determination of the biomass content. The serumvials were placed in a shaker (to keep the biomass in suspension) at 30°C. At time intervals aliquots were taken and centrifuged. The supernatant was analysed for K^+, Ca^{2+}, Mg^{2+}, NH_4^+, Na^+ and ortho-phosphate.

Phosphate uptake experiments. Cells of Acinetobacter strain 210A were precultured under P-limitation at 25°C in shaken Erlenmeyer flasks until growth stopped. At that moment no polyphosphate granules could be seen in strain 210A after staining following the method of Neisser (15). Grown cultures were centrifuged and washed twice with a Tris/HCl buffer, and the pellets were resuspended in a phosphate uptake medium, to obtain a biomass concentration of 0.5 g dry weight per litre. The acetate in this medium served as the energy source for the uptake processes, while streptomycin was added to prevent growth of the cells. During the experiments the dry weight content of the biomass increased by about 8%, which was the result of phosphate and cations uptake. After the phosphate was taken up polyphosphate granules were visible with Neisser staining. Experiments were also carried out in phosphate uptake media from which K^+, Mg^{2+}, Ca^{2+} or ortho-phosphate were omitted. In addition, the ATP content of the cells, exposed to various K^+ concentrations, was measured after 0.5 hours of incubation.

The uptake of phosphate by activated sludge in a mineral medium with varying K^+ concentrations was investigated as well. Sludge was obtained from an intermittently aerated labscale pilot plant which biologically removed phosphate from synthetic waste water (2). When the sludge sample was taken at the end of the anaerobic period phosphate had been released from the cells. To remove adsorbed K^+ from the sludge, it was centrifuged and washed twice at 4°C with a solution of 9 g NaCl per litre demineralized water. The pellet was resuspended in a mineral phosphate uptake medium. All experiments with sludge were carried out in Erlenmeyer flasks at 25°C on a rotary shaker (200 rpm). At time intervals samples were taken to measure the orthophosphate and dry weight.

Continuous cultivation. Acinetobacter strain 210A was continuously cultivated under substrate, K^+- or Mg^{2+}-limitation in a 2 l bioreactor (Applikon, Schiedam, The Netherlands) with automatically controlled pH (7.0) and temperature (25°C). When cultures had reached a steady state, samples were taken and centrifuged. The supernatant was analysed for K^+, Mg^{2+}, orthophosphate and acetate, the pellet was washed with demineralized water and used for the determination of the dry weight content.

Mg-reserve experiments. Cells of Acinetobacter strain 210A, containing 75 mg P per g biomass dry weight, were cultivated in batch in a medium containing acetate as carbon and energy source, harvested, washed with demineralized water and centrifuged as described in the phosphate release experiments. Phosphorus poor cells with only 20 mg P per g biomass dry weight

were obtained as described above in the phosphate uptake experiments. All precultures were grown at 15°C. The pellets were resuspended in a medium without Mg^{2+} with acetate as carbon and energy source. The cell suspensions were shaken in Erlenmeyer flasks at 25°C. At time intervals samples were taken to estimate the biomass dry weight.

Reproduceability. Figs. 1, 3 and 4 and Table 1 show data from one experiment that represents other experiments with the same procedure. Although the rates and quantities varied considerably between the different experiments (the most extreme cases differed by 30%), the stoichiometry between phosphate and the various cations and among the cations themselves remained constant in each experiment. The data presented in Fig. 4d are mean values from experiments in fivefold in which three seperately grown cultures were used that took up 0.85 $\pm$ 0.07 mmol phosphate per g dry biomass.

Chemicals. Streptomycinsulphate was obtained from Sigma Chemical Co., St.Louis, MO. The ATP bioluminescence CLS test-combination including luciferase/luciferin reagent and Na_2ATP was obtained from Boehringer, Mannheim, W-Germany.

Analyses. Ortho-phosphate was determined spectrophotometrically according to Standard Methods (24) using the ascorbic acid method. K^+, Mg^{2+}, Ca^{2+} and Na^+ were quantified using an atomic absorption spectrophotometer. NH_4^+ was analysed with the Nessler reagent according to Standard Methods (24). Acetate was measured after addition of amberlite IR-120H ionexchanger using GLC by means of a Varian 2400 Aerograph (1). Bacterial dry weight was determined after centrifugation of a 50 ml sample of the culture, washing the pellet once with demineralized water and drying at 100°C overnight. Dry weights were also estimated from optical density at 660 nm by using a calibrationcurve (OD_{660} as function of dry weight). The ATP content of the culture was quantified according to Pradét (22). Samples of 0.5 ml culture were boiled for 4 minutes in 5 ml of a solution containing 50 mM Tris/HCl and 4 mM EDTA at pH 7.75 in order to extract ATP from the cells and stop all enzyme activity. Luciferase/luciferin reagent was added to a subsample of this solution and the resulting light-emission was measured with a luminescence-meter (made by the Electronic Design and Maintenance Shop Division of the General Research Department, University of Georgia, Athens, USA). Polyphosphate was stained using the method of Neisser (15).

Release of phosphate and cations. The anaerobic efflux of phosphate, Mg^{2+}, K^+, Ca^{2+} and NH_4^+ by cultures of Acinetobacter strain 210A containing 100 mg phosphorus per g dry biomass is presented in Fig. 1. Mg^{2+} and K^+ were the most important cations released under anaerobic conditions. For comparison the extrusion of phosphate, Mg^{2+} and K^+ by Acinetobacter strain 132, which is unable to accumulate polyphosphate, was also determined. Strain 132 released anaerobically the same amount of K^+ as strain 210A but much less phosphate and Mg^{2+} (Fig. 1). The release of K^+ in strain 210A seems to be independent of the phosphate release, because K^+ was extruded with a higher initial rate than phosphate and Mg^{2+} (Fig.1) and at low pH the efflux of K^+ was less inhibited than the release of phosphate (data not shown). At pH 5.9 and 30°C one g biomass released anaerobically within three hours 0.13 mmol K^+ but only 0.05 mmol phosphate.

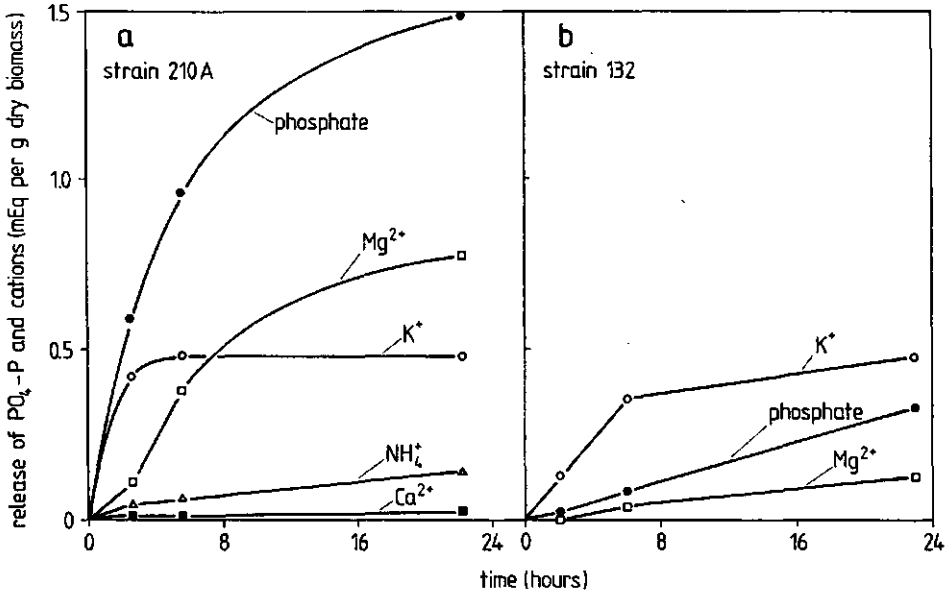

FIG. 1. Release of P_i (1 mEq = 1 mmol), Mg^{2+}, K^+, NH_4^+ and Ca^{2+} by Acinetobacter strain 210A and strain 132 under anaerobic conditions at 30°C and pH 7.5. The presented data were determined in one experiment and are representative for five (strain 210A) or three (strain 132) other experiments.

In a phosphate release medium without a Tris-HCl buffer the pH decreased from 7.4 to 6.4 within 6 hours while 10.5 mg orthophosphate-phosphorus per litre (0.34 mM) was released by cells

of strain 210A (Fig. 2). In a control experiment with the same medium but without biomass the pH decreased from 7.4 to 6.4 by the addition of 0.28 mM KH_2PO_4 plus 0.06 mM $KNaHPO_4$. From these results can be calculated that the pH drop is caused by the extrusion of 1.8 protons per PO_4^{3-} molecule.

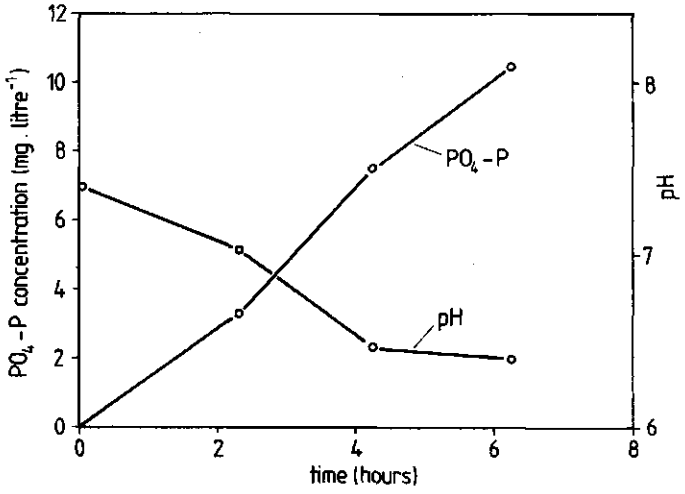

FIG. 2. Decrease of the pH during the release of phosphate by 625 $mg.l^{-1}$ biomass of Acinetobacter strain 210A at 30°C in an unbuffered anaerobic phosphate release medium.

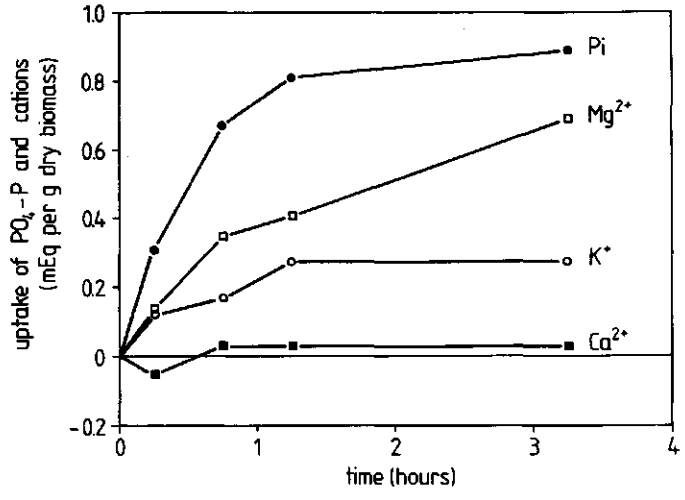

FIG. 3. The uptake of phosphate (1 mEq = 1mmol), Mg^{2+}, K^+ and Ca^{2+} by stationary cultures of Acinetobacter strain 210A in a medium with acetate and streptomycin (pH = 7, 25°C).

Uptake of phosphate and cations. Concomintently with ortho-phosphate cations were taken up by a culture of Acinetobacter strain 210A devoid of polyphosphate (Fig. 3). This experiment has been carried out in a complete phosphate uptake medium. To prevent growth and thus interference between phosphate uptake for polyphosphate formation and phosphate incorporation in newly formed cells (biomass) streptomycin was added to the medium.

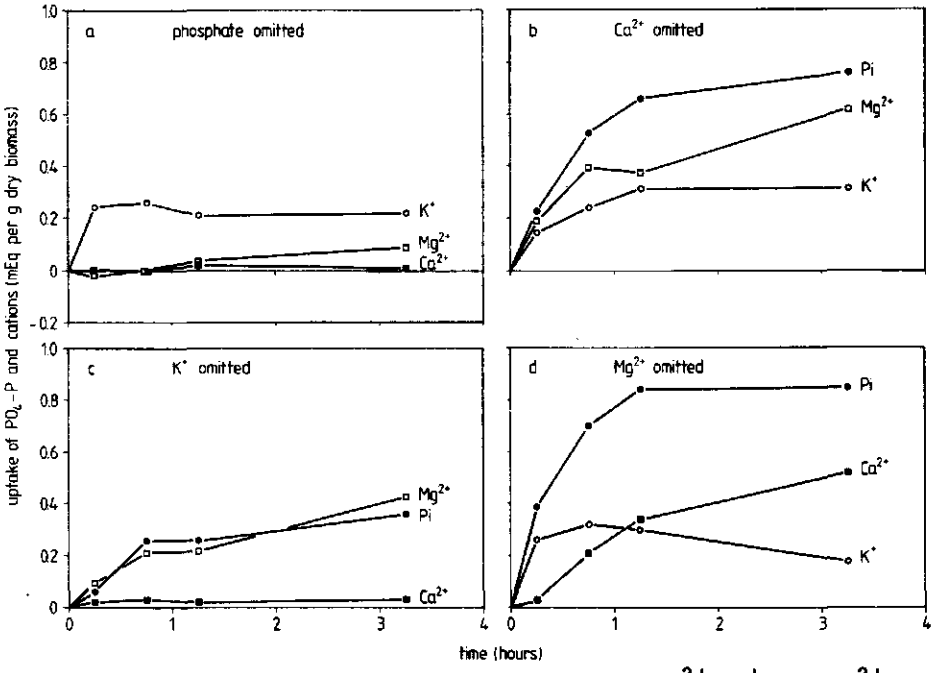

FIG. 4. The uptake of phosphate (1 mEq = 1mmol), Mg^{2+}, K^+ and Ca^{2+} by stationary cultures of Acinetobacter strain 210A in a medium with acetate and streptomycin (pH = 7, 25°C) under different limitations a: without phosphate, b: without Ca^{2+}, c: without K^+, and d: without Mg^{2+}.

To measure the mutualistic relationship between phosphate and the different cations, individual cations were omitted from the complete uptake medium (Figs. 4a-d). Simultaneously with the uptake of ortho-phosphate, K^+ and Mg^{2+} were also incorporated into the cells. All three ions, if present, were absorbed fast in the first hour (Figs. 3 and 4b-d). Later, the rate diminished steadily. The concentration of Na^+ and NH_4^+ did not change much and there was no correlation with phosphate uptake. The largest uptake or release of NH_4^+ and Na^+ mostly occurred in the first 15 minutes (results not presented). Mg^{2+} correlated best with the amount of phosphate that was accumulated, if no phosphate was taken up by the cells, Mg^{2+} uptake was reduced strongly

(Fig. 4a). The equivalents of Mg^{2+} was 60 - 100% of the equivalents of phosphate taken up. The uptake of K^+ was independent of the phosphate absorption. In absence of phosphate in the medium K^+ uptake showed the same behaviour as in the complete medium (Figs. 3 and 4a). The absence of Ca^{2+} did not affect the uptake of phosphate, Mg^{2+} and K^+ (Fig. 4b), while in absence of K^+ less phosphate and Mg^{2+} were absorbed (Fig. 4c). The omission of Mg^{2+} from the medium did not reduce the influx of phosphate, but Ca^{2+} was taken up in large amounts instead of Mg^{2+} (Fig. 4d).

In a separate experiment, cells of Acinetobacter strain 210A containing no polyphosphate were resuspended in the phosphate uptake medium at various initial concentrations of K^+. This cation stimulated the uptake rate of phosphate and increased the final concentration of phosphate in the cells (Table 1). For a maximum phosphate uptake, K^+ concentrations higher than 10 $mg.l^{-1}$ were necessary. Concentrations of 1.0, 1.5 and 3.5 mg K^+ per litre were tested as well, but the amounts of phosphate taken up at these concentration did not differ much (±10%) from those at 0.5 mg K^+ per litre. After 0.5 hours of incubation, the ATP content of the cells was measured. It decreased with increasing K^+ concentrations, namely from 7.2 to 2.6 and 3.0 umol ATP per g dry biomass at a K^+ concentration of 0.5, 10 and 100 $mg.l^{-1}$, respectively.

TABLE 1. The effect of the extracellular K^+ concentration on the uptake of phosphate from a medium with acetate and streptomycin at 25°C and pH 7 by stationary cultures of Acinetobacter strain 210A. The numbers are given in mg PO_4-P per g dry biomass.

Time (h)	K^+-concentrations ($mg.l^{-1}$)		
	0.5	10	100
0.1	0.16	0.21	0.13
0.5	0.40	0.55	0.63
1	0.51	0.72	0.82
2	0.65	0.96	1.11
4	0.74	1.04	1.12

The phosphate uptake by activated sludge showed a similar dependency on the addition of K^+ (Fig. 5), but in these experiments a lower K^+ (= 5 $mg.l^{-1}$) concentration was already sufficient for a maximum phosphate uptake. Concentrations of 12, 25, 40 and 70 mg K^+ per litre were also tested with the same sludge sample. The

phosphate uptake did not differ more than 30% from the uptake measured at 5 and 100 mg K^+ per litre. A different sludge sample, taken one week earlier from the pilot plant, accumulated 30% more phosphate in the presence of 100 mg K^+ per litre, while the same uptake performance as by the first sludge sample was found in absence of K^+.

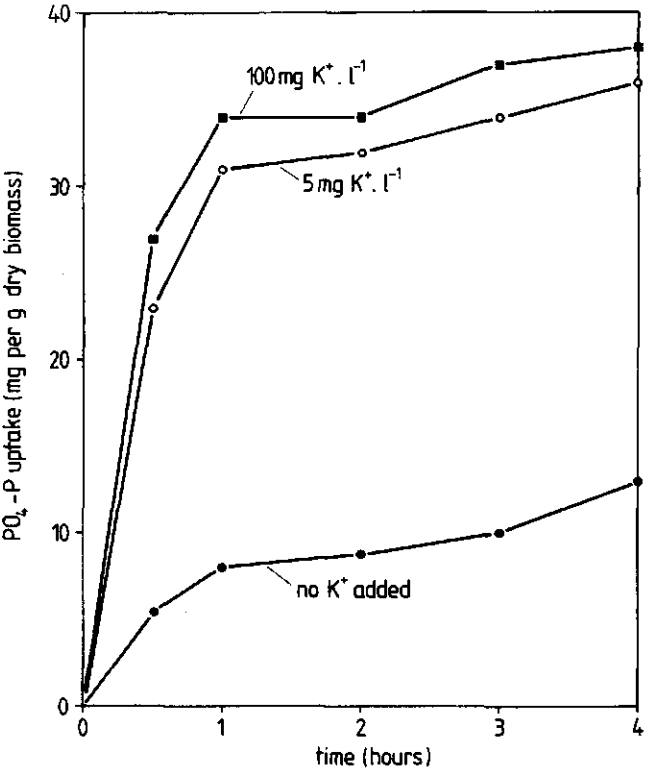

FIG. 5. The uptake of phosphate by activated sludge in an aerobic mineral medium with different initial K^+ concentrations.

Polyphosphate accumulation in continuous cultures under various limitations. Acinetobacter strain 210A was continuously cultivated at different dilutionrates under substrate, K^+- or Mg^{2+}-limitation. The effect of these limitations on the accumulation of phosphate by the cells is presented in Table 2. During cultivation with limited amounts of cations always a steady state was reached in which acetate was still present in considerable amounts while the biomass concentration was not more than half of that found in substrate limited cultures. Cells cultivated under Mg^{2+}-limitation were still able to accumulate considerable amounts of phosphate, while K^+-limita-

TABLE 2. Growth and P-content of Acinetobacter strain 210A in a continuous culture with an acetate medium under substrate, K^+ or Mg^{2+} limitation. The data presented were obtained under steady state conditions at two dilutionrates at 25°C and pH 7.0.

Limiting nutrient	Dilution-rate (h^{-1})	Biomass (mg dry wt. l^{-1})	Acetate (mM)	K^+ ($mg.l^{-1}$)	Mg^{2+} ($mg.l^{-1}$)	P-content in biomass ($mg.g^{-1}$ dry wt.)
acetate (carbon and energy source)	0.09	624	0.2	12	59	59
	0.18	844	0.3	14	53	66
Mg^{2+}	0.06	314	21	85	0.2	41
	0.11	290	27	89	0.3	55
K^+	0.08	362	4.8	1.4	80	14
	0.20	310	20	2.6	77	26

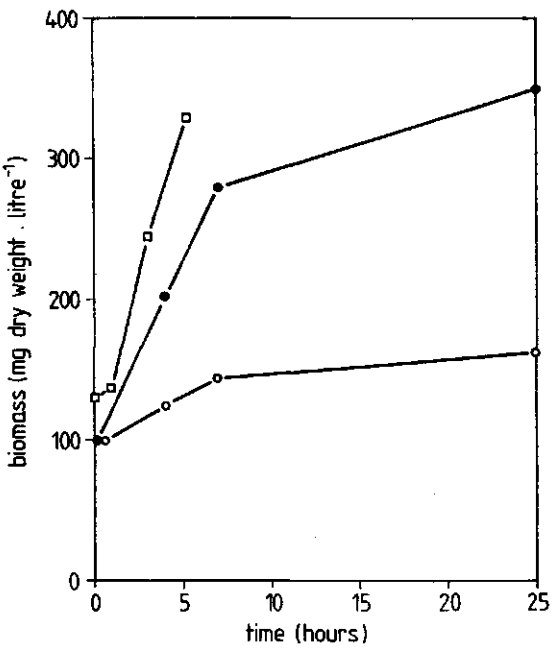

FIG. 6. Growth of cells of Acinetobacter strain 210A with polyphosphate (positive with Neisser staining; containing 75 mg P per g dry biomass), suspended in an acetate medium without Mg^{2+} (●), and cells without polyphosphate (negative with Neisser staining; containing 15 mg P per g dry biomass), suspended in a medium without (○) or with (□) Mg^{2+} (25°C, pH = 7).

tion had a strongly negative effect on this accumulation, and at low dilution rates, where the steady state concentration was lowest, no polyphosphate was accumulated: the cells contained only small amounts of phosphorus and did not colour with Neisser staining.

Polyphosphate granules as a Mg-reserve. Two different cultures of Acinetobacter strain 210A were resuspended in a Mg^{2+}-free growth medium. One culture containing 75 mg phosphorus per g dry biomass was able to grow. The second culture with only 20 mg phosphorus per g dry biomass grew considerably less. Cells from this culture were only able to grow at high rates in a medium to which magnesium was added (Fig. 6).

DISCUSSION

The experiments reported here indicate that Mg^{2+} is the most important counterion of polyphosphate in Acinetobacter strain 210A. In this strain Mg^{2+} was always released and taken up simultaneously with phosphate (Fig. 1 and 4a-c), while strain 132, which doesn't accumulate polyphosphate, released only small amounts of Mg^{2+} under the same conditions. In the phosphate uptake experiments every mmol phosphate was taken up together with 0.6 to 1 mEq Mg^{2+}. This is enough to contrabalance a major part of the negative charge of polyphosphate, since each phosphate group in this molecule carries one negative charge. Release of phosphate by strain 210A was also coupled to a pH decrease in the medium (Fig.2), which was caused by the efflux of 1.8 mmol H^+ per mmol PO_4^{3-}. This approaches the amount of protons per phosphate expected during the netto hydrolysis of Mg-polyphosphate.

The uptake and release of K^+, however, was independent of phosphate uptake and release, since (i) strain 132 extruded under anaerobic conditions the same amount of K^+ as strain 210A (Fig. 1), (ii) K^+ was released faster than phosphate by strain 210A and reached its maximum concentration much earlier (Fig.1), (iii) at a low pH phosphate release was slower than the release of K^+, and (iv) independent of the presence or absence of phosphate strain 210A took up a smaller amount of K^+ as compared to phosphate (Figs. 3, 4a,4b and 4d). The uptake and release of K^+ might not directly be coupled to the accumulation and degradation of polyphosphate but rather to a reestablishment or decline of a specific energetic state of the cell. As was indicated by Mulder et al. (20) the maintenance of a steep K^+ gradient across

the cell-membrane costs energy. Since Acinetobacter is a strict aerobe, energy supply under anaerobic conditions is restricted and a leakage of K^+ can be expected. Although K^+ does not play a quantitatively important role as polyphosphate counterion in Acinetobacter strain 210A, the presence of K^+ in the medium appeared to be a crucial factor for phosphorus accumulation by this strain (Table 1 and 2) as well as in activated sludge (Fig. 5). There could be many reasons for this dependency: K^+ could either play a role in phosphate transport (18), or in energy generation (20), or in stimulation of enzyme-acitivities (10) or may be of importance for the structure of polyphosphate granules. The suboptimal polyphosphate accumulation during growth under K^+-limitation points in the direction of energy shortage which is in accordance to the findings of Mulder et al. (20) and Sall et al. (23). Mulder et al. (20) reported that K^+-limitation causes energy limitation, and Sall et al. (23) found that K^+-limited cells of Corynebacterium diphteriae consumed less oxygen and accumulated less phosphate. However, the batch experiments presented in Table 1 in which phosphate uptake and ATP content in Acinetobacter strain 210A were measured show that energy shortage may not be the only reason for the inhibition of polyphosphate accumulation by K^+-limitation. At low K^+ concentrations less phosphate was taken up and the uptake rate was slower but the cellular ATP content was higher. As ATP is most probably the energy and phosphate donor in polyphosphate synthesis by Acinetobacter sp. (25), an effect of K^+-limitation on phosphate transport and energy generation cannot be the major cause of a decreased polyphosphate accumulation under these conditions. Therefore, K^+ must have at least one other role in polyphosphate accumulation e.g. the stimulation of the activities of enzymes involved in polyphosphate synthesis or a structural role in the formation of polyphosphate granules. Further research is needed to elucidate the relation between polyphosphate and K^+ in Acinetobacter.

Although Mg^{2+} was preferred as a counterion, this cation was not essential for polyphosphate accumulation by Acinetobacter strain 210A. If Mg^{2+} was not available as a counterion it was partly replaced by Ca^{2+} (Fig. 4d). Phosphate accumulation was still possible when Mg^{2+} supply was limited in continuous cultures of Acinetobacter strain 210A (Table 2).

From the results of the experiments on growth of cells with or without polyphosphate in a Mg^{2+}-free medium, it can be concluded that the polyphosphate granules can also act as a Mg^{2+}-reserve. A function of polyphosphate as a cation reserve has always been hypothesized but was difficult to prove, because

most microorganisms accumulating polyphosphate contain relatively small amounts of this compound. As Acinetobacter strain 210A accumulates exceptionally large amounts of polyphosphate the role of this compound as a cation reserve could clearly be demonstrated.

Many cations can serve as counterions in polyphosphate in different microorganisms. In Micrococcus lysodeikticus (11) and in Desulfovibrio gigas (16) Mg^{2+} was the most important polyphosphate counterion, while Mg^{2+} and Ca^{2+} had been found in vacuoles containing polyphosphate in Neurospora crassa (7) and K^{+} was a polyphosphate counterion in Chlorella sp. (5). The findings of Buchan (4) that Ca^{2+} is the predominant polyphosphate counterion in acinetobacters in activated sludge was not confirmed by our experiments. Since Buchan used EDAX, studies have been carried out in our laboratory on the quantitative composition of polyphosphate granules using also the same technique. The first results indicate the presence of high amounts of Mg^{2+} and Ca^{2+} and minimal amounts of K^{+} (unpublished results). One possible explanation of the differences between EDAX studies and release experiments is that Ca^{2+} is a polyphosphate counterion in Acinetobacter sp. which is not released when the polyphosphate is broken down (Ca^{2+} might stay in the cell while ortho-phosphate is released).

In conclusion it can be said that the uptake and release of cations by pure cultures of Acinetobacter strain 210A is in accordance with the results obtained in experiments with activated sludge from waste water treatment plants showing biological phosphate removal. Mg^{2+} has been reported as the most important counterion during phosphate uptake and release, followed by K^{+}, while Ca^{2+} was found to play an insignificant role in this process (3, 6, 17, 19, 26). Arvin et al. (3) concluded that Mg^{2+} is a typical counterion in the polyphosphate formation in sludge, while Ca^{2+} is accumulated in sludge by means of chemical precipitation. Our results with Acinetobacter strain 210A confirm the importance of Mg^{2+} as a counterion of polyphosphate. The uptake and release of K^{+} by activated sludge may not have any relation with phosphate uptake and release. This cation is concentrated aerobically inside the cells, but not in polyphosphate granules, and may be released under energy unfavourable conditions such as anaerobiosis. A shortage of K^{+} in the waste water may have a negative effect on biological phosphate removal (Fig. 5).

ACKNOWLEDGEMENTS

We thank Mr. R.M.L. Rongen for his practical assistance and the Department of Water Pollution Control and the Department of Biochemistry of Wageningen Agricultural University for using their equipment. This research was supported by a special grant from the Agricultural University Wageningen.

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CHAPTER 4

ATP PRODUCTION FROM POLYPHOSPHATE IN ACINETOBACTER STRAIN 210A

Johan W. van Groenestijn, Maria H. Deinema and Alexander J.B. Zehnder

ABSTRACT

In cell-free extracts of Acinetobacter strain 210A polyphosphate:AMP phosphotransferase and adenylate kinase activity was measured. Polyphoshate glucokinase and polyphosphate dependent NAD kinase were not detected. The specific activity of poly-phosphate:AMP phosphotransferase was found to be 43 nmol min^{-1} mg^{-1} protein in presence of 1 mmol l^{-1} AMP. The adenylate kinase reaction had an equilibrium constant ($[ATP][AMP][ADP]^{-2}$) of 0.7, an activity of 54 nmol min^{-1} mg^{-1} protein, and was almost completely inhibited by 0.3 mM P^1,P^5-di(adenosine-5')-pentaphosphate. ATP was formed through the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase in cell-free extracts from bacterial polyphosphate and from chemically prepared polyphosphate (Graham's salt). A spectrophotometric method for the continuous monitoring of polyphosphate:AMP phosphotransferase is also presented.

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Some strains of the genus Acinetobacter may play an important role during enhanced biological phosphate removal from waste water (Fuhs and Chen 1975). These organisms are able to accumulate phosphate intra-cellularly in form of polyphosphate (Ohtake et al. 1985). This polymer can, under certain conditions, account for over 30% of the biomass dry weight (Deinema et al. 1985). In various bacteria polyphosphate act as reserve material for phosphorus. However, more interestingly, it might also be used as an energy reserve in vivo (Kulaev 1979). In the case of Acinetobacter, a strictly aerobic bacterium, phosphate is taken up in excess to form polyphosphate during aerobic growth and is released when the environment becomes anaerobic (Deinema et al. 1985). It is possible, by alternating aerobic and anaerobic conditions, to force some Acinetobacter strains into a cyclic process during which phosphate is accumulated and released again. When these alternating conditions are introduced in waste water treatment plants, the number of Acinetobacter increases and large amounts of phosphate are removed (Buchan 1983, Rensink et al. 1981).

The role of polyphosphate in Acinetobacter has not yet been elucidated, but many hypotheses exist. The most obvious one is that Acinetobacter may use the energy stored in form of polyphosphate during periods of starvation or other adverse conditions. In some other bacteria polyphosphate has been shown to be able to phosphorylate adenylates, pyridine nucleotides or some other organic compounds (a modern and comprehensive review on this subject has been written by Kulaev and Vagabov in 1983). Examples are:
polyphosphate kinase (Kornberg 1957) which catalyses:
$(\text{polyphosphate})_n + \text{ADP} \rightleftharpoons (\text{polyphosphate})_{n-1} + \text{ATP}$,
polyphosphate:AMP phosphotransferase (Dirheimer and Ebel 1965) catalysing:
$(\text{polyphosphate})_n + \text{AMP} \longrightarrow (\text{polyphosphate})_{n-1} + \text{ADP}$,
polyphosphate glucokinase (Szymona and Ostrowski 1964) catalysing:
$(\text{polyphosphate})_n + \text{glucose} \longrightarrow (\text{polyphosphate})_{n-1} + \text{glucose-6-P}$
and polyphosphate dependent NAD-kinase (Murata et al. 1980) catalysing:
$(\text{polyphosphate})_n + \text{NAD} \longrightarrow (\text{polyphosphate})_{n-1} + \text{NADP}$.
Especially polyphosphatekinase and glucokinase has been studied thoroughly. New insights into the reaction mechanism and kinetics of polyphosphatekinase (Robinson and Wood 1986) and glucokinase (Pepin and Wood 1986) has been developed recently.

In the present study it is shown that in cell-free extracts of Acinetobacter strain 210A ATP is formed from native and synthetical polyphosphate via the combined action of two enzymes, namely polyphosphate:AMP phosphotransferase and adenylate kinase.

MATERIALS AND METHODS

Organism. Acinetobacter strain 210A was isolated from activated sludge with the method described by Deinema et al. (1980). This organism was maintained on yeast extract agar slants, subcultured every two months and stored at 4 °C. The strain can be obtained from the authors.

Growth conditions. Polyphosphate rich Acinetobacter cells containing 100 mg phosphorus per g biomass dry weight were cultivated in Erlenmeyer flasks in a shaker at 15 °C. The medium contained per 1000 ml demineralised water 2.29 g Na-butyrate, 0.1 g $Na_2S_2O_3 \cdot 5H_2O$, 1.0 g NH_4Cl, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.44 g KH_2PO_4, 0.06 g $CaCl_2 \cdot 2H_2O$, 6 g Tris(hydroxymethyl)aminomethane and 2 ml of a trace mineral solution (containing 1500 mg $FeCl_3 \cdot 6H_2O$, 50 mg H_3BO_3, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg NaI, 40 mg $MnCl_2 \cdot 4H_2O$, 20 mg $Na_2MoO_4 \cdot 2H_2O$, 40 mg $ZnSO_4 \cdot 7H_2O$ and 50 mg $CoCl_2 \cdot 6H_2O$ per litre demineralised water). The pH was adjusted to 7 with 6 N HCl.
Cells without polyphosphate were cultivated under P-limitation in a medium as described above except that it contained only 34 mg KH_2PO_4 per litre. Polyphosphate free cells did not stain following the method of Neisser (Gurr 1965) and contained the minimum amount of 13 mg phosphorus per g dry weight.

Preparation of cell-free extracts. The cultures were harvested in the late log-phase and centrifuged, washed with demineralised water and centrifuged again. The pellets were resuspended in a 30 mM Tris-HCl buffer pH 7.0, and then sonified with an ultrasonic disintegrator (model B12, Branson Sonic Power Company). The membranes and still intact cells were removed by centrifugation at 35,000 x g for 30 minutes. Under these conditions polyphosphate remained in the supernatant. The polyphosphate rich extract contained 1.94 mg protein per ml, and 360 µg total phosphorus of which 33 µg (as P) was ortho-phosphate. The extract without polyphosphate contained 1.31 mg protein per ml, and 59 µg total phosphorus of which 10 µg (as P) was ortho-

phosphate.

Chemical and biochemical determinations. Ortho-phosphate and total phosphorus (persulphate digestion method) were determined spectrophotometrically according to the Standard Methods (1976). The protein concentration was measured with the method of Lowry using bovine serum albumin as standard. ATP, ADP and AMP concentrations in cell-free reaction mixtures were determined according to Pradét (1967) and the instructions belonging to the Adenylate Energy Charge (AEC) kit of LUMAC/3M. The samples were boiled for 4 minutes in 5 ml of a solution containing 50 mM Tris-HCl and 4 mM EDTA at pH 7.75, in order to stop all enzyme activity. These samples were then divided into three subsamples. The first obtained luciferine and firefly luciferase. The resulting light-emission which was dependent on the ATP concentration was measured with a luminescentionmeter (made by the Electronics Design and Maintenance Shop Division of the General Research Department, University of Georgia, Athens, USA). The second subsample was first pretreated with P-enolpyruvate and pyruvatekinase to convert ADP into ATP and further treated as the first aliquot, while a third subsample was pretreated with adenylate kinase to convert AMP into ADP followed by the same treatment as applied to the second subsample.

Enzyme assays. All assays were carried out at 30 C and pH 7.0. The reaction mixture contained 0.18 ml cell-free extract per ml which resulted in 238 µg protein per ml in the case of extracts without polyphosphate and 353 µg protein and 65 µg bacterial total phosphorus per ml when extracts with polyphosphate were used.

Adenylate kinase,EC 2.7.4.3: 3 mM $MgCl_2$ and 20 mM Tris-HCl, the reaction was started by adding ADP,resulting in a concentration of 0.6 mM. At time intervals samples of 50 µl were taken from the reaction mixture to determine AMP, ADP and ATP concentrations.

Polyphosphate:AMP phosphotransferase (Dirheimer and Ebel 1965): 3 mM $MgCl_2$ and 16 mM Tris-HCl, the reaction was started by adding AMP, resulting in a concentration of 1 mM. At time intervals 25 µl were taken from the reaction mixture to determine AMP and ADP concentrations.

The following assays were carried out in a cuvette containing a reaction mixture with 0.5 mM KH_2PO_4 and 100 mM Tris-HCl pH 7.0. The cuvette was placed in a Beckmann spectrophotometer and the production of $NADPH_2$ was measured continuously at 340 nm. Polyphosphate:AMP phosphotransferase: 8 mM $MgCl_2$, 220 mM

glucose, 0.67 mM NADP, 3.4 U (units) hexokinase (HK)/ml, 1.7 U D-glucose-6-P-dehydrogenase (G6P-DH)/ml and 1 U adenylate kinase/ml, if not sufficient suitable polyphosphate was present Graham's salt was added, the reaction was started by adding AMP, resulting in a concentration of 1 mM. The reaction scheme is given in Fig.1. From this scheme it is evident that the conversion of one AMP to one ADP will result in the synthesis of one $NADPH_2$. Polyphosphate glucokinase,EC 2.7.1.a: 8 mM $MgCl_2$, 0.67 mM NADP 1.7 U/ml G6P-DH, the reaction was started by adding glucose, resulting in a concentration of 220 mM. Polyphosphate dependent NAD kinase: 5.0 mM $MgCl_2$, 1.7 U G6P-DH/ml and 1.2 mM D-glucose-6-P, the reaction was started by adding NAD,resulting in a concentration of 5.0 mM. ATP production in cell-free extracts: 8 mM $MgCl_2$, 220 mM glucose, 0.67 mM NADP, 3.4 U HK/ml and 1.7 U G6P-DH/ml, the reaction was started by adding AMP or ADP.

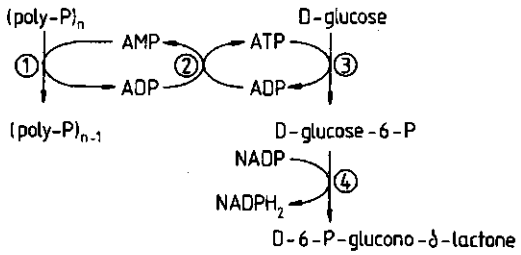

Fig. 1. A method to measure polyphosphate:AMP phosphotransferase (1) continuously. The formed ADP conducts the reactions of the added enzymes which form a part of the detection system: adenylate kinase (2), hexokinase (3) and glucose-6-P-dehydrogenase (4). The produced $NADPH_2$ is measured spectrophotometrically and recorded.

Chemicals. HK (EC 2.7.1.1), G6P-DH (EC 1.1.1.49), adenylate kinase (EC 2.7.4.3), NADP, NAD, AMP, ADP, ATP and D-glucose-6-P were purchased from Boehringer, Mannheim. Ap_5A (P^1,P^5-di(adenosine-5')-pentaphosphate) was obtained from Sigma Chemical Co., St.Louis,MO. The AEC-kit was from LUMAC/3M bv. Synthetic polyphosphate in form of Graham's salt, which represents a mixture of linear sodium polyphosphates [$(NaPO_3)_n$, $\bar{n}=10^2$], was prepared by heating NaH_2PO_4 3 hours at 750 °C followed by cooling in the air outside the furnace (Kulaev 1979).

Measurement of polyphosphate glucokinase, polyphosphate dependent NAD kinase and adenylate kinase

In cell-free extracts no polyphosphate glucokinase and polyphosphate dependent NAD kinase activities were found. In control experiments D-glucose-6-P and NADP were added respectively. In both cases $NADPH_2$ was formed rapidly. Adenylate kinase was measured in cell-free extracts containing no polyphosphate. Its activity amounted to 54 nmol min^{-1} mg^{-1} protein and its equilibrium constant ($[ATP][AMP][ADP]^{-2}$) was 0.7 (Fig.2). This constant is somewhat higher than the constants reported by Bowen and Kerwin (1954) for adenylate kinase (0.3 - 0.4 at a pH of 6.0).

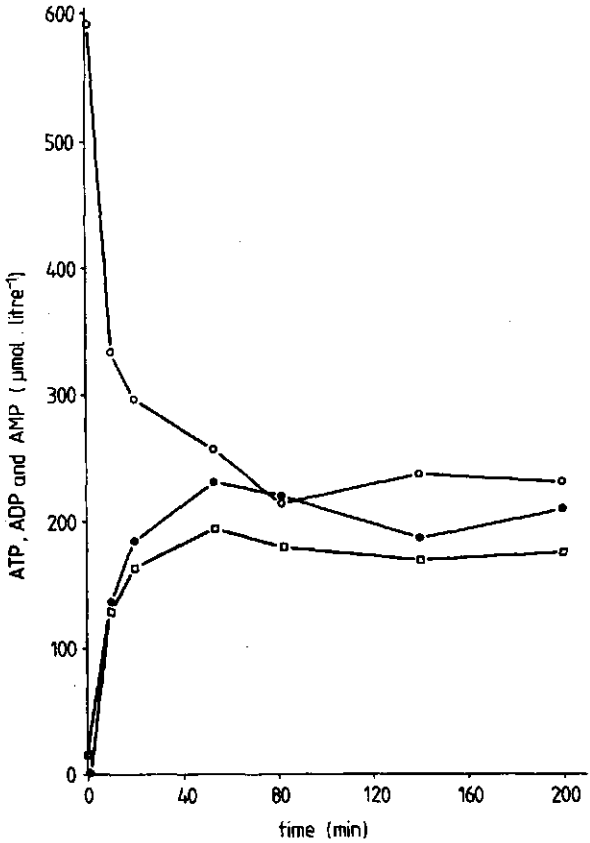

Fig. 2. The adenylate kinase reaction (2 ADP ⇌ AMP + ATP), measured in a cell-free extract of Acinetobacter strain 210A without polyphosphate. At time intervals samples were taken in which the concentrations of AMP (●), ADP (○) and ATP (□) were determined.

Measurement of polyphosphate:AMP phosphotransferase

Cell-free extracts from Acinetobacter strain 210A containing native polyphosphate phosphorylated AMP (Fig.3b). Cell-free extracts without bacterial polyphosphate were not able to catalyse this reaction (Fig.3a). During phosphorylation of AMP the concentration of ADP was always higher than that of ATP. This might be due to the action of adenylate kinase which has an equilibrium constant ($[ATP][AMP][ADP]^{-2}$) of 0.7. The high amounts of AMP added (1 mM), caused therefore a change in relative ADP concentration. Addition of synthetic polyphosphate in the form of 6 mM (as P) Graham's salt plus 8 mM $MgCl_2$ to cell-free extract containing no biological polyphosphate resulted within 440

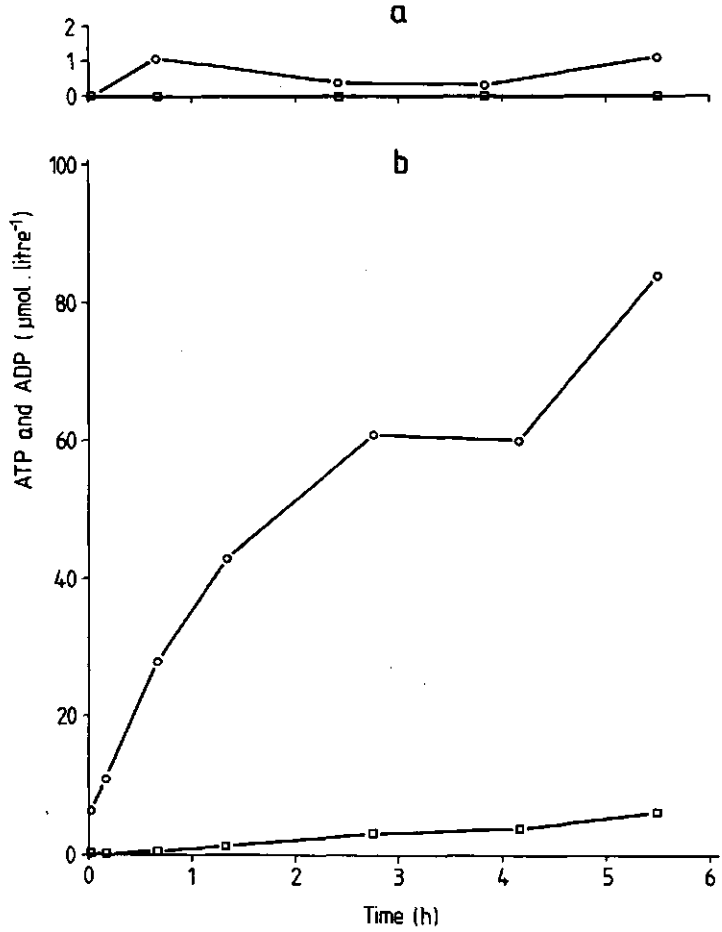

Fig. 3. ADP (○) and ATP (□) formation during incubation of cell-free extracts of Acinetobacter strain 210A with 1 mM AMP (a: without polyphosphate, b: with bacterial polyphosphate).

minutes in an ADP and ATP production of 130 µM and 19 µM, respectively (results not shown). This experiment clearly demonstrated that polyphosphate was the phosphorus donor and agrees with the findings of Dirheimer and Ebel (1965) that for partly purified polyphosphate:AMP phosphotransferase from Corynebacterium xerosis Graham's salt could serve as a substrate.
To establish whether AMP was first converted into ADP and whether ATP was formed subsequently from ADP by adenylate kinase (first ATP and then ADP could also be possible) the following experiments were carried out: In a first experiment AMP, ADP, ATP and $NADPH_2$ were measured simultaneously in cell-free extracts with bacterial polyphosphate. The experiment was started by adding AMP. The results summarized in Table 1 show clearly that AMP was recycled in this process and the equilibrium of the adenylate kinase reaction was not reached since ATP was used up immediately for $NADPH_2$ formation. That meant that ADP was consumed by the adenylate kinase reaction in order to produce ATP and AMP. The decreasing AMP concentration and the increasing ADP concentration in the first hour indicated a conversion of AMP into ADP. During the first hour ADP accumulated, probably because the polyphosphate:AMP phosphotransferase activity was higher than the adenylate kinase activity.

Table 1. The production of ADP and ATP (measured via $NADPH_2$ with the HK/G6P-DH method) after addition of AMP to a polyphosphate containing cell free extract of Acinetobacter strain 210A.

Time (min)	$NADPH_2$ (µM)	ATP (µM)	ADP (µM)	AMP (µM)	ratio $\frac{[AMP]\ [ATP]}{[ADP]^2}$
0	0	0.3	12	3304	6.9
10	4	0.2	151	3382	0.03
20	35	0.1	301	3210	0.004
40	118	2.1	578	2883	0.02
60	245	2.8	658	2455	0.02
100	335	2.4	534	2933	0.02
140	395	1.8	428	2953	0.03

In a second experiment adenylate kinase was specifically inhibited with Ap_5A (Feldhaus et al. 1975). A control experiment revealed that the measurement of ATP by the HK/G6P-DH method was not affected by 0.3 mM Ap_5A. In this assay cell-free extract containing bacterial polyphosphate was incubated with 5 mM AMP and 0.3 mM Ap_5A, and ATP was quantified according to the HK/G6P-

DH method. Very low amounts of $NADPH_2$ were formed (Fig.4) indicating a very low ATP production, while high amounts of ADP accumulated. The results from both experiments showed clearly that in cell-free extracts of Acinetobacter strain 210A ATP are formed from polyphosphate by the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase.

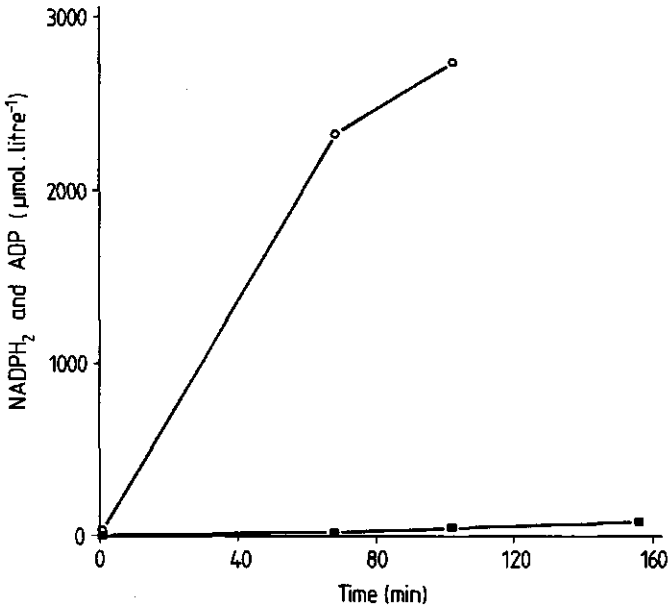

Fig. 4. The accumulation of ADP (O) and the production of ATP (measured as $NADPH_2$ (■)) during the incubation of a polyphosphate containing cell-free extract of Acinetobacter strain 210A with AMP and Ap_5A (inhibitor of adenylate kinase).

In case polyphosphate kinase would also be present in the cell-free extract it could be expected that ATP production would not specifically be inhibited by Ap_5A. Ap_5A has never been reported to inhibit polyphosphate kinase (Feldhaus et al. 1975, Kulaev 1979). The very low ATP production during incubation (Fig.4) suggests that polyphospate kinase plays (if any) merely an inferior role in the conversion of ADP to ATP as compared to the polyphosphate:AMP phosphotransferase/adenylate kinase system.

Continuous monitoring of the polyphosphate:AMP phosphotransferase activity

A method for the continuous measurement of polyphosphate:AMP phosphotransferase activity was developed. The incubation mix-

ture usually used to determine the ATP production in cell-free extracts, was supplied with additional adenylate kinase (commercially available as myokinase). This was necessary because the native activity of adenylate kinase in cell-free extracts of Acinetobacter strain 210A was too low to convert all the ADP produced fast enough to ATP. $NADPH_2$ formation depended strictly on the addition of AMP and became linear after 8 minutes (Fig.5). At that moment the increase of ADP concentration leveled off and reached an equilibrium concentration of 100 µM. Subsequently all the ADP produced was turned over immediately by the adenylate kinase.

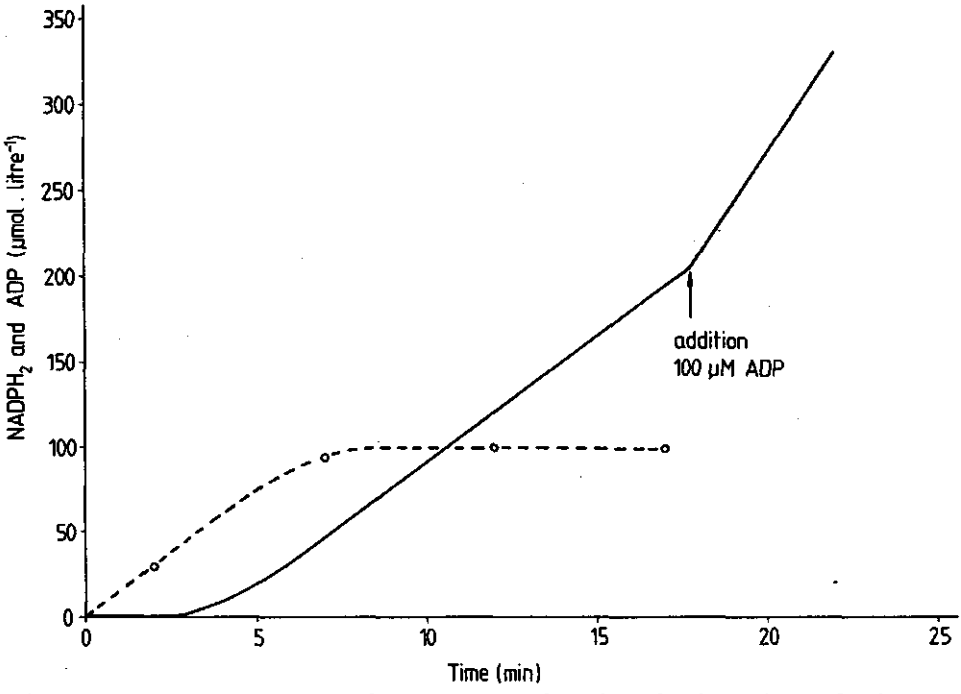

Fig. 5. $NADPH_2$ (———) and ADP (O) production during the polyphosphate:AMP phosphotransferase assay (HK/G6P-DH method). At 18 minutes 100 nmol ADP was added per ml reaction mixture to demonstrate that the activities of the enzymes of the detection system were not saturated.

Polyphosphate:AMP phosphotransferase activity can only be measured if the reaction catalysed by this enzyme is the rate-limiting step; i.e. adenylate kinase, HK and G6P-DH have to be present in excess. The free capacity of these three enzymes can be tested by adding extra ADP at the end of the experiment (in Fig.5 100 µM at 18 minutes). As depicted by Fig. 5 a higher rate of $NADPH_2$ production was indeed measured.

During the assay the phosphate ion concentration in the reac-

tion mixture remained constant and no free phosphate was produced. This means that phosphorylation of one AMP diminishes the polyphosphate molecule by one phosphate group only.

The highest polyphosphate:AMP phosphotransferase activities were found during these HK/G6P-DH assay's, namely 43 nmol min^{-1} mg^{-1} protein as calculated from Fig. 5 as well as from Table 1.

Cell-free extracts without polyphosphate produced no $NADPH_2$. Upon addition of 6 mM (as P) Graham's salt $NADPH_2$ was formed at a rate corresponding to about half of the polyphosphate:AMP phosphotransferase activity measured in cell-free extracts with bacterial polyphosphate.

DISCUSSION

The enzymes polyphosphate dependent NAD kinase and polyphosphate glucokinase were not found in Acinetobacter strain 210A, though these enzymes have been found in many other polyphosphate accumulating bacteria (Murata et al. 1980, Kulaev 1979). The absence of polyphosphate glucokinase was not surprising since Acinetobacter strain 210A is not able to assimilate glucose (Deinema et al. 1980).

The enzymes polyphosphate:AMP phosphotransferase and adenylate kinase enable Acinetobacter cells to form ATP under energy-starvation, as long as polyphosphate is present. This system is in a way superior to polyphosphate kinase since it is able to phosphorylate AMP. The considerable polyphosphate reserve in Acinetobacter strain 210A can therefore be very valuable under conditions when this strict aerobe has no other possibility to obtain energy, e.g. in absence of oxygen. The ability to accumulate polyphosphate and to use it as an energy source under adverse conditions may give Acinetobacter a distinct competitive advantage in an activated sludge system subjected to repeated anaerobic/aerobic cycles. Kulaev and Vagabov (1986) concluded that the general function of high molecular-weight polyphosphates in microorganisms is the regulation of intracellular concentrations of important metabolites including ATP, ADP, other nucleoside polyphosphates, and finally pyro- and particularly ortho-phosphate. In certain aerobic microorganisms polyphosphate is not broken down if energy generation is limited or blocked (Harold 1966). In these organisms polyphosphate might only be a phosphorus storage material, which is valuable in biotopes where phosphate is growth limiting. As Acinetobacter strain 210A is isolated from a phosphate-rich environment it can

be expected that the polyphosphate accumulated by this strain has an other function than phosphorus storage.

The measured activities of polyphosphate:AMP phosphotransferase and adenylate kinase are in accordance to the rates by which phosphate is released by pure cultures of Acinetobacter sp. (1 nmol PO_4-P min^{-1} mg^{-1} dry weight found by Adamse et al. 1984) or activated sludge (0.6 nmol PO_4-P min^{-1} mg^{-1} dry weight found by Rensink et al. 1981).

Dirheimer and Ebel (1965) found a K_m of 2 10^{-2} M AMP for polyphosphate:AMP phosphotransferase in Corynebacterium xerosis and needed in their assay 50 mM AMP to reach an activity of about 17 nmol min^{-1} mg^{-1} protein at 37 C. Because of its low activities at low AMP concentrations, the physiological significance of this enzyme is still unclear (Kulaev 1979). However, from our experiments it appeared that Acinetobacter strain 210A disposes of a polyphosphate:AMP phosphotransferase activity that is already sufficiently high at low AMP concentrations. Assuming a cellular protein content of 500 mg per g dry weight, the specific activity of polyphosphate:AMP phosphotransferase of 43 nmol min^{-1} mg^{-1} protein could be responsible for the production of 1.5 mmol ATP h^{-1} g^{-1} dry weight. When grown under optimal conditions one gram of Acinetobacter strain 210A contains about 100 mg phosphorus (Deinema et al. 1985) of which about 80 mg or 2.6 mmol are incorporated into polyphosphate. At an ATP production rate of 1.5 mmol h^{-1} g^{-1} dry weight, polyphosphate could serve as an energy source for 1.7 hours.

From chemostat experiments with strain 210A (Deinema et al. 1985) 0.6 mmol acetate h^{-1} g^{-1} dry weight are needed for maintenance, which in its best case translates to 6.6 mmol ATP h^{-1} g^{-1} dry weight. This is about four times more than the polyphosphate:AMP phosphotransferase system can supply in vitro. According to Pirt (1975) microorganisms need during growth under carbon limitation 0.5 to 14 mmol ATP h^{-1} g^{-1} dry weight for maintenance. One has to realize, however, that these energies necessary for maintenance were extrapolated from well growing cultures. These values might be significantly different from the maintenance energy required under non-growth conditions (Mason et al. 1986) and under circumstances where no external energy source is available. As a matter of fact microorganisms are actually capable to survive for a very long period of time in their vegetative forms in severely energy-limited systems in nature (e.g. open ocean, deep groundwater systems, etc.). This indicates that the maintenance energy might not be constant but might reach a value close to zero for some organisms under certain adverse circumstances.

Whether Acinetobacter strain 210A can really benefit in vivo from polyphosphate as an energy reserve and whether this reserve is able to cover the maintenance needs for a longer period of time, remains to be answered. Research is in progress in our laboratory concerning these problems.

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CHAPTER 5

POLYPHOSPHATE AS AN ENERGY RESERVE *IN VIVO* IN *ACINETOBACTER* STRAIN 210A

Johan W. van Groenestijn, Maria H. Deinema, and Alexander J.B. Zehnder

ABSTRACT

The regulation of the degradation of inorganic polyphosphate and the use of this compound as an energy reserve *in vivo* in *Acinetobacter* strain 210A has been studied. The response of this organism to various energetic conditions was determined. The ortho-phosphate release and ATP content of strain 210A under anaerobic conditions was compared to another polyphosphate containing *Acinetobacter* sp. and to two acinetobacters and a *Pseudomonas* sp. which are unable to accumulate polyphosphate. It could be concluded that the prerequisites for polyphosphate degradation were a low energy state of the cell and the presence of AMP. The degradation and synthesis of polyphosphate coincided with low and high cellular ATP contents, respectively. This degradation was affected by the pH and was stimulated by alcohols. Under anaerobic conditions cells with polyphosphate released more ortho-phosphate and contained more ATP than cells without polyphosphate. The transmembrane protongradient played an important role in the anaerobic energy metabolism of this strictly aerobic organism. The addition of DNP decreased the cellular ATP concentration and stimulated the polyphosphate degradation.

submitted for publication

INTRODUCTION

Accumulation of polyphosphate is a well known physiological property of many microorganisms, but the function of this biopolymer is not yet totally clear. From the great number of studies on polyphosphate metabolism the general conclusion can be drawn that this inorganic polymer can play an essential role in the metabolism of organisms as an high-energy phosphorus compound functionally alternative to ATP, and that it can be important in the regulation of numerous biochemical processes (Kulaev and Vagabov 1983). Many authors have speculated about the function of inorganic polyphosphate as an energy reserve in vivo. In 1973 Dawes and Senior concluded that the status of polyphosphate as an energy reserve was by no means clearly established. Their major argument against polyphosphate functioning as an energy reserve was based on the observation that in many organisms polyphosphate present in the cell may only account for a small proportion of the total ATP generated. In order to show the energy-storage function of a compound, it is necessary to demonstrate three points (Wilkinson 1959): (1) The compound is accumulated under conditions when the supply of energy from exogenous sources is in excess of that required by the cell for growth and related processes at that particular moment of time; (2) the compound is utilised when the supply of energy from exogenous sources is insufficient for the optimal maintenance of the cell, either for growth and division or for maintenance of viability and other processes; and (3) that the compound is broken down to produce energy in a form utilisable by the cell, and that it is, in fact, utilised for some purpose which gives the cell a biological advantage in the struggle for existence over those cells which do not have a comparable compound. There are a number of polyphosphate accumulating bacteria that don't obey these conditions (Dawes and Senior 1973, Harold 1966). A few authors report the use of pyro- and polyphosphates as an energy reserve in vivo. Pyrophosphate increased the growth rate and yield of Clostridium sp. (Cruden et al. 1983). Pyro- and polyphosphates stimulated growth of anaerobic bacteria of several unidentified species in a minimal medium in which ortho-phosphate was produced. Ortho-phosphate alone did not have this stimulatory effect (Varma and Peck 1983). Zyuzina et al. (1981) reported that Streptomyces levoris used inorganic polyphosphates and not ATP as an energy source during the synthesis of the antibiotic levorin.

Some strains of the strictly aerobic Acinetobacter accumulate aerobically large amounts of polyphosphate (up to 100 mg P per g

dry biomass, Deinema et al. 1980) and were able to degrade this compound in the absence of oxygen (Adamse et al. 1984). Recently, it has been demonstrated that ATP can be produced from polyphosphate in cell-free extracts of Acinetobacter strain 210A by the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase (Van Groenestijn et al. 1987).

This report presents evidence that polyphosphate can act as source of energy in whole cells of Acinetobacter strain 210A and that the phosphorylation of adenylates by polyphosphate depends on the presence of AMP and a low energy state of the cell (low proton motive force and low ATP level).

MATERIALS AND METHODS

Organisms. Acinetobacter strains 210A, B8, 132 and 124 were isolated from activated sludge with the method described by Deinema et al. (1980). These strains were maintained on yeast extract agar slants (5 g glucose, 2.5 g yeast extract and 12 g agar per litre tapwater, pH = 7), subcultured every two months and stored at 4°C. Strains 210A and B8 were able to accumulate large amounts of polyphosphate, while strains 132 and 124 were unable to store this compound. Pseudomonas strain 10, which doesn't contain detectable amounts of polyphosphate, was obtained from the culture collection of our laboratory. It was maintained on nutrient broth agar (8 g nutrient broth and 12 g agar per litre tapwater), subcultured every two months and stored at 4°C.

Media. The cells were precultivated in a standard phosphate rich medium containing butyrate as only carbon and energy source and in one experiment in a phosphate poor (phosphorus limited) medium. Both media were described elsewhere (Van Groenestijn et al. 1987). Butyrate is an optimal substrate for polyphosphate accumulation in Acinetobacter strain 210A.
Experiments were carried out with different phosphate release media which were all based on demineralized water, namely the pH experiment in a medium with 5 g Na-acetate.$3H_2O$, 6 g NaCl and 4 g tris per litre. The desired pH was obtained by adjusting the medium with 6 N HCl. The experiments with different alcohols and the experiments in which the effect of different types of energy starvation was determined, were carried out with a medium containing 6 $g.l^{-1}$ NaCl and 30 mM Tris-HCl. The pH of the medium used for anaerobic incubation was adjusted to 8.0 and the aerobic medium had a pH of 7.5. The anaerobic medium used for

measuring the cellular ATP content contained 30 mM Tris-HCl pH 8.0.

Experimental procedures. Cells were precultivated in Erlenmeyer flasks in a shaker. Acinetobacter strain B8 was grown at 25°C, its optimum temperature for polyphosphate accumulation. All other strains were grown at 15°C, the temperature at which Acinetobacter strain 210A accumulated high amounts of polyphosphate. In the late log-phase one gram biomass dry weight contained 100 mg P (strain 210A), 54 mg P (strain B8), 30 mg P (strain 124), 17 mg P (strain 132) and 20 mg P (Pseudomonas strain 10). These late log-phase cultures were harvested, centrifuged, washed with a 8 mM Tris-HCl buffer pH 7.5 and centrifuged again. The pellets were resuspended in the appropriate release medium resulting in biomass concentrations of 0.5 to 1.0 g dry weight per litre. Media were made anaerobic in serumvials of 30 or 100 ml by flushing them with a gas-mixture of N_2:CO_2 (99%:1%,v/v). The pH of the media which was initially adjusted to 8.0 decreased to 7.5 as a result of addition of carbon dioxide. In experiments where alcohols were used they were directly injected into the vials, containing already the anaerobic cell suspension. Serumvials and Erlenmeyer flasks were incubated on a rotary shaker (200 rpm) at 30°C. At time intervals samples were taken with a syringe. Care was taken to keep the content of the vials anaerobic.

In a seperate set of experiments the effect of acetate addition to aerobically starved cultures was investigated. Cells (0.74 g dry weight per litre) were incubated in shaken Erlenmeyer flasks in a release medium containing NaCl and Tris-HCl but no acetate. At time intervals the medium was analysed for ortho-phosphate, which was initially released by the cells but taken up after the addition of acetate. These experiments were carried out at 25°C, because at this temperature more polyphosphate is accumulated than at 30°C.

Chemical and biochemical determinations. Ortho-phosphate and total phosphorus (persulphate digestion method) were determined spectrophotometrically according to the Standard Methods (1976). The protein concentration was measured with the method of Lowry using bovine serum albumin as standard. Bacterial dry weight was determined after centrifugation of a 50 ml sample of the culture, washing with demineralized water and drying it at 100°C overnight. The concentrations of ATP, ADP and AMP in the cultures were determined according to Pradét (1976) and Van Groenestijn et al. (1987). Samples of 0.5 ml were taken (with a gastight glass syringe in the case of anaerobic cultures to

prevent oxygen contamination) and boiled for 4 minutes in 5 ml of a Tris-HCl/EDTA solution. ATP, ADP and AMP were determined by means of an AEC (adenylate energy charge) kit, based on bioluminescence, using luciferine and luciferase.

Chemicals. The AEC-kit was from LUMAC/3M bv. (Schaesberg, The Netherlands), ∝-dinitrophenol (DNP) and N,N'-dicyclohexylcarbodiimide (DCCD) were purchased from Merck, Darmstadt, Federal Republic of Germany.

RESULTS

ATP levels in cells of Acinetobacter strain 210A

The ATP content of anaerobically incubated cells of Acinetobacter strain 210A varied with the presence of different salts in the medium besides Tris-HCl (Table 1). All nine salts tested (concentration 100 mEq per litre) decreased markedly the ATP-level of the cells as compared to a medium containing Tris-HCl buffer only. K^+- and NH_4^+-salts had a stronger effect than Na^+-salts. Therefore in all experiments the anaerobic cellular ATP-levels were determined in a medium made of demineralized water and Tris-HCl (30 mM).

Table 1. Effect of 100 $mEq \cdot l^{-1}$ of different salts on the ATP content (expressed as µmol ATP per g dry biomass) of Acinetobacter strain 210A two hours after oxygen depletion.

	cation			
	-	Na^+	K^+	NH_4^+
control (only Tris-HCl)	1.76			
anion: Cl^-		0.71	0.40	0.41
NO_3^-		0.41	0.37	0.29
SO_4^{2-}		0.38	0.24	0.28

Regulation of the polyphosphate degradation in Acinetobacter strain 210A

The degradation of polyphosphate and subsequent release of ortho-phosphate by intact cells of Acinetobacter strain 210A took place only when energy generation in the cell was not possible, inhibited or uncoupled (Fig. 1). These conditions were: lack of substrate under aerobic conditions, inhibition of

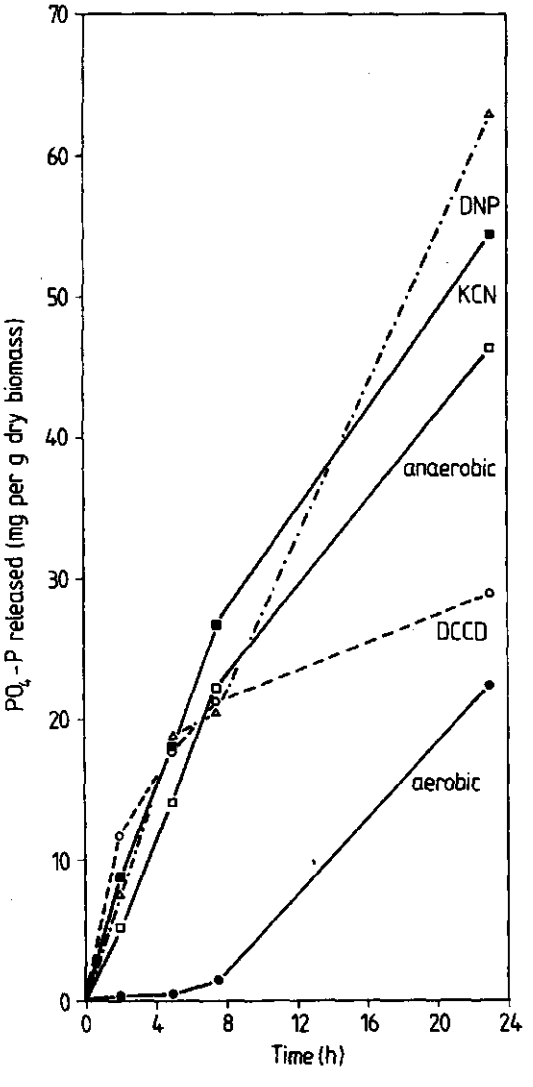

Fig. 1. Release of ortho-phosphate by Acinetobacter strain 210A incubated at pH 7.5 and 30°C under anaerobiosis and different aerobic conditions: with 4 mM DNP, with 1 mM KCN, with 4 mM DCCD and without additions. The presented data are from one experiment which was representative of 4 similar experiments.

respiration by KCN, inhibition of membrane bound ATPase by DCCD, dissipation of the protongradient with the uncoupler DNP or lack of oxygen as electron acceptor (anaerobic conditions). Under aerobic conditions in the absence of an extracellular energy source phosphate release started as soon as the cellular ATP content was low (Fig. 2). Addition of acetate (arrow in Fig. 2 after 12.7 hours) increased rapidly the ATP-level and turned on

again phosphate uptake.

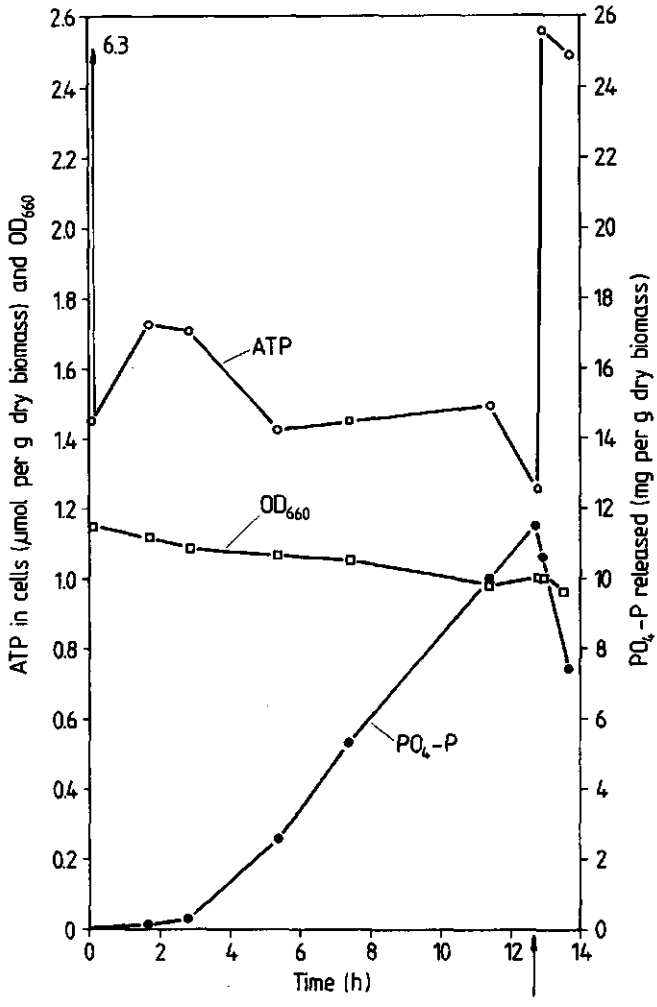

Fig. 2. Release of ortho-phosphate, ATP content and OD_{660} of an aerobically incubated culture of Acinetobacter strain 210A without an extracellular carbon source (pH 7.5, 25°C). At 12.7 h 4 mmol Na-acetate was added per litre. The presented data are from one experiment which was representative of 3 similar experiments.

The rate of phosphate release by the cells depended on the pH, which was optimal at 7.5, while lower pH's had a strong negative effect on this process. The efflux rate was positively affected by the presence of various alcohols. The stimulatory effect of alcohols increased with their molecular weight and with their concentration in the medium (the tested alcohols were methanol, ethanol, propanol-1, butanol-1 and pentanol-1 at a

concentration of 40 mM). The presence of 40 mM pentanol-1 doubled the rate of phosphate release. Pentanol-1 stimulated the phosphate release of Acinetobacter strain 210A up to a concentration of 60 mM. At that concentration the release stopped abruptly after one hour, though the cells still contained polyphosphate. The cause of this inactivation was investigated in seperate experiments. Acinetobacter strain 210A was anaerobically incubated with 60 mM and 80 mM pentanol-1. Cells and medium were analysed for intra- and extracellular adenylates at the moment the phosphate release stopped (between 1 and 2 hours at 60 mM pentanol-1 and at 0.67 hours at 80 mM). When the polyphosphate degradation ceased most of the adenylates had leaked out of the cells. Since AMP is needed for the enzymatic degradation of polyphosphate (Van Groenestijn et al. 1987), its lacking in the cell might have stopped the hydrolysis of the polymer. In fact, after 0.67 hours in the experiment with 80 mM pentanol-1 phosphate release ceased at a time when AMP had completely disappeared from the cells. Besides adenylates and 7.5 mg PO_4-P also 6 mg P from complex compounds and 29 mg protein were detected in 1 l supernatant. As these amounts were low compared with 1 gram biomass dry weight initially containing 0.5 g protein and 100 mg P, it can be concluded that lysis of cells or leakage of

Table 2. Concentration of adenylates ($\mu mol.g^{-1}$ dry biomass) in the total culture and in the waterphase during the anaerobic incubation of Acinetobacter strain 210A with pentanol-1 or the aerobic incubation with DCCD.

		Additions to the medium					
		DCCD		pentanol-1			
		(4 mM)	(4 mM)	(0 mM)	(60 mM)	(60 mM)	(80 mM)
Time elapsed (hours)[a]		2	8	24	1	2	0.67
Adenylates							
ATP	total	0.5	2.7	0.1	3.0	1.2	1.2
	waterphase	0.04	2.7	0.01	0.3	1.2	0.3
ADP	total	2.8	11.7	4.6	7.3	7.1	4.6
	waterphase	0.6	8.7	0.05	1.1	5.5	2.9
AMP	total	N.D.[b]	N.D.	4.8	0	11.5	3.1
	waterphase	N.D.	N.D.	0.05	0.6	12.8	3.3

a: after addition of DCCD or pentanol-1
b: N.D. not determined

high molecular compounds from the cells were quite negligible after 0.67 hours of anaerobic incubation with 80 mM pentanol-1. Anaerobic incubation of Acinetobacter strain 210A with DCCD also caused a slow release of adenylates, while 24 hours incubation without pentanol did not result in a detectable leakage of ATP, ADP or AMP (Table 2).

Polyphosphate as an energy reserve in whole cells of Acinetobacter sp.

Phosphate release and cellular ATP content were measured in anaerobically incubated cultures of Acinetobacter strains 210A, B8, 124 and 132 and Pseudomonas strain 10. Strains 210A and B8 were used because they are able to accumulate the highest amounts of phosphorus compared to the other Acinetobacter strains in our collection. Strains 124 and 132 are typical representatives of acinetobacters which cannot accumulate polyphosphate. Pseudomonas strain 10 served as a control to demonstrate that the behaviour of Acinetobacter strain 124 and 132 was comparable to other fast growing aerobic bacteria not able to accumulate polyphosphate. The strains 124 and 132 showed no or only a very small release of phosphate and the ATP content of the cells immediately dropped to low levels when incubated anaerobically (Fig. 3). Pseudomonas strain 10 reacted the same as Acinetobacter strain 132 (results not shown). The polyphosphate accumulating strains 210A and B8, however, released high amounts of phosphate and were able to maintain a rather high ATP level for several hours. To investigate whether the higher ATP contents of strain 210A and B8 during anaerobiosis were strain specific or a result of the presence of polyphosphate, strain 210A was cultivated under P-limitation or at 35°C. At 35°C strain 210A does hardly accumulate polyphosphate (Van Groenestijn et al. submitted). In both cases, 35°C and P-limited, cultures contained only 15 mg P per g dry biomass. In absence of oxygen phosphate release was negligible and the ATP content of these cells was much lower than compared to cells containing high amounts of polyphosphate (Fig. 3). The addition of DNP to anaerobic cultures of Acinetobacter strain 210A resulted in a higher phosphate release, but lower cellular ATP levels (Fig. 4), indicating that phosphate release is stimulated by low ATP concentrations in the cell.

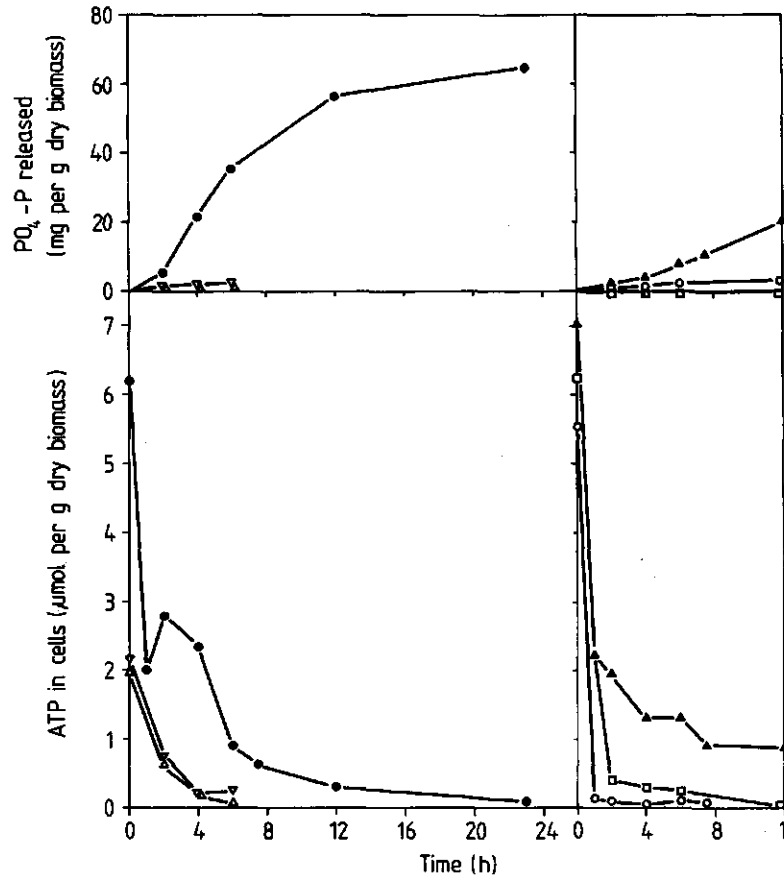

Fig. 3. Release of ortho-phosphate and cellular ATP content in anaerobically incubated cultures of polyphosphate accumulating Acinetobacter strains 210A (●) and B8 (▲), Acinetobacter strains without polyphosphate 132 (○) and 124 (□), and of cultures of Acinetobacter strain 210A which were precultivated at 35°C (no polyphosphate accumulation) (▽) and under P-limitation (no polyphosphate accumulation) (△). The anaerobic incubation was at pH 7.5 and 30°C. The curves are representative of curves determined in sevenfold (210A, 15°C), quadruplicate (132), duplicate (B8) and singular (124; 210A, 35°C; 210A, P-limited).

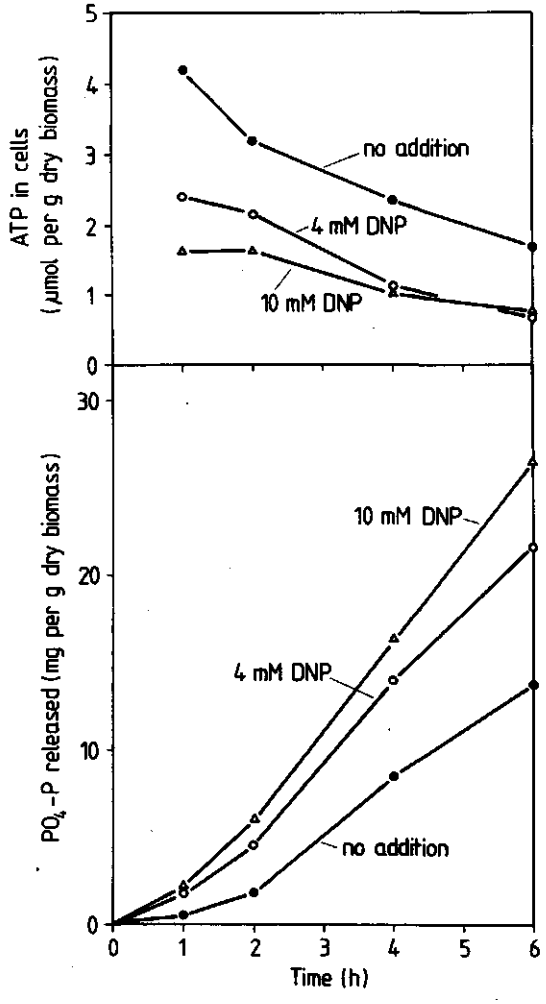

Fig. 4. Effect of 4 mM and 10 mM DNP on the release of ortho-phosphate and cellular ATP content of anaerobically incubated cultures of Acinetobacter strain 210A at pH 7.5 and 30°C. The presented data are mean values from an experiment in duplicate, which was representative of other experiments showing the same effects.

DISCUSSION

Polyphosphate degradation and release of ortho-phosphate by Acinetobacter strain 210A was observed when energy became scarce regardless of the cause of the energy shortage (Fig. 1). However, when sufficient oxygen and energy source were available polyphosphate was not broken down (Fig. 2). As phosphate was

released at a low cellular ATP concentration and taken up at a high ATP concentration (Fig. 2), it can be concluded that the polyphosphate synthesis and degradation is controlled by the energy state of the cells. Similar observations have been made in Micrococcus lysodeikticus in which the polyphosphate accumulated during the exponential phase of growth and disappeared gradually during carbon starvation (Friedberg and Avigad 1968). Rhodopseudomonas spheroides and Anabaena variabilis accumulated polyphosphate under anaerobic conditions in the presence of light, while in the dark, in absence of photophosphorylation, the amount of polyphosphate declined (Carr and Sandhu 1966).

Besides the ATP level also the availability of AMP seems to be crucial for polyphosphate degradation. Polyphosphate hydrolysis stopped when the adenylates had leaked out of permeabilized cells (Table 2). Interestingly, phosphate release ceased completely after 0.67 h of incubation with 80 mM pentanol-1; though only AMP had leaked out entirely into the medium, ATP and ADP were still present in the cells. This indicates that the presence of AMP is an important factor for polyphosphate degradation in vivo and that polyphosphate in anaerobically incubated Acinetobacter strain 210A is mainly degraded by the enzyme polyphosphate:AMP phosphotransferase (as measured in cell-free extracts of this strain by Van Groenestijn et al. 1987), and not by polyphosphatase, which was found in Acinetobacter calcoaceticus (Ohtake et al. 1985).

Cultures of Acinetobacter strains able to accumulate polyphosphate (210A and B8) released anaerobically high amounts of phosphate and were able to maintain an exceptionally high ATP concentration for several hours, while the ATP content of cultures without any detectable polyphosphate immediately dropped to almost zero. A decrease of the ATP content with 90 or more percent within a few minutes after the energy generating system stoppes, seems to be common for many other bacteria (Chapman and Atkinson 1977, Otto et al. 1984). The causal relation between high anaerobic ATP-levels and polyphosphate degradation (and subsequent release of ortho-phosphate), could be explained by production of ATP from polyphosphate in vivo, viz. polyphosphate as an energy reserve. A possible production of ATP by a K^+ efflux (Mulder et al. 1986) during anaerobiosis was considered as negligible, since Acinetobacter strain 132 was not able to maintain high ATP levels during these conditions despite of the release of the same amounts of K^+ as compared to strain 210A (Van Groenestijn et al. submitted).

Besides the production of ATP via polyphosphate:AMP phospho-

transferase and adenylate kinase (Van Groenestijn et al. 1987), additional ATP could be produced via a protongradient generated by the efflux of ortho-phosphate. Acinetobacter strain 210A releases $H_2PO_4^-$ plus a metal ion as the result of polyphosphate degradation (Van Groenestijn et al. submitted). Anaerobically incubated Acinetobacter strain 210A appeared to have a transmembrane protongradient that plays an important role in the anaerobic energy metabolism of this strain, since the addition of DNP, which decreases this gradient, had a clear effect on the cellular ATP-level and the phosphate release rate (Fig. 4). The experiments with DNP also showed that lower cellular ATP contents correlated with faster phosphate releases. An additional indication of the role of the protongradient in ATP generation by anaerobically incubated Acinetobacter strain 210A, is the observation that phosphate is released faster in a medium with DCCD, in the first two hours, as compared to an anaerobic medium (Fig. 1). DCCD inhibits ATPase and therefore the production of ATP by a protongradient (Abrams and Baron 1970), thus resulting in lower cellular ATP concentrations and a faster polyphosphate degradation. The observation that the phosphate release rate decreased after 5 hours of incubation with DCCD can be explained by the leakage of adenylates from the cells (Table 2).

Alcohols have been shown to augment membrane fluidity and reduce membrane order. Membrane fluidity correlates positively with increasing chain length (increasing hydrophobicity) of the alcohols added (Ingram and Buttke 1986). The higher freedom of motion within the membrane can stimulate phosphate transport (Molitoris et al. 1985) or facilitate the leakage of small molecules such as protons. In both cases a higher rate of phosphate release would be expected which actually occurred.

Polyphosphate as an energy reserve may be useful for Acinetobacter sp. under conditions of temporary anaerobiosis by supplying enough energy for the maintenance of a well functioning cell metabolism. This might give these species a competitive advantage over other strictly aerobic bacteria in a habitat where oxygen lacks occasionally. In the process of biological phosphate removal from waste water, in which activated sludge is subjected to alternating cycles of anaerobiosis and aerobiosis, conditions are created where the capacity of polyphosphate accumulation can be beneficial. In fact this kind of sludge is enriched with polyphosphate accumulating Acinetobacter sp. (Fuhs and Chen 1975, Buchan 1983). The role of polyphosphate as an energy reserve under anaerobic conditions in this specific wastewater treatment process has been hypothesized

(Rensink 1981, Marais et al. 1983, Comeau et al. 1986) and is now supported by our findings.

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CHAPTER 6

POLYPHOSPHATE DEGRADING ENZYMES IN ACINETOBACTER SP. AND ACTIVATED SLUDGE

Johan W. van Groenestijn, Michaël M.A. Bentvelsen, Maria H. Deinema and Alexander J.B. Zehnder

ABSTRACT

Polyphosphate degrading enzymes were studied in Acinetobacter and activated sludge. Polyphosphate:AMP phosphotransferase activity in Acinetobacter strain 210A decreased with increasing growth rates. The activity of this enzyme in cell-free extracts of Acinetobacter strain 210A was maximal at a pH of 8.5 and a temperature of 40°C, and was stimulated by $(NH_4)_2SO_4$. The K_m for AMP was 0.6 mM and V_{max} was 60 $nmol.min^{-1}.mg^{-1}$ protein. Cell-free extracts of this strain also contained polyphosphatase, which was able to degrade native polyphosphate and synthetic Mg-polyphosphate, and was strongly stimulated by 300-400 mM NH_4Cl. A positive correlation was found between polyphosphate:AMP phosphotransferase activity, adenylate kinase activity and phosphorus accumulation in six different strains of Acinetobacter. Significant activities of polyphosphatekinase were only detected in strain P, which contained no polyphosphate:AMP phosphotransferase. In samples of activated sludge from different plants the activities of polyphosphate:AMP phosphotransferase and adenylate kinase correlated well with the ability of the sludge to remove phosphate biologically from waste water.

INTRODUCTION

Acinetobacter is important in biological phosphate removal from waste water. Some strains of this strictly aerobic bacterium are able to accumulate large amounts of polyphosphate in from of granules (Fuhs and Chen 1975). Activated sludge in wastewater treatment plants is enriched with Acinetobacter if alternating aerobic and anaerobic conditions are applied. Like activated sludge pure cultures of Acinetobacter sp. degrade polyphosphate and release ortho-phosphate anaerobically, while aerobically ortho-phosphate is taken up and converted into polyphosphate. By the combined action of polyphosphate:AMP phospotransferase, which catalyses
$(\text{polyphosphate})_n + \text{AMP} \longrightarrow (\text{polyphosphate})_{n-1} + \text{ADP}$, and adenylate kinase, which catalyses: $2\ \text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$, ATP is produced from polyphosphate in Acinetobacter strain 210A (Van Groenestijn et al. 1987). This enzyme system allows thus Acinetobacter to use polyphosphate as an energy reserve in times when energy generation becomes difficult or is not possible at all; e.g. in case the electron donor (organic carbon compound) and/or acceptor (oxygen) become limiting or are absent.

Besides polyphosphate:AMP phosphotransferase also other polyphosphate hydrolyzing enzymes have been reported. In a polyphosphate accumulating Acinetobacter strain polyphosphatase was detected (Ohtake et al. 1985). This enzyme was able to degrade synthetic polyphosphate with a chain length of 15 phosphogroups. In Escherichia coli polyphosphatekinase was found (Kornberg 1957), whereas Mycobacterium phlei contained polyphosphate glucokinase (Szymona and Ostrowski 1964) and polyphosphate dependent NAD-kinase was detected in Acetobacter, Achromobacter, Brevibacter, Corynebacterium and Micrococcus species (Murata et al. 1980). Activities of the last three enzymes were absent in cell-free extracts of Acinetobacter strain 210A (Van Groenestijn et al. 1987). Since most Acinetobacter species don't possess a glycolytic pathway (Baumann et al. 1968) a degradation of polyphosphate by means of 1,3-phosphoglycerate:polyphosphate phosphotransferase (Kulaev et al. 1968) is not expected.

In the present study the properties of the polyphosphate degrading enzymes polyphosphate:AMP phosphotransferase and polyphosphatase in Acinetobacter strain 210A are reported. Six different Acinetobacter strains have been analyzed for enzymes that are responsible for the production of ATP from polyphosphate. The biochemical findings obtained with pure cultures were compared to practice, i.e. activated sludge from different wastewater treatment plants in which biological phosphate removal

occurs.

MATERIALS AND METHODS

Organisms. Acinetobacter strain 210A, B8, P, 132 and 124 were isolated from activated sludge with the method described by Deinema et al. (1980). Acinetobacter calcoaceticus (NCIB 8250) was a kind gift of Prof. Fewson (Barrowman and Fewson 1985). These organisms were maintained on yeast extract agar slants (5 g glucose, 2.5 g yeast extract and 12 g agar per litre tapwater, pH = 7.0), subcultured every two months and stored at 4°C.

Activated sludges. Samples of activated sludge were taken from the aerated zones or stages of: labscale fill and draw reactor (Appeldoorn and Deinema 1987), pilot plant P1 (De Vries and Rensink 1985), full scale plant Renkum: train 3 (with an anaerobic and an aerobic zone) and train 2 (completely aerated) (Mulder and Rensink 1987), pilot plant Bunschoten, full scale plant Bunschoten, full scale plant Bunnik (Janssen and Rensink 1987), conventional pilot plant (completely aerated) (Spanjers and Klapwijk 1986).

Growth conditions. Acinetobacter cells were cultivated in shaken Erlenmeyer flasks on a butyrate medium (Van Groenestijn et al. 1987). Strains 210A and P at 15°C and strains B8, 132, 124 and Acinetobacter calcoaceticus at 25°C, the optimal temperatures for the accumulation of polyphosphate in these strains. Cells of Acinetobacter strain 210A without polyphosphate, containing only 13 mg phosphorus per g dry biomass, were cultivated in the same way under phosphorus limitation (Van Groenestijn et al. 1987). In the experiments in which the polyphosphate:AMP phosphotransferase activity in Acinetobacter strain 210A was determined as function of growth rate, cells were continuously cultivated in a 2 l bioreactor (Applikon, Schiedam, The Netherlands) with automatically controlled pH (7.0) and temperature (25°C). The medium used in this experiment was modified by omitting Tris and replacing 2.29 g Na-butyrate per litre by 5.67 g Na-acetate.$3H_2O$ per litre.

Preparation of cell-free extracts. Cell-free extracts of Acinetobacter were prepared by sonication and centrifugation according to Van Groenestijn et al. (1987). The preparation of cell-free extracts from activated sludge was the same, but extended with the following procedure: just before use the frozen ex-

tracts were thawed and centrifuged 10 minutes at 7,000 rpm in a Heraeus Biofuge A centrifuge (West-Germany) to remove precipitated material.

Calculation K_m. Calculation of the K_m for AMP as a substrate for polyphosphate:AMP phosphotransferase was carried out with a computer programme with a non-linear regression fitting procedure using the least squares method.

Chemical and biochemical determination. Ortho-phosphate and total phosphorus (persulphate digestion method) were determined according to Standard Methods (1976). The protein concentration was measured with the method of Lowry using bovine serum albumin as standard. The concentrations of ATP, ADP and AMP in the reaction mixtures were determined according to Pradét (1976) and Van Groenestijn et al. (1987). In this method samples were boiled for 4 minutes in a Tris-HCl/EDTA solution, and ATP, ADP and AMP were determined by means of an AEC (adenylate energy charge) kit, based on bioluminescence, using luciferine and luciferase.

Enzyme assays. All assays were carried out at 30°C.
Polyphosphate:AMP phosphotransferase (assay 1, continuous method): continuous spectrophotometrical method according to Van Groenestijn et al. (1987). The reaction mixture in this assay contains 40 mM $(NH_4)_2SO_4$ due to the addition of enzymes which were suspended in 3.2 M $(NH_4)_2SO_4$. In the pH experiments the Tris-HCl buffer was replaced by equimolar amounts of a MES (2-morpholinoethanesulfonic acid) - NaOH buffer at pH's lower than 7.0. In the experiment in which the activity in different Acinetobacter strains was measured the reaction mixture was supplied with 600 ug Graham's salt per ml.
Polyphosphate:AMP phosphotransferase (assay 2, discontinuous method): 0.18 ml cell-free extract was incubated in 1 ml reaction mixture containing 3 mM $MgCl_2$, 16 mM Tris-HCl (pH=7.0) and 0.3 mM Ap_5A (P^1,P^5-di(adenosine-5')-pentaphosphate), the reaction was started by adding AMP, resulting in a concentration of 1 mM. In the extracts of Acinetobacter strain 210A enough native polyphosphate was present, but cell-free extracts of sludge had to be supplemented with 600 µg Graham's salt per ml. As it is expected that Graham's salt binds Mg^{2+} an additional 1200 µg $MgCl_2.H_2O$ was added per ml to assure an excess of free Mg^{2+} in the reaction mixture. At time intervals samples of 25 µl were taken from the reaction mixture to determine ADP concentrations.
Polyphosphatase (EC 2.7.4.3): 0.99 ml cell-free extract was

mixed with 0.01 ml 50 mM $MgCl_2$, which resulted in a final concentration of 0.5 mM $MgCl_2$ (pH=7.0). At time intervals samples of 100 µl were taken and immediately analysed for ortho-phosphate.

Adenylate kinase: 0.16 ml cell-free extracts per ml reaction mixture in a cuvette, 7 mM $MgCl_2$, 90 mM Tris-HCl (pH=7.0), 200 mM D-glucose, 0.6 mM NADP, 3.4 U (Units) HK and 1.7 U G6P-DH per ml, the reaction was started by adding ADP, resulting in a concentration of 1 mM. The cuvette was placed in a Beckmann spectrophotometer and the production of $NADPH_2$ was measured at 340 nm. A reaction mixture with additional 0.3 mM Ap_5A, a specific inhibition of adenylate kinase (Feldhaus et al. 1975), was used as control.

Polyphosphatekinase (EC 2.7.4.1): 0.18 ml cell-free extract and 600 ug Graham's salt per ml reaction mixture in a cuvette, 8 mM $MgCl_2$, 100 mM Tris-HCl (pH=7.0), 200 mM D-glucose, 0.65 mM NADP, 0.9 mM Ap_5A, 3.4 U HK and 1.7 U G6P-DH per ml, the reaction was started by adding ADP, final concentration 1 mM. The produced $NADPH_2$ was measured spectrophotometrically.

Chemicals. HK, G6P-DH, adenylate kinase, NADP, ATP, ADP and AMP were purchased from Boehringer, Mannheim. Ap_5A was obtained from Sigma Chemical Co., St.Louis, the AEC-kit was from LUMAC/3M, Schaesberg, The Netherlands. MES was purchased from Merck, Darmstadt. Synthetic polyphosphate in form of Graham's salt, which represents a mixture of linear sodium polyphosphates [$(NaPO_3)_n$, $\tilde{n} = 10^2$], was prepared by heating NaH_2PO_4 3 hours at 750°C followed by cooling in the air outside the furnace. K-polyphosphate (Kurrol's salt, $\tilde{n} = 2.10^4$) was prepared by heating KH_2PO_4 2 hours at 260°C (Kulaev 1979). Na-polyphosphate was derived from this salt by cation exchange with DOWEX-50. By adding equivalent amounts of $MgCl_2$ the Na-polyphosphate was precipitated as the insoluble Mg-polyphosphate.

RESULTS

Polyphosphate:AMP phosphotransferase as a function of the growth rate of Acinetobacter strain 210A

The polyphosphate:AMP phosphotransferase activity and the phosphorus content of cells of Acinetobacter strain 210A depended on the growth rate. The enzyme activity decreased from 36 to 25 $nmol.min^{-1}.mg^{-1}$ protein with increasing growth rates, measured at dilutionrates between 0.05 and 0.42 h^{-1}, while the

cellular phosphorus content had an optimum at a growth rate of 0.11 h^{-1}.

Properties of polyphosphate:AMP phosphotransferase in Acinetobacter strain 210A

The effect of pH and temperature on the activity of polyphosphate:AMP phosphotransferase in cell-free extracts of Acinetobacter strain 210A is presented in Fig. 1. The pH optimum was

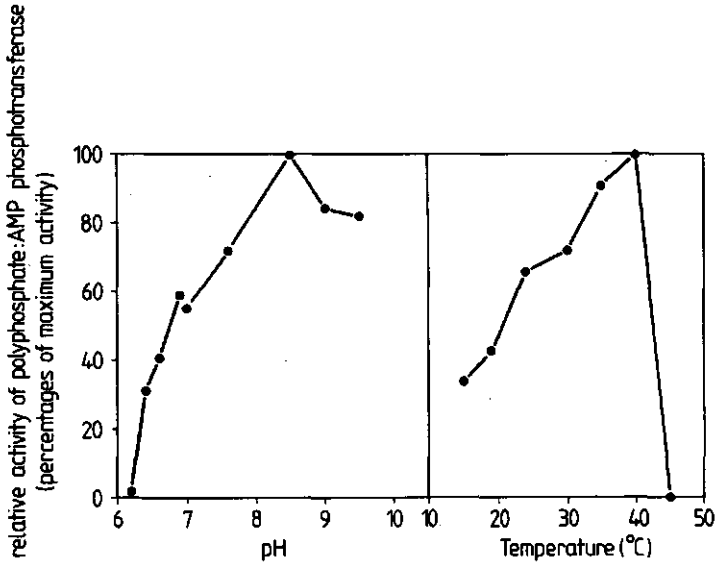

Fig. 1. Effect of pH (at 30°C) and temperature (at pH=7) on polyphosphate:AMP phosphotransferase in cell-free extracts of Acinetobacter strain 210A. At pH's lower than 7 the Tris-HCl buffer in the reaction mixture was replaced by a MES-NaOH buffer.

8.5 and the temperature optimum was 40°C. Two methods exist for the assay of this enzyme: (1) A spectrophotometrical method in which the reactionproduct is continuously converted into ATP, and (2) a discontinuous method in which at time intervals samples are taken from the reaction mixture (see Materials and Methods section). The continuous method resulted in 30 times higher activities than the discontinuous method. One of the differences between the two assay's is the presence of $(NH_4)_2SO_4$ in the spectrophotometrical method. The addition of 40 mM $(NH_4)_2SO_4$ to the reaction mixture of assay 2 stimulated the activity of polyphosphate:AMP phosphotransferase about 3 times The ortho-phosphate concentration, tested in the range of 0.1 to 10 mM did not affect the activity. The activity was dependent on

the AMP concentration and this dependency obeyed the law of Michaëlis and Menten. A K_m of 0.58 ± 0.13 mM and a V_{max} of 60 ± 4 nmol.min^{-1}.mg^{-1} protein was measured following assay 1 at a pH of 7.0 and 30°C.

Properties of polyphosphatase in Acinetobacter strain 210A

Polyphosphatase in cell-free extracts of Acinetobacter strain 210A was stimulated by the presence of NH_4Cl or KCl (Fig. 2).

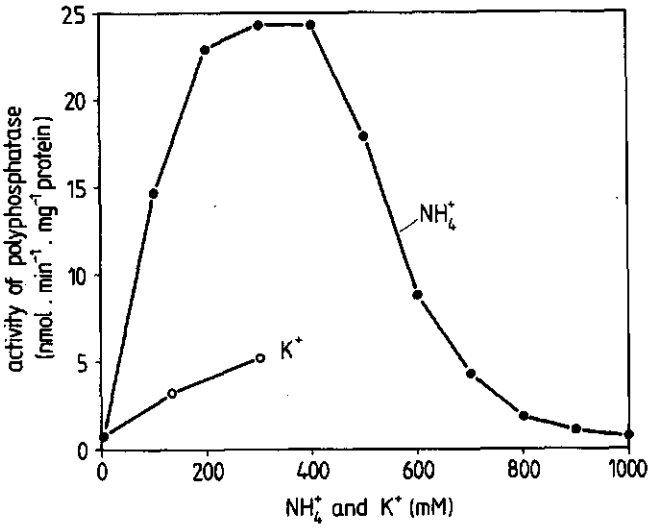

Fig. 2. Polyphosphatase activity in cell-free extracts of Acinetobacter strain 210A as function of the concentration of K^+ and NH_4^+ (added as KCl and NH_4Cl) in the reaction mixture.

The stimulation by NH_4Cl was optimal at 300-400 mM. No activity was found after heating cell-free extracts 5 minutes at 80°C. This is a strong indication that polyphosphate hydrolysis is catalyzed by an enzyme. The substrate for this reaction was native polyphosphate which was already present in the cell extracts. In an extract with a total of 566 µg phosphorus per ml of which 38 µg.ml^{-1} consisted of ortho-phosphate phosphorus, the ortho-phosphate phosphorus content per ml increased to 287 µg after 9 hours of incubation during the polyphosphatase assay. The degradation of synthetic polyphosphates was also studied in cell-free extracts without native polyphosphate. In a cell-free extract of cells cultivated under phosphorus limitation only 83 µg phosphorus per ml was present of which 21 µg.ml^{-1} was in the form of ortho-phosphate phosphorus. The enzyme activity was measured in a reaction mixture with 1 mg polyphosphate-phos-

phorus per ml but no NH_4Cl. Mg-polyphosphate (prepared from Kurrol's salt) was the only polymer hydrolized in this assay (3.2% within 7 hours at an rate of 0.7 $nmol.min^{-1}.mg^{-1}$ protein). Hardly any ortho-phosphate was produced in absence of polyphosphate or in presence of Na- or K-polyphosphate. In a control experiment Mg-polyphosphate was incubated in a reaction mixture but without cell-free extract. Less than 0.1 percent was degraded within 7 hours.

Enzymes involved in polyphosphate degradation in different strains of Acinetobacter sp.

The amount of phosphorus and the activities of polyphosphate:AMP phosphotransferase, adenylate kinase and polyphosphatekinase in different strains of Acinetobacter are shown in Table 1. The activity of polyphosphate:AMP phosphotransferase of the strains correlated positively with phosphorus accumulation, and the adenylate kinase activity correlated best with polyphosphate:AMP phosphotransferase activity. Significant activities of polyphosphatekinase were only detected in strain P, which did not show any polyphosphate:AMP phosphotransferase activity.

Table 1. Phosphorus accumulation and activities of polyphosphate:AMP phosphotransferase, adenylate kinase and polyphosphatekinase in six different strains of Acinetobacter.

Strain	phosphorus in cells (percentage of dry biomass)	enzyme activity ($nmol.min^{-1}.mg^{-1}$protein)		
		polyphosphate:AMP phosphotransferase	adenylate kinase	polyphosphate-kinase
210A	10	43	89	0
B8	6	10	80	1
P	4	0	54	3
NCIB 8250	2.5	2	60	0
124	2.7	1	46	0
132	1.6	0	39	0

Polyphosphate:AMP phosphotransferase and adenylate kinase in activated sludge

The activities of polyphosphate:AMP phosphotransferase and adenylate kinase in cell-free extracts of activated sludge of different wastewater treatment plants are presented in Tables 2 and 3. Sludges that were able to remove phosphorus biologically also produced more ADP from AMP and Graham's salt within 2 hours than conventional sludge, although the activities were low (as-

Table 2. Biological removal of phosphorus from domestic wastewater by different types of activated sludge and the production of ADP from Graham's salt and AMP by cell-free extracts of these sludges.

Type of activated sludge	percentage phosphorus removed from wastewater	ADP production (nmol per mg protein in 2 hours)
Phosphorus accumulating sludge		
pilot plant P1 (Sept. 1986)	99	64
pilot plant P1 (Feb. 1986)	99	25
pilot plant Bunschoten	70	16
Renkum train 3	55	13
Conventional sludge		
Renkum train 2	16	5
conventional pilot plant	not detected	2

Table 3. The removal of phosphorus from wastewater by different types of activated sludge and their activity of adenylate kinase.

wastewater treatment plant	percentage phosphorus removed from wastewater	adenylate kinase ($nmol.min^{-1}.mg^{-1}$ protein)
fill and draw system	100	680
pilot plant P1 (Sept. 1987)	100	146
pilot plant P1 (May 1987)	87	132
full scale plant Bunschoten	81	87
Renkum train 3	55	78
full scale plant Bunnik	90	56
conventional pilot plant (Sept. 1987)	N.D.	64
Renkum train 2	16	40
conventional pilot plant (May 1987)	N.D.	13

N.D. = not detected

say 2, Table 2). Assay 2 was used because of its high sensitivity to low activities of polyphosphate:AMP phosphotransferase. The presence of Graham's salt in the cell-free extracts of the sludge was a prerequisite for this reaction, without this synthetic polyphosphate no ADP was produced. The adenylate kinase activity in the sludge correlated well with the percentage of phosphorus removed from the waste water (Table 3).

DISCUSSION

Polyphosphate:AMP phosphotransferase activities were not paralleled by cellular polyphosphate content at various growth rates of Acinetobacter strain 210A. The induction of higher enzyme activities at low growth rates is a well known property of many catabolic enzymes, especially those involved in early metabolism of substrates (Harder and Dijkhuizen 1983). Polyphosphate:AMP phosphotransferase can be regarded as such an enzyme, because it catalyses the first reaction in the catabolic pathway of the degradation of polyphosphate and the subsequent production of ATP.

The properties of polyphosphate:AMP phosphotransferase in Acinetobacter strain 210A differed from those found for this enzyme in Corynebacterium xerosis. The pH optimum for the enzyme in Acinetobacter strain 210A was 8.5, while the pH optimum found by Dirheimer and Ebel (1965) for polyphosphate:AMP phosphotransferase in Corynebacterium xerosis was 6.5. The physiological function of the enzyme in Corynebacterium xerosis is not yet fully understood, because of its high K_m for AMP, namely 20 mM (Kulaev 1979). The affinity of polyphosphate:AMP phosphotransferase in Acinetobacter strain 210A for AMP, however, is very high. The physiological significance of an enzyme with a K_m of 0.6 mM can hardly be doubted. It has already been demonstrated that this enzyme plays a role in the use of polyphosphate as an energy reserve in Acinetobacter strain 210A (Van Groenestijn et al. submitted). The polyphosphate:AMP phosphotransferase activity in cell-free extracts was stimulated by $(NH_4)_2SO_4$ just as in Corynebacterium xerosis. This $(NH_4)_2SO_4$ effect partly explains the different activities found with assay 1 and assay 2.

Polyphosphatase has been detected in cell-free extracts of Acinetobacter strains 210A in significant amounts. Native bacterial polyphosphate as well as high polymeric synthetic Mg-polyphosphate could be hydrolized by polyphosphatase. Na- and K-polyphosphate could not act as substrates, despite of the small amounts of Mg^{2+} in the reaction mixture. Bacterial polyphosphate in Acinetobacter strain 210A also contains high amounts of Mg^{2+} (Van Groenestijn et al. submitted). Mg-polyphosphate may be the actual substrate that is accepted by the enzyme, like Mg-pyrophosphate by mouse duodenal pyrophosphatase (Nayadu and Miles 1969). The need for Mg^{2+} by polyphosphatase has been found in other organisms (Harold and Harold 1965, Kulaev and Konoshenko 1971). The stimulation of polyphosphatase by NH_4Cl is a specific NH_4^+ effect and not a general salinity effect, because equal

concentrations of KCl caused a less strong stimulation. NH_4^+ may change the enzyme or the substrate (e.g. cation composition of polyphosphate). An effect of NH_4^+ on polyphosphatase has never been reported in biology, but the activating effect of K^+ on this enzyme from Aerobacter aerogenes has been observed earlier by Harold and Harold (1965). Kulaev and Konoshenko (1971) found also a stimulation of polyphosphatase from Neurospora crassa with a factor 3 by 200 mM KCl. Polyphosphatase may have a role in the degradation of polyphosphate in cells of Acinetobacter strain 210A growing under phosphorus limitation. Under these conditions polyphosphate can be used as an phosphorus reserve (Van Groenestijn and Deinema 1985). As the cells do not contain any detectable amounts of AMP under phosphorus limitation (unpublished results), a degradation of polyphosphate by polyphosphate:AMP phosphotransferase is unlikely.

Increased levels of adenylate kinase in different strains of Acinetobacter correlated with the polyphosphate:AMP phosphotransferase activity and phosphorus accumulation by these strains. This correlation reflects the role of adenylate kinase in the degradation of polyphosphate in combined action with polyphosphate:AMP phosphotransferase. Both enzymes play a role in the production of ATP from polyphosphate in acinetobacters in vitro and in vivo and therefore probably also in biological phosphate removal by activated sludge (Van Groenestijn et al. 1987, Van Groenestijn et al. submitted). This role was indicated by the positive correlation between the activities of polyphosphate:AMP phosphotransferase and adenylate kinase and the ability of the sludge to remove phosphate biologically from waste water. Very high adenylate kinase activities were found in the intermittently fed and aerated labscale fill and draw system, which contained a sludge with more than 60% acinetobacters that was able to release accumulated phosphate very fast under anaerobic conditions (Appeldoorn and Deinema 1987). The higher phosphate release rate in this sludge (71 mg phosphorus.h^{-1}.g^{-1} dry biomass) as compared to the rates measured in pure cultures of Acinetobacter strain 210A (8 mg phosphorus.h^{-1}.g^{-1} biomass, Van Groenestijn et al. submitted) might be caused by the higher adenylate kinase activity in the sludge. Further research is needed to explain the higher activities. Because of its high activities and its good correlation with biological phosphate removal adenylate kinase might be used as an indicator for monitoring this process.

In conclusion it can be said that among the three enzymes that can degrade polyphosphate in Acinetobacter (polyphosphate:AMP phosphotransferase, polyphosphatase and polyphosphate-

kinase), polyphosphate:AMP phosphotransferase plays, in combined action with adenylate kinase, the most important role in anaerobic ATP production in Acinetobacter strains that accumulate aerobically large amounts of polyphosphate. A link between the findings obtained with pure cultures and practice has been made by showing the correlation between the activities of polyphosphate:AMP phosphotransferase and adenylate kinase in activated sludge and the ability of the sludge to remove phosphate biologically from waste water. The suitability of the adenylate kinase activity as an indicator for this process has been suggested. The study presented in this paper shows the validity of pure culture studies for the application.

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CHAPTER 7

SUMMARY + SAMENVATTING

SUMMARY

Biological phosphate removal from waste water is a biotechnological alternative to chemical phosphorus precipitation. This process is obtained by recycling the sludge through anaerobic and aerobic zones. In the anaerobic parts phosphate is released by the sludge and during anaerobiosis phosphate is taken up. Biological phosphate removal is dependent on the enrichment of activated sludge with polyphosphate accumulating Acinetobacter. Like activated sludge, pure cultures of strictly aerobic Acinetobacter sp. absorbe phosphate (up to 100 mg phosphorus per g dry biomass) during aerobic conditions and release it anaerobically. The aim of this study was to gather knowledge on the uptake and release of phosphate by Acinetobacter and the metabolic functions of polyphosphate.

The accumulation of polyphosphate by pure cultures of Acinetobacter strain 210A depended on the presence of an intra- or extracellular energy source (chapter 2). The highest amount of polyphosphate was found in cells in which energy supply was not limited, namely at low growth rates under sulphur limitation, and in the stationary phase of growth when either the nitrogen or the sulphur source was depleted. Accumulation of polyphosphate was also possible during endogenous respiration. When this respiration was blocked with KCN the phosphate uptake stopped, while the inhibition of the protein synthesis with streptomycin enhanced the accumulation of phosphate, which indicated the competition between protein synthesis and polyphosphate synthesis for energy. There was a pronounced effect of the temperature on phosphorus accumulation but this effect varied from strain to strain.

The role and behaviour of cations in the accumulation and release of phosphate was studied (chapter 3). PO_4^{3-} was released together with 1.8 protons. Mg^{2+} appeared to be the most important counterion of polyphosphate in Acinetobacter strain 210A. It was released and taken up simultaneously with phosphate. Mg^{2+} was not an essential polyphosphate counterion. If Mg^{2+} was depleted, stationary cultures of Acinetobacter strain 210A took up the same amount of phosphate with Ca^{2+} as the most important counterion. In the presence of Mg^{2+} stationary cultures did not need Ca^{2+} for their phosphate absorption, but the presence of K^{+}

seemed to be crucial for this process, although this cation did not play a quantitatively important role as a polyphosphate counterion. In addition, the influx and efflux of K^+ was independent of phosphate uptake and release.Continuous cultivation at low growth rates under K^+-limitation did not result in polyphosphate accumulation, while under substrate or Mg^{2+}-limitation large amounts of polyphosphate were present in the cells. The same effect was found in activated sludge. 5 mg K^+ per litre was needed for a satisfactory biological phosphate removal in the aerobic zone of a wastewater treatment plant. Granules of Mg-polyphosphate in Acinetobacter strain 210A could serve as a Mg^{2+}-reserve. Cells with these granules were able to grow in a medium free of Mg^{2+}, whereas cells without granules were not, they only grew in the presence of extracellular Mg^{2+}.

Polyphosphate in cell-free extracts of Acinetobacter strain 210A could be degraded by the enzymes polyphosphatase or polyphosphate:AMP phosphotransferase (chapters 4 and 6). Polyphosphate glucokinase, polyphosphate dependent NAD-kinase and polyphosphatekinase were not detectable. Polyphosphate:AMP phosphotransferase was also found in Acinetobacter strain B8, but not in Acinetobacter strain P, which contained only polyphosphatekinase. Both strains were able to accumulate large amounts of polyphosphate. In strains that cannot accumulate this biopolymer, no or very small activities of polyphosphatekinase and polyphosphate:AMP phosphotransferase were found. All strains showed activities of adenylate kinase. It was demonstrated that by the combined action of polyphosphate:AMP phosphotransferase and adenylate kinase a continuous regeneration of ATP from AMP or ADP was possible as long as polyphosphate was present. Polyphosphate:AMP phosphotransferase could use native and synthetic polyphosphate as substrate and showed a maximum activity at a pH of 8.5. Its activity was stimulated by $(NH_4)_2SO_4$, the K_m for AMP appeared to be 0.6 mM, and V_{max} was 60 $nmol.min^{-1}.mg^{-1}$ protein. Polyphosphatase in cell-free extracts of strain 210A was able to hydrolyse native polyphosphate and synthetic Mg-polyphosphate. The K- and Na-form, however, were not degraded. The activities of polyphosphate:AMP phosphotransferase and adenylate kinase in activated sludge correlated well with the ability of the sludge to remove phosphate biologically from waste water.

Degradation of polyphosphate in vivo in Acinetobacter strain 210A occurred if the energy supply in the cell was stopped, for example under anaerobiosis or in the presence of KCN, α-dinitrophenol or N-N'-dicyclohexylcarbodiimid (chapter 5). The degradation and synthesis of polyphosphate was dependent on the ATP concentration in the cells. Lower ATP concentrations caused a

faster phosphate release. This release was stimulated by alcohols. The transmembrane protongradient seemed to play an important role in the anaerobic energy metabolism of this strictly aerobic bacterium. Addition of α-dinitrophenol, a protonionophore, decreased the cellular ATP concentration and stimulated the polyphosphate degradation. The role of polyphosphate as an energy reserve in vivo has been demonstrated by experiments in which five strains were incubated anaerobically. Cells of Acinetobacter strains 210A and B8, which were able to accumulate polyphosphate, released large amounts of ortho-phosphate anaerobically and contained high levels of ATP. Cells of two other strains of Acinetobacter and one strain of Pseudomonas which didn't accumulate polyphosphate, showed a much smaller release of phosphate and contained only low ATP concentrations. Cells of strain 210A cultivated under phosphorus limitation or at 35°C did not contain detectable amounts of polyphosphate. As a result their ATP level was low and they released only small or negligible amounts of phosphate under anaerobic conditions

Mg-polyphosphate in Acinetobacter sp. is a multifunctional compound. It can serve as: (1) an energy reserve if it is degraded by a reaction with AMP, catalyzed by polyphosphate:AMP phosphotransferase, (2) a phosphorus reserve if it is hydrolyzed by polyphosphatase, and (3) a Mg^{2+} reserve whereby Mg^{2+} can be replaced by Ca^{2+} as a counterion. The most important role of polyphosphate in wastewater treatment plants with biological phosphate removal, and probably also in natural environments, is its use as an energy reserve to sustain temporary anaerobiosis. This property might explain the enrichment of activated sludge subjected to alternating anaerobic and aerobic conditions with polyphosphate accumulating Acinetobacter sp..

SAMENVATTING

Biologische defosfatering van afvalwater is een biotechnologisch alternatief voor de chemische fosfaatverwijdering. Dit proces wordt verkregen door het slib te recirculeren door anaerobe en aerobe zones. In de anaerobe gedeelten geeft het slib fosfaat af en tijdens aerobie wordt er fosfaat opgenomen. Biologische defosfatering is gebaseerd op de verrijking van aktief slib met polyfosfaat ophopende acinetobacters. Reinculturen van strict aerobe acinetobacters nemen fosfaat op tijdens aerobie en onder anaerobe omstandigheden wordt het fosfaat weer afgegeven, zoals dat ook bij aktief slib wordt waargenomen. Het doel van het hier beschreven onderzoek is het verzamelen van kennis over de fosfaat opname en afgifte door Acinetobacter en de functies van polyfosfaat in deze bacterie. Dit onderwerp is van groot belang door de zeer grote hoeveelheden fosfor die Acinetobacter sp. kan opnemen (tot 100 mg fosfor per g droge biomassa) en de rol die dit geslacht speelt bij de biologische defosfatering. Dit proefschrift beschrijft de regulatie van de ophoping en afbraak van polyfosfaat in Acinetobacter sp., alsmede de enzymen die bij de afbraak betrokken zijn en de mogelijke functies van dit biopolymeer in deze bacteriën.

De ophoping van polyfosfaat door reinculturen van Acinetobacter stam 210A hing af van de aanwezigheid van een intra- of extracellulaire energiebron (hoofdstuk 2). De grootste hoeveelheden polyfosfaat werden gevonden in cellen waarin de aanvoer van energie niet beperkend was, namelijk bij lage groeisnelheden tijdens zwavellimitatie of in de stationaire fase na uitputting van de stikstof- of zwavelbron. Ophoping van polyfosfaat was ook mogelijk tijdens endogene respiratie. De opname van fosfaat stopte wanneer deze respiratie geblokkeerd werd met KCN, terwijl deze opname werd bevorderd door de eiwitsynthese te remmen met streptomycine. Dit effect duidde op een energie concurrentie tussen eiwit- en polyfosfaatsynthese. De temperatuur had een duidelijk effect op de fosfor ophoping, maar dit effect varieerde van stam tot stam.

Hoofdstuk 3 beschrijft het onderzoek naar de rol en het gedrag van kationen bij de ophoping en afgifte van fosfaat. PO_4^{3-} werd samen met 1.8 protonen afgegeven. Mg^{2+} bleek het belangrijkste tegenion te zijn van polyfosfaat in Acinetobacter stam 210A. Dit werd steeds gelijktijdig met fosfaat afgegeven en opgenomen. Mg^{2+} was geen essentieel tegenion, want stationaire culturen van Acinetobacter stam 210A konden wanneer Mg^{2+} uitgeput was, dezelfde hoeveelheden fosfaat opnemen met Ca^{2+} als belangrijkste tegenion. De fosfaat opname door stationaire

culturen was niet afhankelijk van de aanwezigheid van Ca^{2+}, mits er Mg^{2+} aanwezig was, maar de aanwezigheid van K^+ leek cruciaal voor dit proces, hoewel dit kation kwantitatief geen belangrijke rol speelde als polyfosfaat tegenion. De influx en efflux van K^+ was onafhankelijk van de fosfaat opname en afgifte. Continue cultivering bij lage groeisnelheden onder K^+-limiterende omstandigheden resulteerde niet in polyfosfaat ophoping, terwijl tijdens substraat of Mg^{2+}-limitatie wel grote hoeveelheden polyfosfaat aanwezig waren in de cel. In aktief slib werd een zelfde effect waargenomen. Voor een goede biologische defosfatering in de aerobe zone van een zuiveringsinstallatie was 5 mg K^+ per liter nodig. Korrels met Mg-polyfosfaat in Acinetobacter stam 210A konden als een Mg^{2+} reserve dienen. Cellen met deze korrels waren in staat om in een medium zonder Mg^{2+} te groeien, terwijl cellen zonder korrels alleen konden groeien als er extracellulair Mg^{2+} aanwezig was.

Het polyfosfaat in celvrije extracten van Acinetobacter stam 210A kon worden afgebroken met de enzymen polyfosfatase en polyfosfaat:AMP fosfotransferase (hoofdstukken 4 en 6). Polyfosfaat glucokinase, polyfosfaat-afhankelijk NAD-kinase en polyfosfaatkinase konden niet worden aangetoond. Polyfosfaat:AMP fosfotransferase werd ook gevonden in Acinetobacter stam B8, maar niet in Acinetobacter stam P, deze laatste stam bevatte wel polyfosfaatkinase. Beide stammen kunnen grote hoeveelheden polyfosfaat ophopen. In stammen die dat niet kunnen werden geen of zeer lage polyfosfaatkinase en polyfosfaat:AMP fosfotransferase activiteiten gevonden. Alle stammen bezaten een adenylaat kinase activiteit. Door middel van de gecombineerde werking van polyfosfaat:AMP fosfotransferase en adenylaat kinase kon er continu ATP worden geregenereerd uit AMP of ADP zolang er polyfosfaat aanwezig was. Zowel synthetisch als bacterieel polyfosfaat kon als substraat dienen voor polyfosfaat:AMP fosfotransferase. Dit enzym vertoonde een maximale activiteit bij een pH van 8.5 en werd gestimuleerd door $(NH_4)_2SO_4$. De K_m voor AMP bleek 0.6 mM te zijn, en de V_{max} was 60 $nmol.min^{-1}.mg^{-1}$ eiwit. Polyfosfatase in celvrije extracten van stam 210A kon bacterieel en synthetisch Mg-polyfosfaat hydrolyseren, maar geen Na- en K-polyfosfaat. De activiteit van dit enzym werd sterk gestimuleerd door NH_4^+. De polyfosfaat:AMP fosfotransferase en adenylaat kinase activiteiten in aktief slib correleerde goed met het vermogen van dit slib om fosfaat biologisch uit afvalwater te verwijderen.

De afbraak van polyfosfaat in vivo in Acinetobacter stam 210A trad op wanneer de energie voorziening in de cel stopte, bijvoorbeeld tijdens anaerobie of in de aanwezigheid van KCN, α-dinitrofenol of N-N'-dicyclohexylcarbodiimide (hoofdstuk 5). De

afbraak en opbouw van polyfosfaat bleek afhankelijk te zijn van de ATP concentratie in de cel. Een lagere ATP concentratie veroorzaakte een snellere fosfaat afgifte. De afgifte werd gestimuleerd door alcoholen. De transmembraan protonengradient leek een belangrijke rol te spelen in het anaerobe energie metabolisme van deze strict aerobe bacterie. De toevoeging van -dinitrofenol, een protonionofoor, verlaagde de ATP concentratie in de cel en stimuleerde de polyfosfaat afbraak. De rol van polyfosfaat als een energie reserve *in vivo* werd gedemonstreerd door vijf stammen anaeroob te incuberen. Cellen van Acinetobacter stammen 210A en B8, die polyfosfaat kunnen ophopen, gaven tijdens anaerobie grote hoeveelheden ortho-fosfaat af en bevatten hoge ATP niveau's. Cellen van twee andere Acinetobacter stammen en een Pseudomonas stam, die geen polyfosfaat kunnen ophopen, lieten een veel lagere fosfaat afgifte zien en bevatten slechts lage ATP concentraties. Ook cellen van stam 210A die onder fosforlimitatie of bij 35°C waren gekweekt, waardoor zij geen aantoonbare hoeveelheden polyfosfaat bevatten, gaven kleine of verwaarloosbare hoeveelheden fosfaat af en bezaten lage ATP niveau's.

Mg-polyfosfaat in Acinetobacter sp. is een multifunctionele verbinding. Het kan dienen als (1) een energie reserve, waarbij het wordt afgebroken door een reactie met AMP, gekatalyseerd door polyfosfaat:AMP fosfotransferase, (2) een fosfor reserve waarbij het wordt gehydrolyseerd met behulp van polyfosfatase, en (3) een Mg^{2+} reserve waarbij Mg^{2+} door Ca^{2+} als tegenion kan worden vervangen. De belangrijkste functie die dit biopolymeer vervult in biologisch defosfaterende zuiveringsinstallaties en waarschijnlijk ook in natuurlijke milieus, is een rol als energie reserve om tijdelijke anaerobe situaties te doorstaan. Deze eigenschap zou de verklaring kunnen zijn voor de verrijking van aktief slib dat onderworpen is aan afwisselend anaerobie en aerobie met polyfosfaat ophopende Acinetobacter sp..

CURRICULUM VITAE

De auteur werd op 28 mei 1958 geboren te Tiel, alwaar hij in 1976 aan de Rijksscholengemeenschap het Atheneum-B diploma behaalde. In dat jaar werd begonnen met een studie Milieuhygiëne aan de Landbouwhogeschool te Wageningen. Het doctoraalpakket bestond uit Waterzuivering, Microbiologie en Proceskunde. Praktijkervaring werd opgedaan bij het Zuiveringsschap West-Overijssel te Zwolle en bij het National Environmental Engineering Research Institute te Nagpur in India. In 1984 werd het doctoraalexamen met lof behaald. Van februari 1984 tot januari 1988 is de auteur werkzaam geweest bij de vakgroep Microbiologie van de Landbouwuniversiteit te Wageningen. Het onderzoek dat in deze periode is verricht heeft geleid tot dit proefschrift.