Metabolic behaviour of *Acinetobacter* spp. in enhanced biological phosphorus removal – a biochemical model

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Abstract

A biochemical model is presented that explains the behaviour of *Acinetobacter* spp. in enhanced biological phosphorus removal activated sludge systems. The model modifies and extends the proposals of Comeau *et al.* (1985). Two key parameters are identified in controlling poly-P and PHB synthesis and degradation, the ATP/ADP and NADH/NAD ratios. The predicted behaviour appears to be consistent with that observed.

Introduction

Since the phenomenon of enhanced biological phosphorus removal in activated sludge systems first was observed (Srinath, Sastry and Pillay, 1959), attempts have been made to elucidate the mechanisms governing it. Over the years various hypotheses and models have been proposed; each successive model has been influenced in some degree by the models preceding it, but often the factors on which a model conceptualization has been based are not clear. In this paper we wish to trace briefly but critically, the evolution of these models. We then propose yet another model which we hope will resolve some of the difficulties that have become apparent with previous models.

The research papers that either directly or indirectly influenced the conceptualization of the respective models are extensive and it is not possible to include a critical evaluation of each; only those papers of major direct influence will be mentioned. For a detailed review of this topic, and related ones, the reader is referred to Marais, Loewenthal and Siebritz (1983).

Model evolution

Early observations

Harold (1966), in an authoritative survey, reported that phosphorus accumulation in the form of polyphosphate (poly-P) is widespread among micro-organisms, in bacteria, yeasts, fungi and photosynthetic algae. Harold, however, could not advance a substantive hypothesis as to what function was served by these accumulations. He noted that temporary limitations of the nutrients sulphur and nitrogen could result in accumulation of poly-P in certain bacteria.

Levin and Shapiro (1965) demonstrated accumulation of phosphorus in a mixed liquor sample from an activated sludge system, under aerobic conditions. Further, they noted that in the mixed liquor samples phosphorus (P) is released to the bulk solution when the dissolved oxygen level falls. Shapiro (1967) showed that this release of P under anaerobic conditions could be reversed on subsequent aeration.

Fuhs and Chen (1975) conducted a wide-ranging investigation into the phenomena of P release and uptake. They observed the following:

- Samples taken from a full-scale anaerobic/aerobic activated sludge plant (showing enhanced P removal) and from a laboratory-scale aerobic plant fed on an artificial sewage (not showing excess P removal) were dosed with radioactively labelled glucose and aerated. The results indicated that the organism assembly receiving artificial substrate under completely aerobic conditions metabolized the glucose via the Entner-Doudoroff pathway, whereas the organism assembly from the anaerobic/aerobic plant indicated metabolism via the Embden-Meyerhof pathway. They then changed operation of the laboratory scale plant from aerobic to anaerobic/aerobic; after two weeks operation the system still showed no P release or uptake. They concluded that this system had a different species composition from the full-scale plant.

- P removal appeared to be mediated by either a single micro-organism or several closely related forms. From a series of isolation tests Fuhs and Chen concluded that *Acinetobacter* spp. probably was the principal organism mediating the P removal.

- *Acinetobacter* spp. accumulated poly-P and poly-β-hydroxybutyrate (PHB). Fuhs and Chen speculated that PHB storage could serve as an energy source for poly-P formation.

- In studying the nutritional requirements of an *Acinetobacter* strain isolated from an enhanced P removal activated sludge system they found this strain could not use glucose or similar compounds but grew on lower fatty acids, LFA. (In this paper the terminology lower fatty acids (LFA) and volatile fatty acids (VFA) are used interchangeably). They concluded that anaerobic conditions preceding aerobiosis in sewage treatment could well be related to the appearance of *Acinetobacter* spp. – they saw the principal function of the anaerobic state as enabling a facultative anaerobic microflora to become established which produces lower fatty acids to serve as substrate for *Acinetobacter* spp. Furthermore, from pure culture studies, they concluded that the anaerobic state takes no part in enhanced P removal *per se* and can be excluded provided LFA are supplied to *Acinetobacter* spp. in the aerobic state. They found that if acetate was added to a pure culture of *Acinetobacter* spp. under aerobic conditions, P uptake took place.

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Barnard (1976) in reviewing observations on P removal in full-scale plants concluded that, contrary to Fuhs and Chen, an anaerobic state at a point in the plant was essential for P removal, that an anaerobic/aerobic sequence with substrate fed to the anaerobic zone were explicit requirements to obtain excess P removal. However, he could identify no correlation between the P release in the anaerobic reactor and P uptake and removal.

Model 1 (Nicholls and Osborn, 1979)

Nicholls and Osborn (1979) from a study of the literature and their own work noted a consistent association between poly-P accumulation and organic carbon accumulation in the form of poly-β-hydroxybutyrate (PHB). They concluded that these high energy polymers were stored for survival purposes under the set of imposed anaerobic/aerobic conditions. They did not implicate any particular bacterial genus in poly-P and PHB accumulation, but noted that in their systems *Acinetobacter*, a poly-P and PHB accumulator, was abundantly present. They proposed a biochemical model in which they attempted to explain the functions of and the connections between PHB and poly-P accumulations. They hypothesized the following model:

In the anaerobic phase:

(i) Carbohydrate (glucose) is taken up by poly-P organisms and metabolized, via the glycolytic (Embden-Meyerhof) pathway, to acetyl-CoA and electrons and protons.

(ii) The acetyl CoA has two functions: First, to act as an electron and proton sink by its reduction to PHB. Second, to act as an energy source for ATP synthesis via deactivation to acetic acid.

(iii) The role of poly-P is to serve as a source of P in the formation of ATP in (ii) above.

(iv) The ATP generated in (ii) above is utilized for cell maintenance via hydrolysis to ADP + P_i; such P_i is released to the medium.

In the aerobic phase:

- The stored PHB is utilized as a carbon and energy source for cell function and an energy source for poly-P generation and storage.

In support of their model Nicholls and Osborn set out quantitative biochemical pathways.

Model 2 (Rensink, 1981)

Rensink accepted the work of Fuhs and Chen (1975) that *Acinetobacter* spp. was the principal organism implicated in enhanced phosphorus removal and that these organisms only utilize LFA as substrate. Also, he accepted Fuhs and Chen's hypothesis that the purpose of the anaerobic state was to enable an assemblage of facultative organisms to develop which produced lower fatty acids to serve as substrate for the *Acinetobacter* spp. Furthermore he presupposed that *Acinetobacter* spp. is a slow growing obligate aerobe and in a purely aerobic environment would not be competitive for substrate with other obligate aerobes; however, by having the propensity for PHB storage in the anaerobic zone, competition for substrate in the aerobic zone is removed enabling the *Acinetobacter* spp. to survive in the anaerobic/aerobic cycle. With these ideas as background, taking due cognizance of the model of Nicholls and Osborn, he proposed the following conceptual model:

In the anaerobic phase:

(i) Lower fatty acids (for example acetate) serve as substrate for *Acinetobacter*.

(ii) The lower fatty acids (LFA) are stored as PHB, such a process requiring energy (ATP).

(iii) The energy (ATP) requirements in (ii) above are supplied by breakdown (hydrolysis) of poly-P to free phosphate, the phosphate being released to the bulk solution.

In the aerobic phase:

- Rensink follows the proposals of Nicholls and Osborn.

Rensink did not propose any biochemical pathways for PHB synthesis from acetate or for poly-P breakdown and P release.

The models of Rensink and Nicholls and Osborn differ in the following aspects:

- LFA as substrate for *Acinetobacter* (Rensink); – glucose as substrate for poly-P organisms (Nicholls and Osborn).
- Function of poly-P is as – an energy source (Rensink); – a phosphorus source (Nicholls and Osborn).
- Function of PHB is as – a carbon sink for *Acinetobacter* to survive in competition in the aerobic zone (Rensink); – a proton and electron sink for poly-P organisms to survive an anaerobic stress (Nicholls and Osborn).

Model 3 (Marais, Loewenthal and Siebritz, 1983)

Marais *et al.* (1983) proposed that poly-P accumulation in poly-P organisms serves as an energy source for two purposes; maintenance during the anaerobic phase, and, PHB synthesis in the anaerobic phase thereby partitioning substrate in the anaerobic phase for their exclusive use subsequently in the aerobic phase, in this fashion gaining a positive advantage over non-poly-P organisms in anaerobic/aerobic systems.

Marais *et al.* concurred with Rensink that poly-P serves as an energy source for PHB storage. They investigated theoretical biochemical pathways under anaerobic conditions, for PHB synthesis from the substrates glucose and acetate respectively. With *glucose* as substrate, accepting that the poly-P organisms possess the glycolytic (Embden-Meyerhof) pathway, they proposed biochemical pathways whereby PHB could be synthesized. With *acetate* as substrate, however, they found it not possible to put forward pathways for PHB synthesis – conversion of acetate as far as acetoacetate only appeared to be feasible. The problem was that with acetate as substrate no source of protons and electrons, to reduce acetoacetate to PHB, was available. They were unable, therefore, to find an explanation for PHB storage under anaerobic conditions with acetate as substrate.

In some measure these speculations appeared to be supported by the work of Siebritz, Ekama and Marais (1983). They found that the mass of P removal in anaerobic/aerobic systems was proportional to an influent COD fraction called the "readily

biodegradable COD", a fraction that normally contains insignificant amounts of LFA. However, Wentzel, Dold, Ekama and Marais (1985) provided an explanation. They showed that in the anaerobic phase with addition of acetate as substrate:

- the mass of P release was proportional to the mass of acetate added; and
- the rate of P release was zero order.

With addition of sewage as substrate they showed that

- the P release, uptake and removal were proportional to the readily biodegradable COD concentration;
- the rate of P release was first order with respect to the readily biodegradable COD concentration; and
- the rate of P release was significantly lower than with acetate.

These observations suggested that the P release with sewage as influent was governed by the rate of LFA generation by facultative organisms, as suggested by Fuhs and Chen (1975), from readily biodegradable substrate. The work of Meganck *et al.* (1985) and Brodisch (1985) supported these conclusions; they showed that anaerobic/aerobic systems developed organisms which converted sugars, and similar organic compounds, to LFA in the anaerobic zone.

Juni (1978) reported that *Acinetobacter* spp. do not possess the glycolytic (Embden-Meyerhof) pathway. However, some species (or strains) possess the Entner-Doudoroff pathway, a pathway which becomes inoperative when oxygen is not present. Consequently the organisms can metabolize glucose only under aerobic conditions. Hence, the pathways proposed by Nicholls and Osborn (1979) and Marais *et al.* (1983) for PHB synthesis from glucose in the anaerobic zone, were no longer tenable. Evidence also became available that in completely aerobic systems receiving normal sewage, up to 60 per cent of the organisms cultured aerobically from mixed liquor samples were *Acinetobacter* spp. (Lötter *et al.*, 1986a). This would indicate that there are strains or species of *Acinetobacter* which are *not* slow growing (as suggested by Rensink, 1981) and can compete successfully for substrate in purely aerobic systems. Furthermore these *Acinetobacter* must have been able to metabolize glucose or similar compounds aerobically via the Entner-Doudoroff pathway as the LFA at best formed only a minor fraction of the biodegradable COD. However no poly-P inclusions or enhanced P removal was observed in these purely aerobic systems. Lötter *et al.* (1986b) showed further that strains, isolated from systems exhibiting enhanced P removal and from completely aerobic systems which did not show enhanced P removal, both had the propensity to accumulate poly-P and PHB under standard culture conditions. They concluded that the propensity to accumulate poly-P and PHB is inherent in these strains and is induced when the appropriate anaerobic/aerobic conditions are imposed. The slow increase in P release, uptake and removal often observed when changing from completely aerobic to anaerobic/aerobic operation they attributed to the time factor necessary to develop relevant enzyme systems. These observations appear to negate Rensink's hypothesis that the *Acinetobacter* spp. were slow growing under aerobic conditions and that PHB accumulation in the anaerobic phase was essential for their survival under anaerobic/aerobic conditions.

Model 4 (Comeau, Hall, Hancock and Oldham, 1985)

In 1985 Comeau *et al.* proposed a conceptual model that resolved some of the difficulties and inconsistencies raised above. Comeau *et al.* (1985), following Rensink, accepted that acetate was the substrate and that poly-P serves as an energy source for activation of acetate to acetyl-CoA in the anaerobic state. To overcome the problem that troubled Marais *et al.*, namely that in the anaerobic state with acetate as substrate no source of electrons and protons was available to reduce acetyl-CoA to PHB, they propsosed that these can be supplied from the tricarboxylic acid (TCA) cycle. However, they did not elaborate on the biochemical pathways by means of which this can be achieved. They noted further that acetate would be taken up by the organism in the undissociated acetic acid (HAc) form, as generally accepted in biochemistry. This causes the proton motive force (pmf) across the cytoplasmic membrane, essential to cell function (for function see Comeau *et al.*, 1985), to be dissipated. To re-establish the pmf they proposed that the poly-P serves as the energy source for translocation of protons. Comeau *et al.* showed further that the cations Mg^{2+}, K^{+} and Ca^{2+} are released with phosphate in the molar ratio cation/phosphate released of 0,27; 0,28 and 0,02 respectively. In terms of charge, one positive cation charge is released with every phosphate.

They described the biochemical pathways only in outline.

Qualitatively the model of Comeau *et al.* provides a framework for explaining anaerobic/aerobic behaviour of enhanced P release and uptake. However, the model is incomplete in that it does not provide quantitative information on the pathways and control mechanisms governing the biochemical reactions under different sets of imposed conditions of substrate and oxygen tension. Furthermore an analysis of the mechanism proposed for maintaining the pmf in the anaerobic zone gives rise to charge and proton imbalances across the cytoplasmic membrane.

In the rest of this paper we will be concerned with modifying and extending the model of Comeau *et al.*, to overcome the objections raised above. In this endeavour we will accept as our typical poly-P organism the genus *Acinetobacter*. Although Brodisch and Joyner (1983) have shown that a number of genera are associated with biological phosphorus removal the genus *Acinetobacter* has been identified to be principally responsible for P release and P removal in appropriately designed activated sludge systems (Buchan, 1983).

Preliminary considerations

Characteristics of *Acinetobacter* spp.

Acinetobacter spp. are ubiquitous in nature and can be readily isolated from soil, water and sewage (Warskow and Juni, 1972; Juni, 1978). All *Acinetobacter* spp. are gram negative, non-motile, catalase positive and oxidase negative (Henriksen, 1973; Juni, 1978; Fricke *et al.*, 1982). The genus requires oxygen for catabolic metabolism, Juni (1978); there are however some species within the genus which can utilize nitrate as an electron acceptor where oxygen is not present (Lötter, 1985). A characteristic of the genus is that they utilize sugars exclusively via the Entner-Doudoroff pathway – a pathway that is inoperative under anaerobic conditions. As a consequence they cannot produce energy from fermentation due to the absence of the glycolytic or similar metabolic pathways – they are able to utilize sugars only under aerobic conditions. Furthermore, the genus is

capable of storing phosphorus as poly-P (Deinema *et al.*, 1980; Buchan, 1983) and organic carbon as poly-β-hydroxybutyrate (Lawson and Tonhazy, 1980). It is this propensity to store P and organic carbon which, with the appropriate process configuration, is utilized to effect excess P removal. Each of these metabolic processes and their regulation is discussed below.

Carbon metabolism

Carbon metabolism of *Acinetobacter* spp. in activated sludge incorporates three main metabolic pathways, namely:

- the tricarboxylic acid cycle;
- the glyoxylate cycle; and
- poly-β-hydroxybutyrate synthesis and degradation.

The pathways and the relevant controls are discussed below.

Tricarboxylic acid (TCA) cycle:

The main steps of the TCA cycle are shown in Fig. 1. The figure

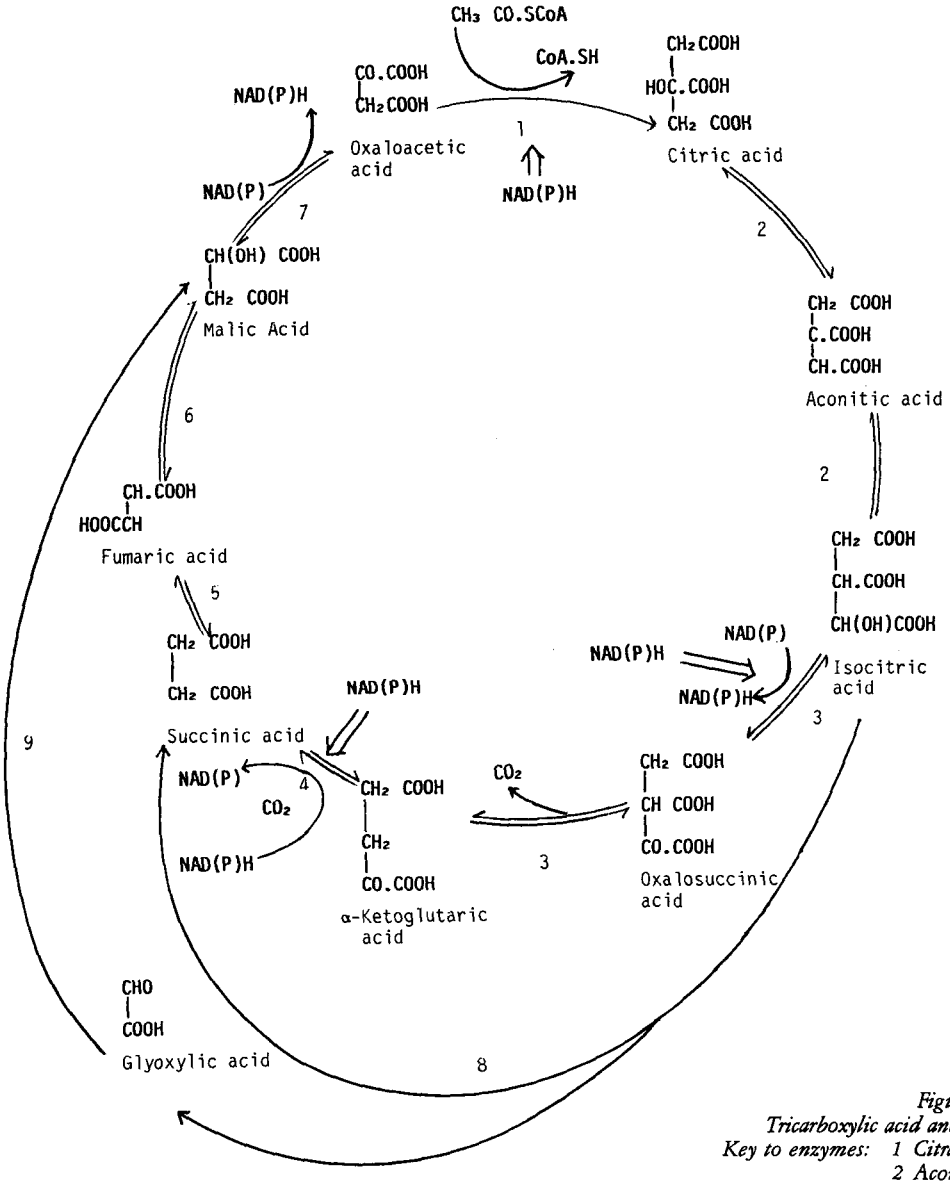


Figure 1
Tricarboxylic acid and glyoxylic acid cycles
Key to enzymes: *1 Citrate synthase*
2 Aconitase
3 Isocitrate dehydrogenase
4 α-ketoglutarate dehydrogenase
5 Succinate dehydrogenase
6 Fumarase
7 Malate dehydrogenase
8 Isocitrate lyase
9 Malate synthase
⇒ *Indicates inhibition.*
Tricarboxylic acid cycle 1, 2, 3, 4, 5, 6, 7
Glyoxylic acid cycle 1, 2, 8-9, 5, 6, 7

illustrates that, for an input of one molecule of acetyl-CoA, in a complete cycle of the pathway two molecules of carbon dioxide are lost and eight electrons (and protons) are captured by reduction of three molecules of nicotinamide adenine dinucleotide (NAD) to the reduced form (NADH) and one molecule flavin adenine dinucleotide (FAD) to the reduced form (FADH). Under aerobic conditions these reduced coenzymes donate the electrons and protons to oxygen and adenosine triphosphate (ATP) is generated from this redox reaction. Organic intermediates are formed at different stages of the TCA cycle; these become available to the organism if required in which event the full cycle is not completed. Thus the function of the TCA cycle under aerobic conditions is for energy generation as well as supplying carbon intermediates for anabolism. Under anaerobic conditions no terminal electron acceptor is available and, unless some alternative electron sink is found, then the NADH/NAD (or NADPH/NADP) ratio increases, and ATP/ADP ratio decreases. The TCA cycle is regulated by these ratios; high values for these ratios have an inhibitory effect on the cycle. This inhibition has been observed in *Acinetobacter* spp. High values of NADH/NAD inhibit the enzymes citrate synthase (Weitzman and Jones, 1968; Weitzman and Dunmore, 1969) and α-ketoglutarate dehydrogenase (Weitzman, 1972); high values of ATP/ADP inhibit the enzymes isocitrate dehydrogenase (Parker and Weitzman, 1970) and citrate synthase (Weitzman and Dunmore, 1969); two isoenzymes of isocitrate dehydrogenase have been identified in *Acinetobacter* spp. and are termed isoenzyme I and isoenzyme II – isoenzyme I is regulated by NADPH/NADP (Self and Weitzman, 1972) and isoenzyme II by NADH/NAD (Weitzman, 1972).

Glyoxylate cycle

This cycle is shown in Fig. 1. The principle function of this cycle is anaplerotic, that is, to supply intermediates to the TCA cycle from two carbon (C2) units. The two key enzymes in this cycle, isocitrate lyase and malate synthase, have been detected in *Acinetobacter* spp. (Sturm *et al.*, 1970). In most organisms regulation of the cycle is effected by three carbon (C3) compounds. In *Acinetobacter* spp. it is the four carbon (C4) intermediates of the TCA cycle which regulate activity of the enzyme isocitrate lyase (Bell and Herman, 1967; Herman and Bell, 1970) and thus regulate the glyoxylate cycle. As the TCA cycle is controlled by the NADH/NAD and ATP/ADP ratios, and as the TCA cycle intermediates regulate the glyoxylate cycle, this cycle is indirectly controlled by the NADH/NAD and ATP/ADP ratios.

Poly-β-hydroxybutyrate (PHB) synthesis and degradation

Synthesis and degradation pathways of PHB are shown in Fig. 2. Regulation of these pathways has not been studied in *Acinetobacter* spp. However, studies on these pathways in numerous other bacterial species invariably show the same system of regulation (Dawes and Senior, 1973); we therefore assume that

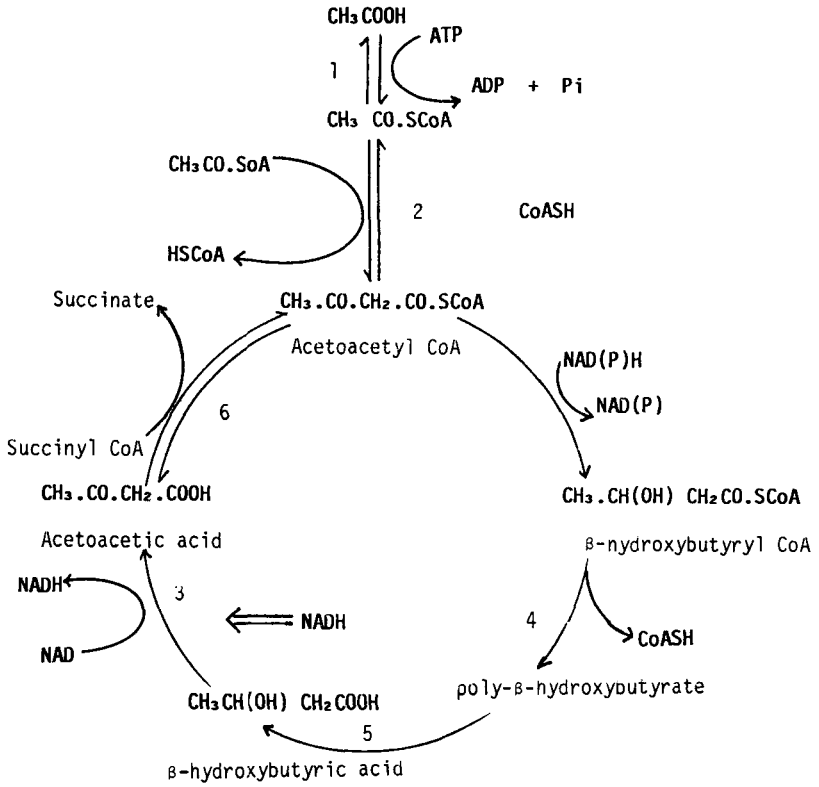


Figure 2
Synthesis and degradation of poly-β-hydroxybutyrate
Key to enzymes: *1 thiokinase*
2 β-ketothiolase
3 β-hydroxybutyrate dehydrogenase
4 β-hydroxybutyryl CoA polymerase
5 poly-β-hydroxybutyrate depolymerase
6 Acetoacetate succinyl CoA : CoA transferase
⇒ *indicates inhibition.*

this holds also for *Acinetobacter* spp. The synthesis and degradation of PHB proceeds via separate metabolic pathways, as follows:

- Synthesis of PHB

Referring to Fig. 2, synthesis of PHB takes place via activation of acetate to acetyl-CoA (mediated by the enzyme thiokinase), condensation of two melecules of acetyl-CoA to form acetoacetyl-CoA (catalysed by the enzyme ketothiolase) and reduction of acetoacetyl-CoA to hydroxybutyryl-CoA (catalysed by the enzyme β-hydroxybutyrate dehydrogenase) which is then polymerized to form PHB (catalysed by the enzyme hydroxybutyryl-CoA polymerase). The synthesis of PHB acts as a sink for protons and electrons and organic carbon. The synthesis pathway is stimulated by a high NADH/NAD ratio (or NADPH/NADP) and also by high concentrations of acetyl-CoA (Dawes and Senior, 1973). An inhibitory effect on the synthesis pathway has been observed, that of a high concentration of CoASH via its action on the enzyme ketothiolase (Dawes and Senior, 1973). One therefore expects this synthesis pathway to be functional where a high concentration of organic carbon is available to the organism, and no external electron sink (i.e. an anaerobic state) is present.

- Degradation of PHB

Referring to Fig. 2, the degradation pathway proceeds via the hydrolysis of PHB to free β-hydroxybutyrate (catalysed by the enzyme PHB depolymerase), oxidation of this acid to acetoacetate (catalysed by NAD β-hydroxybutyrate dehydrogenase) and activation of acetoacetate to acetoacetyl-CoA (catalysed by acetoacetate succinyl-CoA CoA transferase) which, with activated cleavage, forms two molecules of acetyl-CoA (catalysed by ketothiolase). These end products now may enter the TCA cycle. Regulation of this degradation pathway is via inhibition of NAD β-hydroxybutyrate dehydrogenase (which catalyses the oxidation of the free acid to acetoacetate) by high concentrations of pyruvate and/or a high NADH/NAD ratio (Dawes and Senior, 1973). One therefore expects this PHB degradation pathway to be functional under conditions where extracellular organic substrate concentration is low and a terminal electron acceptor (i.e. an aerobic state) is present.

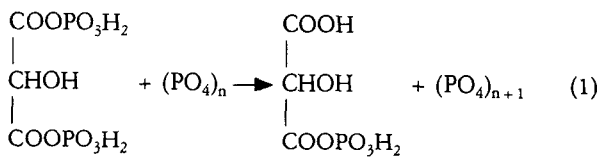
Phosphorus metabolism

Phosphorus is accumulated by *Acinetobacter* spp. as polyphosphate and stored in intracellular metachromic granules which may occupy up to 60 per cent of the total micro-organism volume (Buchan, 1981). Polyphosphate contains a large negative charge which probably is stabilized by the cations Mg^{2+}, K^+ and Ca^{2+}. Polyphosphate (poly-P) metabolism within *Acinetobacter* spp. occurs via two pathways:

- a synthesis pathway; and
- a degradation pathway.

Synthesis of Poly-P

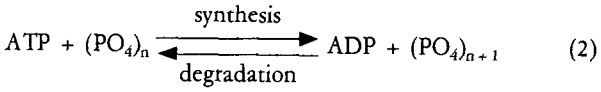
Two pathways of poly-P synthesis have been observed in micro-organisms, neither of which have been studied specifically in *Acinetobacter* spp. The first pathway is via phosphorylation of the polyphosphate by 1,3 diphosphoglycerate (catalysed by the enzyme diphosphoglycerate: polyphosphate transferase) – a pathway not widely observed. The reaction is as follows:



$$\begin{array}{l}COOPO_3H_2\\ |\\ CHOH\\ |\\ COOPO_3H_2\end{array} + (PO_4)_n \longrightarrow \begin{array}{l}COOH\\ |\\ CHOH\\ |\\ COOPO_3H_2\end{array} + (PO_4)_{n+1} \qquad (1)$$

The enzyme catalysing this reaction has been observed only in a narrow spectrum of micro-organisms including *Escherichia coli* (Kulaev *et al.*, 1971; Nesmeyanova *et al.*, 1974), *Micrococcus lysodeikticus*, *Propionibacterium shermanii* and *Neurospora crassa* (Kulaev and Bobyk, 1971; Kulaev, 1973).

The second poly-P synthesis pathway is via phosphorylation of the poly-P by ATP (catalysed by the enzyme ATP: polyphosphate phosphotransferase). The reaction is as follows:



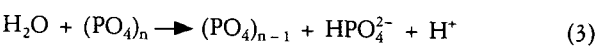
$$ATP + (PO_4)_n \underset{\text{degradation}}{\overset{\text{synthesis}}{\rightleftharpoons}} ADP + (PO_4)_{n+1} \qquad (2)$$

This pathway has been identified in a wide variety of organisms (Harold, 1966; Suzuki, Kancho and Ikeda, 1972; Felter and Stahl, 1970; Kulaev, 1975) and is the principal mechanism of poly-P synthesis. Regulation of this pathway is via the ATP/ADP ratio; poly-P synthesis is inhibited by a low ATP/ADP ratio (Kornberg, 1957; Zaitseva and Belozerskii, 1960; Felter and Stahl, 1970), a condition likely to be encountered where ATP is low, i.e. under anaerobic conditions where no external electron acceptor is present, irrespective of the substrate concentration surrounding the organism.

Degradation of poly-P

The degradation of poly-P to free phosphate radicals has been observed to take place via three reaction pathways in micro-organisms. First, the reverse reaction of that set out in the reaction Eq (2), also catalysed by the enzyme ATP : polyphosphate phosphotransferase (Kornberg, 1957; Harold and Harold, 1965; Kulaev, 1975; Shabalin, Vagabov, Tsiomenko, Zemlenukhia and Kulaev, 1977). This degradation pathway is inhibited by a high ATP/ADP ratio (Harold and Harold, 1965), a situation likely to arise when the ATP level is high, that is, under aerobic conditions with internal stored PHB or external substrate (e.g. acetate).

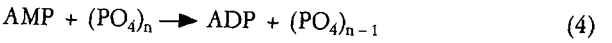
Second, the hydrolysis of poly-P to release the free phosphate radical (catalysed by the enzyme polyphosphatase; Harold, 1964; Severin, Lusta, Nesmeyanova and Kulaev, 1976) as follows:



$$H_2O + (PO_4)_n \longrightarrow (PO_4)_{n-1} + HPO_4^{2-} + H^+ \qquad (3)$$

Regulation of this pathway is effected by the cellular phosphate concentration (Felter and Stahl, 1970; Nesmeyanova, Dmitriev and Kulaev, 1974). A high cellular phosphate concentration will inhibit this reaction.

Third, phosphorylation of AMP by poly-P (catalysed by the enzyme polyphosphate : AMP phosphotransferase) as follows



$$AMP + (PO_4)_n \longrightarrow ADP + (PO_4)_{n-1} \qquad (4)$$

The reaction pathway has been observed in mycobacteria (Harold, 1966; Kulaev, 1975). The regulation of the reaction pathway has not been studied.

Having established the mechanisms of carbon and phosphorus metabolism it is possible now to interlink these

pathways under the conditions imposed by the activated sludge system to describe *Acinetobacter* spp. behaviour.

Biochemical model

The development of the model, set down below, is formulated for the environmental conditions imposed on *Acinetobacter* spp. that result in excess P removal, i.e. an anaerobic-aerobic sequence. Fundamental in the model development is the effect of the anaerobic and aerobic phases (in the anaerobic/aerobic sequence) on the intracellular ratios NADH/NAD and ATP/ADP, and the influence of these ratios in the biochemical regulation of carbon and phosphorus metabolic pathways.

Anaerobic phase

In this phase no external electron acceptor is available (neither oxidized nitrogen species nor oxygen); further, the organisms are in an environment containing a relatively high concentration of readily assimilable organic substrate.

Consequences of no external electron acceptor are that the NADH/NAD ratio increases, and no oxidative phosphorylation (ATP generation) takes place. The lack of oxidative phosphorylation causes the ATP/ADP ratio to decrease. The effects of these changes in the ratios of NADH/NAD and ATP/ADP are to inhibit and stimulate the TCA cycle respectively (cf. regulation of TCA cycle above). Clearly if an electron sink is available to the NADH (causing a decrease in the NADH/NAD ratio) inhibition of the TCA cycle is removed and further NADH generation by the TCA cycle is possible. Decrease in the ATP/ADP ratio stimulates ATP production via hydrolysis of stored poly-P and transfer of the high energy phosphoryl group to ADP.

Consequence of the high organic carbon concentration is that, provided both an electron and energy source are avaiable, i.e. provided the NADH/NAD and ATP/ADP ratios are sufficiently high, the organism can take up the carbon and store it as PHB. The source of electrons will vary depending on substrate. The most widely observed substrate to *Acinetobacter* spp. is acetate, consequently the metabolic fate of this substrate is considered first:

(i) A high external concentration of acetate allows passive diffusion of acetate into the cell, i.e. without expenditure of energy (Konings, Hellingwerf and Robillard, 1981).

(ii) Activation of acetate to acetyl-CoA, by coupled ATP hydrolysis, decreases the ATP/ADP level to such a degree that ATP formation is stimulated via poly-P degradation and the transfer of the phosphate and energy to ADP, see Eq. 2 (thermodynamic control of this degradation process, and its reversal, are considered in Appendix I). The hydrolysis of poly-P to form ATP and the subsequent utilization of ATP increases the intracellular concentration of free phosphate. With poly-P degradation the cations stabilizing the negative charge on the poly-P chain, also are released increasing the intracellular cation concentration. The phosphate and cations (M^+) are subsequently released to the bulk solution. The biochemical interaction between the concomitant acetate uptake and phosphate and cation release is described after (iv) below.

(iii) The high concentration of acetyl-CoA and a concomitant high NADH/NAD ratio stimulates PHB synthesis. Referring to Fig. 2 it is evident in the synthesis of PHB that NADH is oxidized to NAD, with the electrons (and protons) reducing acetoacetyl-CoA to β-hydroxybutyryl-CoA. PHB thus acts as an electron sink and this decreases the NADH/NAD ratio, removing the inhibition of the TCA cycle (and associated glyoxylate cycle) for further electron generation which in turn increases the NADH/NAD ratio. This interaction between the TCA cycle and PHB synthesis, mediated by the NADH/NAD ratio, ensures electron and proton generation to store all acetate taken up as PHB. Summarizing, for acetate uptake under anaerobic conditions a fraction of the acetate is reduced to PHB, and a fraction is oxidized in the TCA cycle to supply the electrons and protons for the reductive process via NADH formation. Stoichiometrically for acetate as carbon source about 89 per cent of the carbon is stored as PHB and 11 per cent reappears as CO_2; however, *all* the electrons and protons of the acetate taken up reappear in PHB. A diagram of the stoichiometry is set out in Fig. 3.

(iv) Formation of PHB lowers the intracellular concentration of acetate, allowing continued diffusion of acetate into the cell.

The mechanisms outlined above are in agreement with observed enzyme control systems as set out earlier in this paper. However, the mechanisms do not explain the biochemical links between acetate uptake and phosphate and cation release. Two fundamental biochemical principles link the release of phosphate and cations to the uptake of acetate across the cytoplasmic membrane. First, the generation of intracellular phosphate and cations from poly-P hydrolysis needed to provide energy for acetate storage (see (ii) above); second, uptake of acetate in the acetic acid form dissipates the proton motive force (pmf), the force being vital as a potential energy source for translocation of molecules across the cytoplasmic membrane of the cell. This pmf ($\Delta\mu_H$) has both a pH and a charge ($\Delta\psi$) component according to Eq. (5), Padan *et al.* (1976).

$$\Delta\mu_H = \Delta\psi + \frac{2{,}3RT}{F}\Delta pH \qquad (5)$$

For continued acetate uptake *inter alia*, the dissipated pmf must be restored (Comeau *et al.*, 1985). It is proposed that this is achieved by the concomitant release of phosphate and cation, and uptake of hydroxyl ions and protons from the bulk solution, as set out in Fig. 4. Referring to this figure:

- phosphate release occurs via hydroxyl mediated antiport protein carrier, a transport system commonly observed in microorganisms (Harold and Spitz, 1975);
- the cation (M^+) release occurs via a proton mediated antiport protein carrier; and
- acetic acid uptake is by passive diffusion.

If the pmf is to be maintained, then, from Eq. (5), the pH and charge difference between inside and outside the cell must be maintained. The question is whether the proposed mechanisms set out in Fig. 4 satisfy these requirements. In terms of the mechanisms in Fig. 4 the extracellular changes in alkalinity, acidity and charge are set down in Table 1 and the intracellular changes in Table 2. These tables show that charge neutrality is maintained both extracellularly and intracellularly. With regard to alkalinity and acidity, intracellularly there is no change; extracellularly the alkalinity and acidity each increase by one mole

for every mole of acetic acid uptake. In the pH region 6,8 to 7,2 the phosphate system dominates with a pK value of about 7. Consequently for equal increases in alkalinity and acidity one can show that the pH change also will be zero or insignificant in this pH region (Wentzel *et al.*, 1986a). This is confirmed by experimental observation (Wentzel *et al.*, 1986b).

TABLE 1
EXTRACELLULAR (BULK SOLUTION) ALKALINITY, ACIDITY AND CHARGE CHANGES FOR THE PROPOSED MODEL UNDER ANAEROBIC CONDITIONS WITH UPTAKE OF ONE MOLE OF ACETIC ACID

Process	Net change in Alk. (mol)	Acid (mol)	Charge (mol)
HAc moves into cell and condensed into PHB	0	−1	0
$H_2PO_4^-$ moves out of cell	+1	+2	−1
OH^- moves into cell	−1	+1	+1
M^+ moves out of cell	0	0	+1
H^+ moves into cell	+1	−1	−1
Sum of changes	+1	+1	0

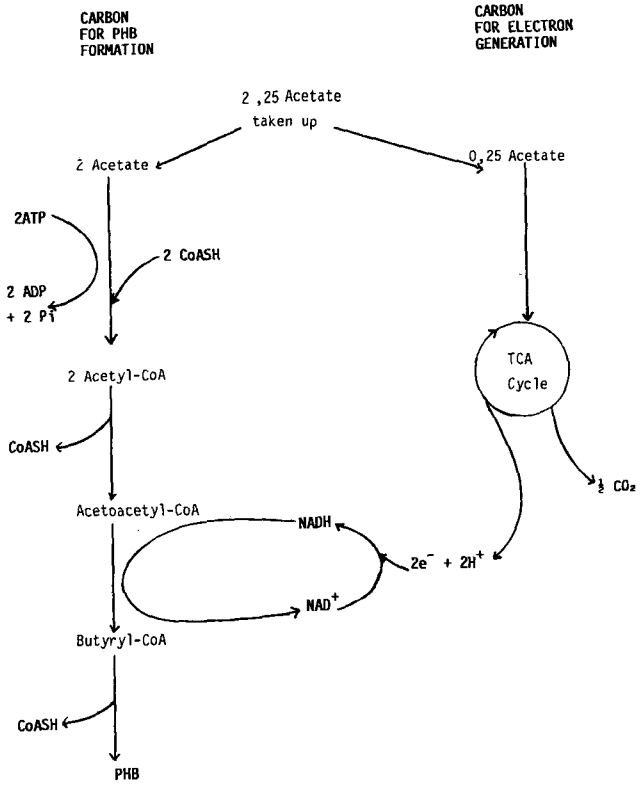


Figure 3
Stoichiometry of acetate utilisation in PHB formation under anaerobic conditions where acetate is the substrate.

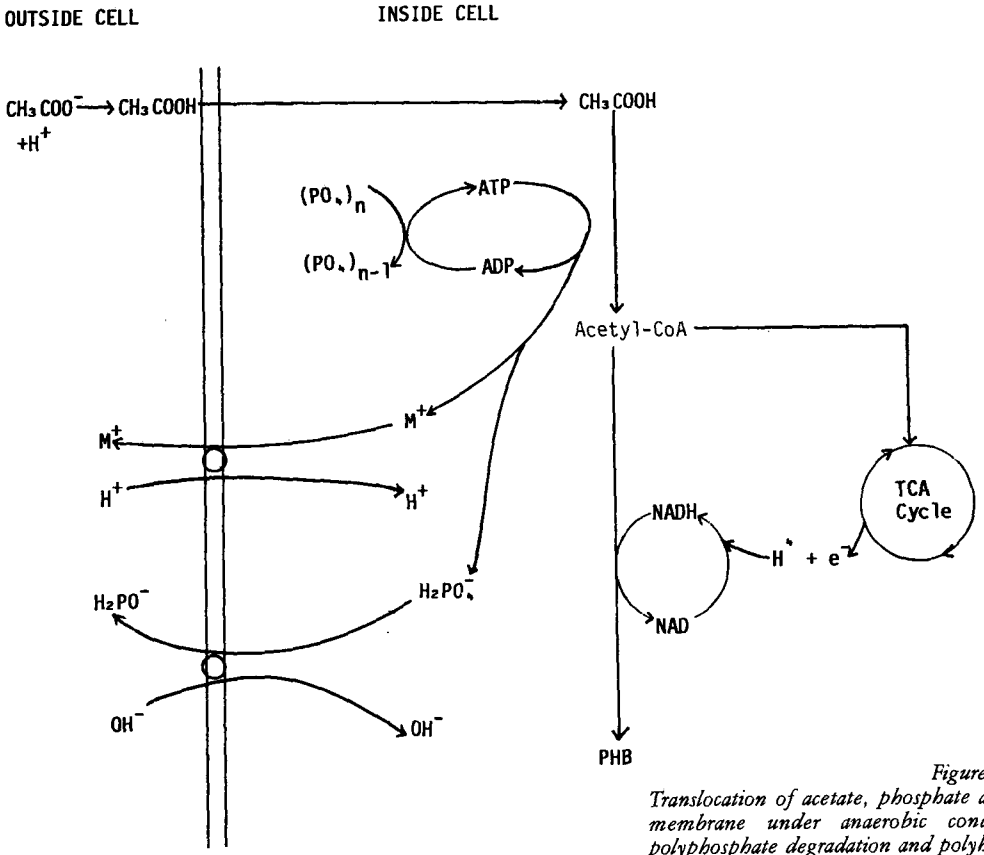


Figure 4
Translocation of acetate, phosphate and cations across the cytoplasmic membrane under anaerobic conditions. For completeness the polyphosphate degradation and polyhydroxybutyrate synthesis to attain this translocation, are shown in outline.

TABLE 2
INTRACELLULAR ALKALINITY, ACIDITY AND CHARGE CHANGES FOR THE PROPOSED MODEL UNDER ANAEROBIC CONDITIONS WITH UPTAKE OF ONE MOLE OF ACETIC ACID

Process	Net change in Alk. (mol)	Acid (mol)	Charge (mol)
HAc into cell	0	+1	0
HAc into PHB synthesis	0	−1	0
*Poly-P hydrolysis: $(PO_4)_n \longrightarrow (PO_4)_{n-1} + H_2PO_4^- + M^+$	+1	+2	0
H_2PO_4 out of cell	−1	−2	+1
OH^- into cell	+1	−1	−1
M^+ out of cell	0	0	−1
H^+ into cell	−1	+1	+1
Sum of changes	0	0	0

*Poly-P hydrolysis to free phosphate involves ATP formation and subsequent utilization as intermediate steps.

The anaerobic biochemical model above was developed for acetate as substrate. For other observed organic substrate sources, e.g. propionate, butyrate, etc. the mechanisms are similar. However, for these the generation of NADH may not require the TCA cycle. For example, for propionate the observed generation of NADH is as follows:

$$\text{Propionate} + \text{HSCoA} \xrightarrow[\text{ATP}\ \text{ADP} + \text{Pi}]{\text{NAD}\ \text{NADH}} \text{acetyl-CoA} + CO_2 \quad (6)$$

(Hodgson and McGarry, 1968).

The generation of NADH maintains the NADH/NAD ratio at a level which inhibits the TCA cycle, and stimulates PHB synthesis (cf. section on carbon metabolism). ATP requirements are supplied by poly-P cleavage, as in the case of acetate.

Aerobic phase

In this phase an external electron acceptor is available in the form of molecularly dissolved oxygen; furthermore the organisms are in an environment where organic substrate in the bulk liquid surrounding them is limited (readily available (soluble) COD having been assimilated in the preceding anaerobic phase). However, the organisms do possess stored PHB. A consequence of the presence of an external electron acceptor is a reduction in the NADH/NAD ratio, and, with concomitant oxidative phosphorylation (ATP generation), the ATP/ADP ratio increases. The decrease in the NADH/NAD ratio stimulates the degradation of PHB, the TCA cycle and associated glyoxylate pathway (cf. aerobic section on carbon metabolism). Degradation of PHB to acetate provides a carbon and energy source for cell function.

With regard to poly-P degradation/synthesis, the high ATP/ADP ratio stimulates poly-P synthesis, as shown in Eq. 2. A further important result of a high ATP/ADP ratio is that it enables the organism to establish any required pmf and to utilize ATP for molecule translocation, i.e. the difference between extracellular and intracellular pH is no longer the determining factor for transport function whereas in the anaerobic state it is (see above).

The proposed mechanisms of phosphate and cation uptake for poly-P synthesis are given in Fig. 5. Phosphate uptake occurs via the hydroxyl mediated antiport and cation uptake via the proton mediated antiport. Intracellular and extracellular changes in alkalinity, acidity and charge for the proposed mechanisms are given in Tables 3 and 4 respectively. Charge neutrality is maintained both extracellularly (Table 3) and intracellularly (Table 4). With regard to alkalinity and acidity: intracellularly there is no change; extracellularly the alkalinity and acidity decrease by one mole and two moles respectively for each mole of phosphate taken up. As for the anaerobic zone the phosphate system (pK ~ 7) dominates. It can be shown that for a decrease in alkalinity by one mole, and a decrease in acidity by two moles, the pH of the mixed liquor from the anaerobic zone will increase in the aerobic zone if the anaerobic pH ≥ 6,8 (Wentzel *et al.*, 1986a). Such pH behaviour conforms with observations in aerobic zones of *Acinetobacter* spp. enhanced culture activated sludge systems (Wentzel *et al.*, 1986b).

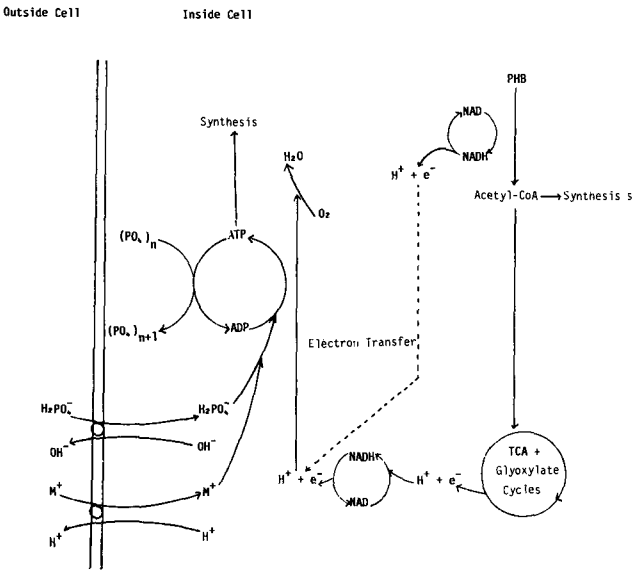


Figure 5
Translocation of phosphate and cations across the cytoplasmic membrane under aerobic conditions. For completeness the polyphosphate synthesis and polyhydroxybutyrate degradation to attain this translocation are shown in outline.

Implications

In terms of the model set out above, the behaviour of *Acinetobacter* spp. in sequential anaerobic/aerobic activated sludge systems can be predicted and evaluated against observations in the literature on these systems.

TABLE 3
EXTRACELLULAR (BULK SOLUTION) ALKALINITY, ACIDITY AND CHARGE CHANGES FOR THE PROPOSED MODEL UNDER AEROBIC CONDITIONS USING PHB AS SUBSTRATE

Process	Net change in Alk. (mol)	Acid (mol)	Charge (mol)
$H_2PO_4^-$ into cell	−1	−2	+1
OH^- out of cell	+1	−1	−1
M^+ into cell	0	0	−1
H^+ out of cell	−1	+1	+1
Sum of changes	−1	−2	0

In the anaerobic zone:

- Decrease in LFA in the bulk solution (Rensink, 1981).
- Increase in phosphate concentration in the bulk solution (extensively reported).
- Increase in cation concentration in the bulk solution, in particular Mg^{2+} and K^+ (Comeau *et al.*, 1985).
- Molar ratio of LFA decrease to phosphate increase in the bulk solution approximately 1:1 (Fukase *et al.*, 1982; Wentzel *et al.*, 1985).
- Charge ratio of cation increase to phosphate increase in the bulk solution is one mole positive charge increase per mole phosphate increase (Comeau *et al.*, 1985).
- Increase (synthesis) of intracellular PHB (Fukase *et al.*, 1982; Hart and Melmed, 1982).
- Decrease (degradation) of intracellular poly-P (Murphy and Lötter, 1986).
- No net change in bulk solution pH (Wentzel *et al.*, 1986b).

In the aerobic zone:

- Decrease in phosphate concentration in bulk solution (extensively reported).
- Decrease in cation concentration in the bulk solution, in particular Mg^{2+} and K^+ (Comeau *et al.*, 1985).
- Charge ratio of cation decrease to phosphate decrease in the bulk solution is one mole positive charge decrease per mole phosphate decrease (Comeau *et al.*, 1985).
- Decrease (degradation) in intracellular PHB (Hart and Melmed, 1982).
- Increase (synthesis) in intracellular poly-P (Murphy and Lötter, 1986).
- Increase in bulk solution pH (Wentzel *et al.*, 1986b).

It is evident that the behavioural patterns predicted by the model in the anaerobic/aerobic sequence conform with observations.

The model can also be applied to describe *Acinetobacter* spp. behaviour in systems and environments that do not include anaerobic/aerobic sequences.

Completely aerobic environment:

Pure cultures of *Acinetobacter* spp. developed aerobically have been shown to accumulate poly-P and PHB. In the culture conditions, *Acinetobacter* spp. invariably are placed in an environment where both a suitable external carbon source (e.g. acetate) in high concentration and an electron acceptor (oxygen) are present. In terms of the model these conditions should stimulate poly-P and PHB accumulation. The presence of an external electron acceptor momentarily decreases the NADH/NAD ratio. The initial decrease in NADH/NAD ratio and the presence of a high substrate concentration both stimulate the TCA cycle. The substrate taken up is utilized for two purposes:

- as a carbon source for anabolism; and
- metabolism by the TCA cycle for energy production and production of specific carbon compounds.

The high concentration of acetate entering the TCA cycle increases the ATP/ADP ratio to a level sufficiently high to stimulate poly-P synthesis and also increases the NADH/NAD ratio. Thus, if the concentration of acetate and thus acetyl-CoA is high, PHB synthesis will be stimulated, with the TCA cycle operation supplying protons and electrons for the reduction of acetate to PHB.

Let us now consider what happens in a completely aerobic system with, say, a sludge age of 10 to 20 days. In the mixed liquor the substrate concentration is low due to competition be-

TABLE 4
INTRACELLULAR ALKALINITY, ACIDITY AND CHARGE CHANGES FOR THE PROPOSED MODEL UNDER AEROBIC CONDITIONS USING PHB AS SUBSTRATE

Process	Net change in Alk. (mol)	Acid (mol)	Charge (mol)
$H_2PO_4^-$ into cell	+1	+2	−1
OH^- out of cell	−1	+1	+1
M^+ into cell	0	0	+1
H^+ out of cell	+1	−1	−1
*Poly-P synthesis: $(PO_4)_n + H_2PO_4^- + M^+ \longrightarrow (PO_4)_{n+1}$	−1	−2	0
Sum of changes	0	0	0

*Poly-P synthesis involves the intermediate step of ATP formation by oxidative phosphorylation, and the transfer of the high energy phosphoryl group to the poly-P chain.

tween organisms for the substrate. In such systems Lötter *et al.* (1986a) have shown that *Acinetobacter* spp. are present in appreciable concentrations and concluded that the *Acinetobacter* spp. are competitive for substrate (via the Entner-Doudoroff pathway) with other aerobes, even though the substrate concentration is low. However, no poly-P or PHB accumulation has been observed. This behaviour can be explained in terms of the model as follows: the low substrate concentration causes that the ATP/ADP ratio is not elevated sufficiently for poly-P synthesis, and NADH/NAD ratio is kept low; low substrate concentration and low NADH/NAD both inhibit PHB synthesis.

If the *Acinetobacter* spp. from the aerobic long sludge age system above are isolated and cultured aerobically they accumulate poly-P and PHB (Lötter *et al.* 1986b). Clearly the propensity to accumulate poly-P and PHB is an inherent characteristic of the organism; it requires only a stimulus to trigger the biochemical pathways necessary for poly-P and PHB accumulation. This stimulus, we have seen is a high ATP/ADP ratio (for poly-P accumulation) and a high NADH/NAD ratio plus a high substrate concentration (for PHB accumulation). If the extracellular substrate concentration is high, as in pure cultures, or the intracellular substrate concentration (i.e. PHB) is high, as in the aerobic zone of anaerobic/aerobic systems, then the ATP/ADP ratio is elevated sufficiently for poly-P synthesis. If the extracellular substrate concentration is high and an external electron acceptor is present, then both PHB and poly-P are accumulated.

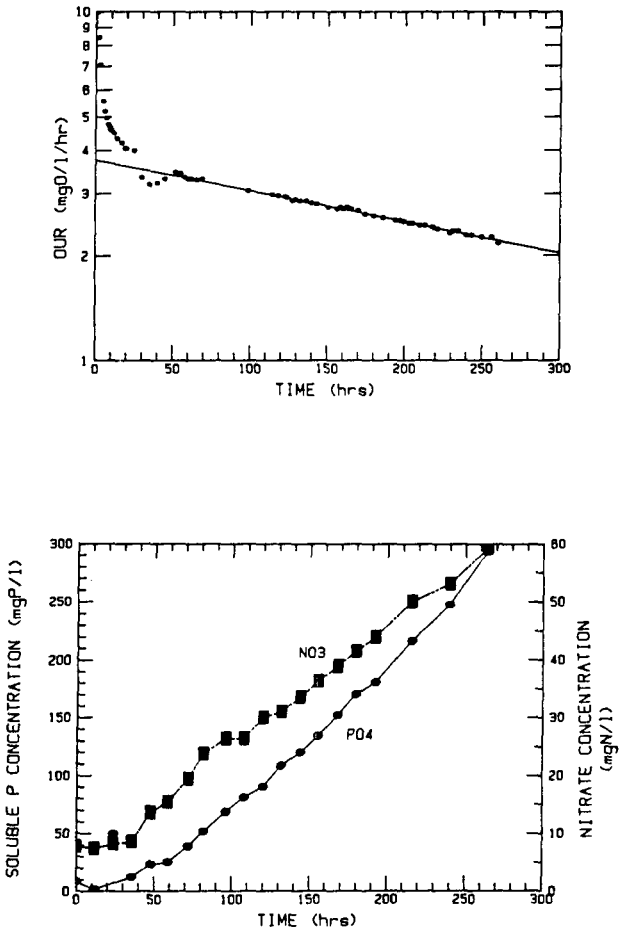


Figure 6
Aerobic batch test on mixed liquor samples drawn from aerobic zone of three-stage Bardenpho enhanced culture system:
(a) Oxygen utilisation rates (OUR) with time
(b) Phosphorus (P) and nitrate (NO_3) concentrations with time.

Endogenous respiration

In order to investigate the endogenous respiration phenomenon in *Acinetobacter* spp. a high concentration of the organism was developed in the system by addition of acetate as substrate to the anaerobic reactor of an anaerobic/aerobic sequence (Wentzel *et al.*, 1986b). An analysis of the population structure indicated that 99 per cent of the organisms cultured aerobically from system samples were *Acinetobacter* spp. (Lötter *et al.* 1986a). When a sample from the aerobic zone of the system was aerated under batch conditions and the phosphate (P) concentration of the bulk solution and oxygen utilization rates (OUR) were monitored with time the response showed a two-phase behaviour: Initially there was a rapid decrease in OUR and a slower decrease in P concentration followed by a slow decrease in OUR with a simultaneous slow increase in P concentration, see Fig. 6(a) and (b).

The initial phase of P concentration decrease clearly is a continuation of the reaction in the aerobic reactor of the system i.e. PHB utilization with some of the energy produced being used for poly-P synthesis. By the end of the first phase the *Acinetobacter* has utilized all the PHB and completed its storage of poly-P. The second phase is more interesting as it reflects complete endogenous behaviour. The slow decrease in OUR, and simultaneous slow increase in P concentration in the bulk solution in this phase was analysed as follows: Assuming that the decrease in OUR is proportional to the decrease in active mass, the rate of decrease in active mass was found to be 0,04 mg VASS/mgVASS/d. With regard to the observed increase in P concentration this must be due to decrease in P stored by the sludge mass, and release of the P to the bulk solution. Relating the magnitude of P release to the stored P concentration indicates that the stored P decreased at a rate approximately equal to the rate of decrease of the active mass, i.e. the observed increase in P concentration probably was the release of stored P to the bulk solution due to *death* or endogenous mass loss of the *Acinetobacter* spp., *not* from poly-P cleavage.

During endogenous respiration the *Acinetobacter* spp. are placed in an environment where no substrate, intracellular or extracellular, is present, but an external electron acceptor (oxygen) is present. In terms of the model the lack of substrate will switch off any synthesis pathways, thereby removing ATP requirements for synthesis. Thus ATP is required only for cell maintenance. In cell maintenance generally a fraction of the cell protoplasm is utilized for energy (ATP) production – giving rise to endogenous mass loss. (Endogenous mass loss associated with maintenance can be viewed either as a decrease in the individual cell mass, or, as death of a fraction of the organism population releasing protoplasm as a substrate to the bulk liquid, in this fashion providing an energy source for the other cells for maintenance. In long sludge age systems, say 20 days, the mass of organisms receive only a small input of energy per day (± 5%) from the influent – this situation is not different from that where no substrate is fed. Under such starvation conditions it is unlikely that the cells synthesized would have "spare" protoplasm for maintenance – protoplasm released from death (lysis) of weaker cells is the more likely source for maintenance energy). This

energy generation from cell protoplasm, together with the low ATP requirement, maintains the ATP/ADP ratio in the *Acinetobacter* spp. at a sufficiently high level to inhibit poly-P degradation. Thus the loss of mass through endogenous action has associated with it the release of poly-P stored by this mass to the bulk solution – this P release is not due to poly-P cleavage for energy generation.

Now consider "endogenous respiration" under anaerobic conditions. If the *Acinetobacter* spp. are placed in an environment where no external electron acceptor is available they are unable to utilize the organic material released from cell death for energy generation. In this situation the cell maintenance requirements decrease the ATP/ADP ratio to a level that invokes poly-P cleavage for energy production. The rate of P release under these conditions will be higher than under aerobic conditions, because uncleaved poly-P is released due to cell death and P is released from poly-P cleavage for maintenance energy. This has been observed when the aerated batch is allowed to become anaerobic. (The situation is rather complicated by the possibility that the protoplasm released due to cell death may be converted to acetate whereupon it becomes available to the *Acinetobacter* for PHB storage by cleavage of poly-P).

Thus under endogenous conditions P is released under both aerobic and anaerobic conditions but at different rates. Because the P releases due to these two endogenous processes are not associated with PHB storage the released P cannot be regained. This will be so also under anoxic conditions (see below) and provides an explanation for the phenomenon of "secondary release" sometimes reported on full-scale plants (Barnard, 1984).

Anoxic environment

Under anoxic conditions oxidized nitrogen (e.g. nitrate) serves as the external electron acceptor. Lötter (1985) demonstrated that approximately 50 per cent of *Acinetobacter* spp. strains isolated are capable of nitrate reduction. Recently Lötter *et al.* (1986b) found that of the *Acinetobacter* spp. able to reduce nitrate the majority could only do so as far as nitrite, while only a minority could reduce nitrate (and nitrite) to nitrogen gas. Thus when explaining *Acinetobacter* spp. behaviour under anoxic conditions three "groups" of *Acinetobacter* must be considered; those unable to utilize nitrate as an external electron acceptor, those able to reduce nitrate to nitrite only, and those able to reduce nitrate to nitrogen. It is of interest to speculate on the behaviour of these three *Acinetobacter* "groups" in two situations commonly encountered in enhanced P removal activated sludge systems, the primary anoxic ractor and the secondary anoxic reactor. In order to describe the behaviour as expeditiously as possible we accept that the reactors are plug flow, not completely mixed; this allows one to separate out the different phases of behaviour.

Primary anoxic reactor

In the activated sludge systems, designed for nitrogen and excess P removal (modified Bardenpho and UCT systems, Marais *et al.*, 1983), mixed liquor passes from the anaerobic reactor to the primary anoxic reactor. In the anaerobic reactor LFA have been sequestered by *Acinetobacter* spp. and stored as PHB at the expense of poly-P. The *Acinetobacter* spp. entering the primary anoxic zone thus have a high intracellular substrate concentration (PHB), a low intracellular poly-P concentration and a low extracellular substrate concentration in the bulk liquid. Consider the behaviour of each of the three *Acinetobacter* groups under such conditions:

- *Acinetobacter* spp. unable to reduce nitrate:
  This group of *Acinetobacter* spp. recognize the anoxic environment as anaerobic and behave accordingly. No oxidative phosphorylation takes place so that the ATP/ADP ratio decreases. This decrease stimulates poly-P breakdown to produce energy (ATP) for cell maintenance. If LFA leak from the anaerobic to the anoxic reactor, PHB is synthesized with concomitant P release (as in the anaerobic reactor). This group of *Acinetobacter* thus release P in the primary anoxic reactor.

- *Acinetobacter* spp. able to reduce nitrate to nitrite:
  This group of *Acinetobacter* spp. are able to produce ATP via oxidative phosphorylation by reducing nitrate to nitrite using stored PHB as substrate. However, in the reduction of nitrate to nitrite only two molecules of ATP are generated per electron pair donated to nitrate compared with three ATP per electron pair for oxygen reduction (Payne, 1981). Thus the energy yield per electron pair in reducing nitrate to nitrite is much lower than in reducing oxygen. With the intracellular substrate available (PHB), this group of *Acinetobacter* probably will increase the ATP/ADP ratio sufficiently for poly-P synthesis. However, due to the lower energy (ATP) yield per electron pair, the poly-P synthesis per mass of substrate (PHB) utilized will be less under anoxic than under aerobic conditions. Furthermore, recognizing that both denitrification and aerobic oxidation are zero order reactions with respect to oxygen and nitrate concentrations, P uptake for these *Acinetobacter* spp. under anoxic conditions is likely to be at a slower rate than if the organism were under aerobic conditions.

- *Acinetobacter* spp. able to reduce nitrate to nitrogen:
  This group of *Acinetobacter* spp. are able to produce ATP via oxidative phosphorylation by reducing nitrate to nitrogen using stored PHB as substrate. The energy yield per electron pair in reducing nitrate to nitrogen is equivalent to that in reducing nitrate to nitrite i.e. 2ATP/electron pair. Thus this group of *Acinetobacter* spp. will exhibit a similar P behavioural pattern as the group able to reduce nitrate only to nitrite i.e. a relatively slow P uptake. However, the two *Acinetobacter* groups will differ in nitrate removal capacity because the electron accepting capacity in nitrate/nitrite reduction is only a quarter of that in the nitrate/nitrogen reduction. The *Acinetobacter* spp. reducing nitrate to nitrite only thus will reduce more nitrate per electron pair (equivalent to COD utilized) than the *Acinetobacter* spp. reducing nitrate to nitrogen.

The combined behaviour of the three *Acinetobacter* spp. groups gives rise to the observed overall behaviour. The observed behaviour is likely to be a rapid decrease in nitrate, an associated decrease in PHB and either a slow increase or decrease in P concentration depending on the relative fractions of *Acinetobacter* spp. present.

The speculated interaction between the three groups of *Acinetobacter* can account for the apparently contradictory observations of behaviour in primary anoxic zones; that of P release or uptake. These speculations are being experimentally investigated.

Secondary anoxic reactors

A secondary anoxic reactor is found in modified 5 stage Bardenpho processes, the reactor being placed immediately downstream of the aeration reactor. In the main aeration reactor the stored PHB is likely to have been very nearly or completely utilized and poly-P storage similarly completed. In terms of the model on entry of mixed liquor to the secondary anoxic zone: Those *Acinetobacter* unable to reduce nitrate will respond as if the reactor is anaerobic i.e. P release due to endogenous death and poly-P cleavage for maintenance energy as in the section on endogenous "respiration" under anaerobic conditions; those *Acinetobacter* spp. capable of reducing nitrate to nitrite and those capable of reducing nitrate to nitrogen gas will behave in the same fashion as *Acinetobacter* spp. in the primary anoxic reactor provided PHB is available. Should PHB be reduced to zero, these *Acinetobacter* spp. will behave as under aerobic endogenous conditions, that is, the fraction of their mass released on death is used to supply ATP for cell maintenance purposes and the poly-P associated with the metabolized protoplasm mass is released giving rise to an observed P release.

In the secondary anoxic reactor if nitrate becomes depleted, i.e. an anaerobic state, all three groups of *Acinetobacter* spp. will behave as described for anaerobic endogenous conditions.

We can now apply the above behavioural patterns to explain observed plant response:

- In the 3 stage Bardenpho or UCT processes with completely mixed reactors, in the aerobic reactor, release of P due to endogenous respiration effects is virtually certain to be swamped by poly-P accumulation from PHB metabolism, and a net removal of P from the liquid will be observed.

- In the 5 stage Bardenpho process, i.e. one that includes a secondary anoxic zone, the behaviour in the secondary anoxic zone will be governed to a large extent by the mass of PHB still stored in the sludge entering that zone, and the concentration of nitrate:

If the nitrate and PHB concentrations are adequate then poly-P storage will continue and probably will exceed P release due to endogenous metabolism and other effects. If stored PHB is low the poly-P storage may be either more, equal or less than the release phenomena and correspondingly, P uptake, no change, or P release will be observed. If there is no stored PHB then certainly P release will be observed. This will also be the case when no nitrate is present, whether PHB is present or not. P release associated with energy production for maintenace cannot be taken up subsequently on aeration. The behaviour deduced above has been reported: Barnard (1984) has observed P release in some secondary anoxic reactors of full-scale modified Bardenpho plants, and that the released P was not taken up in the subsequent reaeration reactor. This phenomenon he termed "secondary" release. It would appear that endogenous ("secondary") P release takes place throughout the system, its manifestation usually being swamped by other biological phosphorus processes. This implies that the longer the sludge age the greater the release, consequently, for the same substrate mass utilized by the poly-P organisms the net removal of P from the system should diminish as the sludge age increases. Observations on enhanced *Acinetobacter* spp. activated sludge systems appear to support this (Wentzel *et al.*, 1986b).

Space does not allow detailed discussion of the many different situations that can arise in the main aeration, secondary anoxic and reaeration reactors.

Nutrient limitation

If any essential nutrient becomes limiting and a suitable carbon substrate is available, poly-P and PHB accumulation is likely to occur. This is because the synthesis pathways will be inhibited by the limiting nutrient, this inhibition decreasing the ATP and substrate requirements for synthesis. The TCA cycle will not be inhibited by nutrient deficiency so that the NADH/NAD ratio will be maintained at a high level and oxidative ATP production will continue elevating the ATP/ADP ratio. The high ATP/ADP will stimulate poly-P accumulation, while the high substrate concentration and NADH/NAD ratio will stimulate PHB accumulation (with the proviso that the substrate concentration is sufficiently high). Poly-P accumulation under limitation of the nutrients sulphur and nitrogen has been observed, the so-called "luxury uptake" (Harold, 1966), and appears to find explanation in terms of this model. If however the limiting nutrient is one of the cations required to stabilize poly-P charge, i.e. Mg^{2+} or K^{+}, then synthesized poly-P will be unstable and no poly-P accumulation will take place. This behaviour has been explicitly verified in enhanced *Acinetobacter* spp. activated sludge systems (Wentzel *et al.*, 1986b).

Conclusion

Acinetobacter spp. are able to compete successfully with other organisms for substrate in completely aerobic activated sludge systems. In such systems *Acinetobacter* spp. are able to metabolize glucose via the Entner-Doudoroff pathway. When an anaerobic reactor is introduced into the system the anaerobic reactor selects an assemblage of facultative organisms able to ferment sugars to LFA via the glycolytic (Embden-Meyerhof) pathway. *Acinetobacter* spp. do not possess the glycolytic pathway and thus are not able to produce energy for survival in the anaerobic zone by e.g. glucose fermentation. Consequently the *Acinetobacter* spp. would be at a distinct disadvantage in the anaerobic/aerobic system due to this lack of the glycolytic pathway and would be outcompeted by the facultative organisms in such a system. Without some mechanism of competitive compensation they would disappear from the anaerobic/aerobic activated sludge system. This competitive compensation is the ability to store poly-P and PHB. During the anaerobic state poly-P serves as an energy source for the maintenance of cell functions and PHB synthesis. PHB synthesis in the anaerobic phase effectively removes the *Acinetobacter* from competition for substrate enabling the organism to withstand the selective pressures of the anaerobic/aerobic sequences.

The biochemical model presented here describes the pathways whereby *Acinetobacter* spp. accumulate and degrade poly-P and PHB, and how these pathways are regulated. Identification of the regulatory system allows one to predict the response of the organism under a variety of imposed conditions. Principally regulation appears to be via the ATP/ADP and NADH/NAD ratios. These ratios are affected by the substrate concentration, intracellular or extracellular (PHB and, say, acetate respectively), and the presence or absence of external electron acceptors (aerobic, anoxic or anaerobic). In terms of these parameters it appears to be possible to explain the observed behaviour of biological excess phosphorus removal as observed in plants and other situations. These are briefly summarized below:

1. Under aerobic conditions:

(i) If acetate concentration is high both PHB *and* poly-P storage

takes place; this is observed in pure culture tests.

(ii) If acetate concentration is relatively low *Acinetobacter* spp. grow without PHB or poly-P accumulation; this situation is relevant to completely aerobic activated sludge systems.

(iii) If acetate concentration is very low or zero, but stored PHB is present, poly-P accumulation will take place; this situation is relevant to the main aeration basin of anaerobic/anoxic/aerobic systems (Bardenpho or UCT).

(iv) If neither acetate nor PHB is present, maintenance energy is obtained by utilization of substrate generated by the death of *Acinetobacter* spp. (endogenous mass loss); poly-P is released apparently uncleaved to the bulk solution proportionally to the protoplasm mass of organisms that died; this situation might be observed explicitly in the reaeration zone [and in the secondary anoxic zone if nitrate is present – see 3(ii)] and is one component of the so-called secondary release. Speculatively it would appear that death of *Acinetobacter* and associated release of uncleaved poly-P takes place throughout the system, i.e. whether acetate is available or not; but because of its low rate its manifestation is swamped by other biological phosphate processes. This secondary P release is never associated with any form of substrate storage and will not give rise to any form of subsequent P uptake.

2. Under anaerobic conditions:

(i) If acetate is present, PHB is stored by cleaving stored poly-P.

(ii) If acetate is not present, irrespective of whether PHB is present or not, maintenance energy is obtained by cleavage of stored poly-P. This is another component of secondary release and again is not associated with subsequent P uptake.

3. Under anoxic conditions (nitrate present):

(i) If PHB is present but acetate is not, as in the primary anoxic zone, two situations can apply: *Acinetobacter* not able to use nitrate react as in 2(ii); *Acinetobacter* that can use nitrate react as in 1(iii). Hence a release and uptake of P takes place simultaneously giving rise to a net P release or uptake, depending on the relative concentrations of organisms and the rates of reaction.

(ii) If neither PHB nor acetate is present, as in the secondary anoxic zone, then: *Acinetobacter* not able to use nitrate react as in 2(ii); *Acinetobacter* that can use nitrate react as in 1(iv). However, in the secondary anoxic zone, if no nitrate is present, maintenance energy cannot be obtained by oxidation of the protoplasm released due to death; instead it is obtained by cleavage of poly-P giving rise to secondary release as in 2(ii). When no nitrate is present the protoplasm released due to death may be converted to acetate or other LFA by non-poly-P facultative organisms, so that P release due to PHB formation can take place and subsequently on aeration give rise to an uptake of P, usually limited. This behaviour has been observed in reaeration zones on occasion.

Appendix I

Thermodynamic control of poly-P metabolism

Poly-P metabolism can follow two pathways, namely a synthesis pathway leading to phosphate uptake and a degradation pathway leading to phosphate release.

The basic equation governing both these pathways is the reversible reaction:

$$ATP + (PO_4)_n \underset{\text{degradation}}{\overset{\text{synthesis}}{\rightleftharpoons}} ADP + (PO_4)_{n+1} \qquad (A.1)$$

From an energy point of view the direction of the reaction under non-equilibrium isothermal conditions is determined by the value of the Gibb's free energy change for the reaction (ΔG_R); a negative value for ΔG_R effecting the forward reaction and a positive value the reverse. It is of fundamental interest to delineate thermodynamic conditions favouring the forward and reverse reactions. This can be done from an analysis of the equation for ΔG_R, as follows:

The equation for the free energy change (ΔG_R) of the forward reaction (A.1), i.e. the synthesis reaction, is

$$\Delta G_R = \Delta G_R^{\ominus} + RT\,\ell n \frac{(PO_4)_{n+1}\cdot(ADP)}{(PO_4)_n\cdot(ATP)} \qquad (A.2)$$

where

(X) = activity of species X
R = universal gas constant
T = temperature (K)
$\Delta G_R^{\ominus}$ = standard free energy change for the equilibrium reaction
$= \Delta G_F^{\ominus}$ (products) – $\Delta G_f^{\ominus}$ (reactants) (A.3)
$\Delta G_f^{\ominus}$ = standard free energy of formation of one mole of the substance under standard conditions

Referring to Eq. (A.2) the poly-P species, $(PO_4)_n$ and $(PO_4)_{n+1}$, are solids and their activities are therefore unity; the equation for ΔG_R therefore reduces to:

$$\Delta G_R = \Delta G_R^{\ominus} + RT\,\ell n \frac{(ADP)}{(ATP)} \qquad (A.4)$$

The value for $\Delta G_R^{\ominus}$ in Eq. (A.3) is determined as

$$\Delta G_R^{\ominus} = \Delta G_R^{\ominus}(PO_4)_{n+1} + \Delta G_R^{\ominus}(ADP) - \Delta G_R^{\ominus}(PO_4)_n - \Delta G_R^{\ominus}(ATP) \qquad (A.5)$$

Noting that the phosphoryl bonds between ADP and P and between $(PO_4)_n$ and $(PO_4)_{n+1}$ are identical, the standard free energy changes for hydrolysis and condensation will be closely equal i.e. $\Delta G_f^{\ominus}$ for (ATP-ADP) will closely equal $\Delta G_f^{\ominus}$ for $[(PO_4)_{n+1} - (PO_4)_n]$. Thus $\Delta G_R^{\ominus}$ in Eq. (A.5) closely equals zero and Eq. (A.4) reduces to

$$\Delta G_R = RT\,\ell n \frac{(ADP)}{(ATP)} \qquad (A.6)$$

This equation forms the basis for linking poly-P formation or degradation to the intracellular ATP/ADP ratio. If ATP > ADP then ΔG_R will be negative and the forward reaction (i.e. poly-P synthesis) is favoured. This situation arises in an aerobic state where organic substrate is not limiting. Conversely if ATP > ADP then ΔG_R will be positive favouring the reverse reaction i.e. poly-

P degradation and concomitant ATP formation. The ATP thus formed is used for two purposes: for cell function, and PHB synthesis, thereby maintaining ATP < ADP. This situation arises when an obligate aerobic poly-P organism, with an intracellular ATP demand, is placed in an anaerobic environment.

The key role of the ATP/ADP ratio in thermodynamic control of poly-P metablism concurs with observed regulation of the enzyme mediating the reaction (A.2) (cf. section on poly-P metabolism in paper).

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References

BARNARD, J.L. (1976) A review of biological phosphorus removal in the activated sludge process. *Water SA* **2**(3) 136-144.

BARNARD, J.L. (1984) Activated primary tanks for phosphate removal. *Water SA* **10**(3) 121-126.

BELL, E.J. and HERMAN, N.J. (1967) Effect of succinate on isocitrate lyase synthesis in *Mima polymorpha*. *J. Bacteriol.* **93** 2020-2021.

BRODISCH, K.E.U. and JOYNER, S.J. (1983) The role of micro-organisms other than *Acinetobacter* in biological phosphate removal in the activated sludge process. *Wat. Sci. Tech.* **15**(3/4) 117-125.

BRODISCH, K.E.U. (1985) Interaction of different groups of micro-organisms in biological phosphate removal. *Wat. Sci. Tech.* **17** 89-97.

BUCHAN, L. (1981) The location and nature of accumulated phosphorus in seven sludges from activated sludge plants which exhibited enhanced phosphorus removal. *Water SA* **7**(1) 1-7.

BUCHAN, L. (1983) Possible biological mechanism of phosphorus removal. *Wat. Sci. Tech.* **15** 87-103.

COMEAU, Y., HALL, K.J.,HANCOCK, R.E.W. and OLDHAM, W.K. (1985) Biochemical model for enhanced biological phosphorus removal. *Proc. UBC Conference* on new directions and research in waste treatment and residuals management. June, Vancouver, Canada.

DAWES, E.A. and SENIOR, P.J. (1973) The role and regulation of energy reserve polymers in micro-organisms. *Adv. Microbiol Physiol.* **10** 135-266.

DEINEMA, M.H., HABETS, L.H.A., SCHOLTEN, J., TURKSTRA, E. and WEBERS, H.A.A.M. (1980) The accumulation of polyphosphate in *Acinetobacter* spp. *Fems Microbiol. Letters* **9** 275-279.

FELTER, S. and STAHL, J.C. (1970) Recherche sur les enzymes du metabolisme des polyphosphate dans la levure. *Bull. Soc. Chim. Biol.* **52** 75-87.

FRICKE, B., BERGMAN, R., SORGER, H. and AURICH, H. (1982) Optimierung von Kulturbedingungen für *Acinetobacter calcoaceticus* beim Wachstum aus n-Alkanen in einem Laborfermentor. *Zeitschr. Allgemein. Mikrobiol.* **22** 365-372.

FUHS, G.W. and CHEN, M. (1975) Microbiological basis of phosphate removal in the activated sludge process for the treatment of waste water. *Microbiol. Ecology* **2** 119-138.

FUKASE, T., SHIBATA, M. and MIJAYI, X. (1982) Studies on the mechanism of biological phosphorus removal. Trans. by C.J. Mardon, *Japan J. Water Pollut. Res.* **5** 309-317.

HAROLD, F.M. (1964) Enzymic and genetic control of polyphosphate accumulation in *Aerobacter aerogenes*. *J. Gen. Microbiol.* **35** 81-90.

HAROLD, F.M. (1966) Inorganic polyphosphate in biology: structure, metabolism and function. *Bacteriol Rev.* **30** 772-794.

HAROLD, F.M. and HAROLD, R.L. (1965) Degradation of inorganic polyphosphate in mutants of *Aerobacter aerogenes*. *J. Bacteriol.* **89** 1262-1270.

HAROLD, F.M. and SPITZ, E. (1975) Accumulation of arsenate, phosphate and aspartate by *Streptococcus faecalis*. *J. Bacteriol.* **122** 266-277.

HART, M.A. and MELMED, L.N. (1982) Microbiology of nutrient removing activated sludge. *Wat. Sci. Tech.* **14** 1501-1502.

HENRIKSEN, S.D. (1973) *Moraxella*, *Acinetobacter* and the *Mimeae*. *Bacteriol. Rev.* **37** 522-561.

HERMAN, N.J. and BELL, E.J. (1970) Metabolic control in *Acinetobacter* spp.: I. Effect of C4 versus C2 and C3 substrates on isocitrate lyase synthesis. *Can. J. Microbiol.* **16** 769-774.

HODGSON, B. and McGARRY, J.D. (1968) A direct pathway for the metabolism of propionate in cell extracts from *Moraxella lwoffi*. *Biochem. J.* **107** 19-28.

JUNI, E. (1978) Genetics and physiology of *Acinetobacter*. *Ann. Rev. Microbiol.* **32** 349-371.

KORNBERG, S.R. (1957) Adenosine triphosphate synthesis from polyphosphate by an enzyme from *Escherichia coli*. *Biochem. Biophys. Acta* **26** 294-300.

KONINGS, W.N., HELLINGWERF, K.J. and ROBILLARD, G.P. (1981) Transport across bacterial membranes. In *Membrane transport*. Eds. F.L. Bonting and J.J.H. de Pont. Elsevier North Holland Biomedical press, Amsterdam.

KULAEV, I.S. (1973) The enzymes of polyphosphate metabolism in protoplasts and some sub-cellular structures of *Neurospora crassa*. In *Yeasts, Moulds and Plant protoplasts*. Eds. J.P. Villaneuva, I. Garcia-ana, S. Gascon and F. Uruburu. Academic Press, New York.

KULAEV, I.S. (1975) Biochemistry of inorganic polyphosphates. *Rev. Physiol. Biochem. Pharmacol.* **73** 136-158.

KULAEV, I.S. and BOBYK, M.A. (1971) Demonstration of a new enzyme 1,3 Diphosphoglycerate:polyphosphate phosphotransferase in *Neurospora crassa*. *Biokhimiya* **36** 426-429.

LAWSON, E.N. and TONHAZY, N.E. (1980) Change in morphology and phosphate uptake patterns of *Acinetobacter calcoaceticus* strains. *Water SA* **6** 105-112.

LEVIN, G.V. and SHAPIRO, J. (1965) Metabolic uptake of phosphorus by waste-water organisms. *J. Wat. Poll. Contr. Fed.* **37**(6) 800-821.

LÖTTER, L.H. (1985) The role of bacterial phosphate metabolism in enhanced phosphorus removal from the activated sludge process. *Wat. Sci. Technol.* **17** 127-138.

LÖTTER, L.H., WENTZEL, M.C., EKAMA, G.A. and MARAIS, G.v.R. (1986a). An investigation into the heterotrophic bacterial population of various activated sludge plants. In preparation.

LÖTTER, L.H., WENTZEL, M.C., LOEWENTHAL, R.E., EKAMA, G.A. and MARAIS, G.v.R. (1986b). A study of selected characteristics of *Acinetobacter* spp. isolated from activated sludge in anaerobic/anoxic/aerobic and aerobic systems. *Water SA* **12**(4) 203-208.

MARAIS, G.v.R., LOEWENTHAL, R.E. and SIEBRITZ, I.P. (1983) Review: observations supporting phosphate removal by biological excess uptake. *Wat. Sci. Tech.* **15** 15-41.

MEGANCK, M., MALNOU, D., LE FLOHIC, P., FAUP, G.M. and ROVEL, J.M. (1985) The importance of acidogenic microflora in biological phosphorus removal. *Wat. Sci. Tech.* **17** 199-212.

MURPHY, M. and LÖTTER, L.H. (1986) The effect of acetate on polyphosphate formation and degradation in activated sludge with particular reference to *Acinetobacter calcoaceticus:* A microscopic study. *Water SA* **12** (2) 63-66.

NESMEYANOVA, M.A., DMITRIEV, A.D. and KULAEV, I.S. (1974) Regulation of the enzymes of phosphorus metabolism and the level of polyphosphate in *Escherichia coli* K-12 by exogenous orthophosphate. *Mikrobiologiya* **43** 227-234.

NICHOLLS, H.A. and OSBORN, D.W. (1979) Bacterial stress: Prerequisite for biological removal of phosphorus. *J. Wat. Poll. Contr. Fed.* **51** 557-569.

PADAN, E., ZILBERSTEIN, D. and ROTTENBERG, H. (1976) The proton electrochemical gradient in *Escherichia coli*. *Eur. J. Biochem.* **63** 533-541.

PARKER, M.C. and WEITZMAN, P.D.J. (1970) Regulation of NADP-linked isocitrate dehydrogenase activity in *Acinetobacter*. *FEMS Letters* **7**(4) 324-326.

PAYNE, W.J. (1981) *Denitrification*. Publ. John Wiley and Sons, New York.

RENSINK, J.H. (1981) Biologische defosfatering en procesbepalende factoren. In *Symp. Boek – Defosfatering*; nieuwe ontwikkelingen en praktijkervaringen in Nederland en Zweden. NVA Symp. (in Dutch).

SHABALIN, Yu.A., VAGABOV, V.M., TSIOMENKO, A.B., ZEMLENUKHIA, O.A. and KULAEV, I.S. (1977) Polyphosphate kinase activity in yeast vacuoles. *Biokhimiya* **42** 1642-1648.

SHAPIRO, J. (1967) Induced rapid release and uptake of phosphate by micro-organisms. *Science* **155** 1269-1271.

SELF, C.H. and WEITZMAN, P.D.J. (1972) The isocitrate dehydrogenases of *Acinetobacter lwoffi*. *Biochem. J.* **130** 211-219.

SEVERIN, A.J., LUSTA, K.A., NESMEYANOVA, M.A. and KULAEV, I.S. (1976) Membrane bound polyphosphatase of *E. coli*. *Biokhimiya* **41** 357-362.

SIEBRITZ, I.P., EKAMA, G.A. and MARAIS, G.v.R. (1983) A parametric model for biological excess phosphorus removal. *Wat. Sci. Tech.* **15** 127-152.

SRINATH, E.G., SASTRY, C.A. and PILLAY, S.C. (1959) Rapid removal of phosphorus from sewage by activated sludge. *Water and Waste Treatment* **11** 410.

STURM, R.N., HERMAN, N.J. and BELL, E.J. (1970) Metabolic control in *Acinetobacter* spp.: Effect of C4 versus C2 substrate and α-ketoglutarate dehydrogenase *Can. J. Microbiol.* **16** 817-820.

SUZUKI, H., KANCKO, J. and IKEDA, Y. (1972) Properties of polyphosphate kinase prepared from *Mycobacterium smegmatis*. *Biochim. Biophys. Acta.* **268** 381-390.

WARSKOW, A.L. and JUNI, E. (1972) Nutritional requirements of *Acinetobacter* strains isolated from soil, water and sewage. *J. Bacteriol.* **112** (2) 1014-1016.

WEITZMAN, P.D.J. and JONES, D. (1968) Regulation of citrate synthase and microbial taxonomy. *Nature* **219** 270-272.

WEITZMAN, P.D.J. and DUNMORE, P. (1969) Citrate synthase: Allosteric regulation and molecular size. *Biochim. Biophys. Acta* **171** 198-200.

WEITZMAN, P.D.J. (1972) Regulation of α-ketoglutarate dehydrogenase activity in *Acinetobacter*. *FEMS Letters* **22** 323-326.

WENTZEL, M.C., DOLD, P.L., EKAMA, G.A. and MARAIS, G.v.R. (1985) Kinetics of biological phophorus release. *Wat. Sci. Tech.* **17** 57-71.

WENTZEL, M.C., LOEWENTHAL, R.E. and MARAIS, G.v.R. (1986a) pH control in mixed weak acid/base systems. In preparation.

WENTZEL, M.C., LOEWENTHAL, R.E. and MARAIS, G.v.R. (1986b) Development of enhanced poly-P cultures in activated sludge systems. In preparation.

ZAITSEVA, G.N. and BELOZERSKII, A.N. (1960) Formation and utilization of polyphosphate catalysed by an enzyme isolated from *Azotobacter vinelandii*. *Dokl. Akad. Nauk.* SSSR, 950-953.