INTRODUCTION

Biological nutrient removal (BNR) is commonly the most economical means of removing nitrogen and phosphorus from wastewaters to control eutrophication in lakes and estuaries, and to prevent the gradual deterioration of water quality. Loss of recreational and economical value of a water body is unavoidable if controlled discharge of wastewater streams is not practiced. However, there are current gaps in the knowledge of enhanced biological phosphorus removal (EBPR) that have restricted its widespread adoption compared to nitrogen removal. These gaps need to be filled before it will receive the widespread implementation it deserves.

Removal of nitrogen is successfully and widely practiced because the biological and biochemical mechanisms behind the processes are well-known, and they are not as complicated as those of EBPR. For these reasons, EBPR has been left on the back burner although it has significant economical advantages and its implementation would make nitrogen removal more efficient while also reducing some of the stress on nitrogen removal. Currently nitrogen removal goals that need to be attained are getting much stricter and it is becoming more imperative that phosphorus removal be accomplished, because phosphorus is nearly always the limiting nutrient for freshwater bodies, and is seasonally or regionally limiting for estuarine bodies of water. There is a need to increase the number of wastewater treatment plants that practice EBPR in addition to nitrogen removal, but a better understanding of its economic and process optimization advantages is needed by the wastewater treatment profession. One way to accomplish this would be through a better understanding of the biochemical mechanisms and microbial population dynamics that determine the reliability and efficiency of EBPR, and through the utilization of this information to improve the design and operation of BNR plants. Such knowledge will also contribute to better structure of modeling tools that are used for design and educational purposes. One such model is the IWA Activated Sludge Model No.3, and it lacks the necessary information to accurately describe and predict the phosphorus removal capacity of a BNR plant design since it is not based on a complete biochemical model for EBPR. The current body of knowledge is limited to observational

studies that lack detailed biochemical explanations backed well planned experiments, and this has introduced uncertainties and inaccuracies into the biochemical and design models. The accuracy of design and strength of the scientific background need to be reinforced to enable accurate modeling and economically optimal design.

This study was performed to gather information that will aid in sound and economical design and upgrade of BNR plants. Hence it mainly covers a biochemical survey of the underlying metabolisms of active populations in BNR biosolids. BNR biomass with BPR capability (i.e. anaerobic release of phosphorus with concomitant uptake of volatile fatty acids (VFAs) available in the feed, and subsequent aerobic uptake of phosphorus in excess of the released amount for EBPR) were cultivated in continuous flow reactor (CFR) systems, configured as either University of Cape Town (UCT) and anoxic/oxic (A/O) systems. Following an acclimation period at 20°C, low temperature stress (5°C) was imposed on one UCT system for investigation of the response of the consortium responsible from EBPR activity under cold temperature. Data collection was planned such that sufficient information would be available at the end of the study to evaluate the involvement of glycogen in EBPR, since one of the most debated issues of EBPR is the source of reducing equivalents that are necessary for the sequestering and polymerization of available VFAs into polyhydroxyalkanoate (PHA) units. Once a stable population with EBPR capabilities is established in each system, activities of the enzymes that are hypothesized to be taking part in the EBPR metabolism were measured. Also, ^{13}C-NMR was used to monitor the flux of labeled carbon in and out of pools of cellular storage. Combining the gathered information, accurate mass balances of carbons and reducing equivalents were calculated, eventually leading to determination of the biochemical pathways utilized by the cultivated EBPR consortium.

In summary, the main objective of this study was to investigate the biochemical mechanisms underlying the enhanced biological phosphorus removal (EBPR) systems, while determining the extent of interactions between bacterial carbon and energy storage pools and phosphorus removal under different conditions. In doing this, a substantial amount of contribution was made to both environmental engineering and biochemistry

literature, as the study dealt with both the engineering and biochemical aspects of EBPR. Additionally, anaerobic stabilization of COD, which has been a long debated but empirically established phenomenon, was addressed during this study. The correct incorporation of anaerobic stabilization of COD into process design has the potential to reduce design aeration requirements and to result in economic savings during both construction and operation of BNR plants.

CHAPTER I: LITERATURE REVIEW

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INTRODUCTION

Phosphorus is an essential part of the nutrient cycle in nature. It is also essential for growth of the microorganisms in an activated sludge wastewater treatment plant (WWTP), as it makes up 1.5 to 2.0 per cent of the cells. However, due to increasing anthropogenic sources of phosphorus in the environment, it has become a nuisance leading to eutrophication of lakes and deterioration of water quality. EBPR systems employed as an integral part of the nutrient removal WWTPs successfully remove phosphorus from the incoming wastewaters. Since 1980s a great deal of research efforts have been dedicated to exploring the underlying reactions of EBPR. The current knowledge puts the research community into two camps, if not more: i) followers of Comeau, (Comeau *et al*., 1986; Comeau *et al*., 1987; Wentzel *et al*., 1991) ii) followers of Mino (Liu *et al*., 1996; Maurer *et al*., 1997; Brdjanovic *et al*., 1998; Christensson *et al*., 1998)). Currently, there are other researchers who support hypotheses built on the ideas of both researchers, such as Pereira *et al.* (1996), Louie *et al*. (2000), Hesselmann *et al*. (2000) and others.

Activated sludge is such a closely-knit, and perfectly operating symbiotic microbial community that isolating one process from another through pure culture studies has not been possible without loosing phosphorus removal functions of the cultivated microbial community. Besides, culture dependent techniques used for identifying phosphorus removing organisms has also been unsuccessful, and current approach is to use genetic markers and nucleotide dependent techniques (Cloete and Steyn, 1988; Hiraishi *et al*., 1989; Bond *et al*., 1995; Auling *et al.*, 1991; Kavanaugh and Randall, 1994). Thus,

current approach is to examine EBPR and underlying mechanisms as a whole. Other processes co-existing in a nutrient removal plant such as nitrification, and carbon oxidation have impacts on successful operation of phosphorus removal mechanisms, and they are made an integral part of such research. With this in mind, this study will deal with the metabolic interactions arising from the actions of EBPR biomass and leading to biological phosphorus removal, and inevitably with the possible bearing of the findings on EBPR design and operation.

Although there are many aspects of phosphorus removal that need to be explored in order to draw a complete picture of the process, this study will mainly deal with the interactions of the two internal storage polymers shown to play a significant role in EBPR: PHAs and glycogen. In doing this, an attempt will be made to develop a biochemical model. The ideas of the above mentioned two camps of EBPR research will be visited, and conclusions will be drawn based on the combined results of the individual experiments that were performed. Furthermore, in spite of the fact that glycogen was hypothesized to take part in EBPR in the mid 1980s (Mino *et al*., 1987), much of the related research did not include glycogen as a significant parameter that needed to be monitored. Thus, no reliable data on glycogen were available until recently. Major reason for this was the absence of a reliable method for the measurement of cellular glycogen, and the researchers chose to measure total carbohydrates instead, if they ever attempted to measure glycogen. Although bacteriology literature suggests the Anthrone Method for glycogen measurements, practical difficulties in its application prevent it from being a reliable method that generates accurate and precise data. Thus this research will in itself fill such a gap in EBPR research through development of a method for reliable glycogen measurements, and by accumulation of glycogen data under numerous operational conditions.

BACKGROUND

Biochemical Background on Metabolic Pathways

The biochemical reactions that will be relevant for the scope of this research are those included in glycogen degradation and synthesis, Embden-Meyerhoff-Parnass (EMP), Entner-Doudoroff (ED) and pentose phosphate (PP) pathways, gluconeogenesis, TCA and glyoxylate cycles, succinate-propionate pathway, and branched TCA cycle.

Glycolysis and Gluconeogenesis:

As summarized in White (1995) and illustrated in Figure 1, glycolysis occurs in two stages: C6 glucose molecule is initially split into two C3 phosphoglyceraldehyde (PGALD) molecules, and the remaining part consists of oxidation of PGALD to pyruvate. PGALD is also the merging point of the three glycolytic pathways that take glucose to pyruvate: EMP, ED, and PP. Thus the enzyme that catalyzes the phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP), phosphofructokinase (PFK) can be a good indicator of the activity of the EMP pathway. On the other hand the enzyme glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the first step of both the ED and PP pathways, but not the EMP pathway.

In order for microorganisms to synthesize hexoses needed for the production of cell wall mucopeptide, storage glycogen and other compounds necessary for growth, they need to reverse the carbon flow from pyruvate. This “new production” of glucose is called “gluconeogenesis”. However, three of the enzymes that take part in EMP glycolysis are not reversible, and they need to be bypassed for the completion of the glucose production route. One of these enzymes is pyruvate kinase, which cannot be reversed due to the requirement for a large free energy. For this purpose, phosphoeneolpyruvate (PEP) is generated by decarboxylation and phosphorylation of oxaloacetate through the action of PEP carboxykinase (Moat and Foster, 1995). The second enzyme that needs to be

bypassed is PFK, and it is achieved by fructose-1,6-bisphosphatase, which dephosphorylates F1,6BP to yield F6P. This is one of the key regulating steps that control the relative rates of glycolysis and gluconeogenesis, which in turn control the overall net direction of the carbon flux. The last hurdle of gluconeogenesis is the dephosphorylation of G6P to yield glucose, and the enzyme that proceeds at this step is G6Pase. The overall reaction from pyruvate to glucose can be written as follows, and it shows the great extent of energy that needs to be put into it:

$$2\text{pyruvate} + 4\text{ATP} + 2\text{GTP} + 2\text{NADH} + 2H^{+} + 4H_2O \rightarrow \text{glucose} + 2NAD^{+} + 4\text{ADP} + 2\text{GDP} + 6\text{Pi}$$

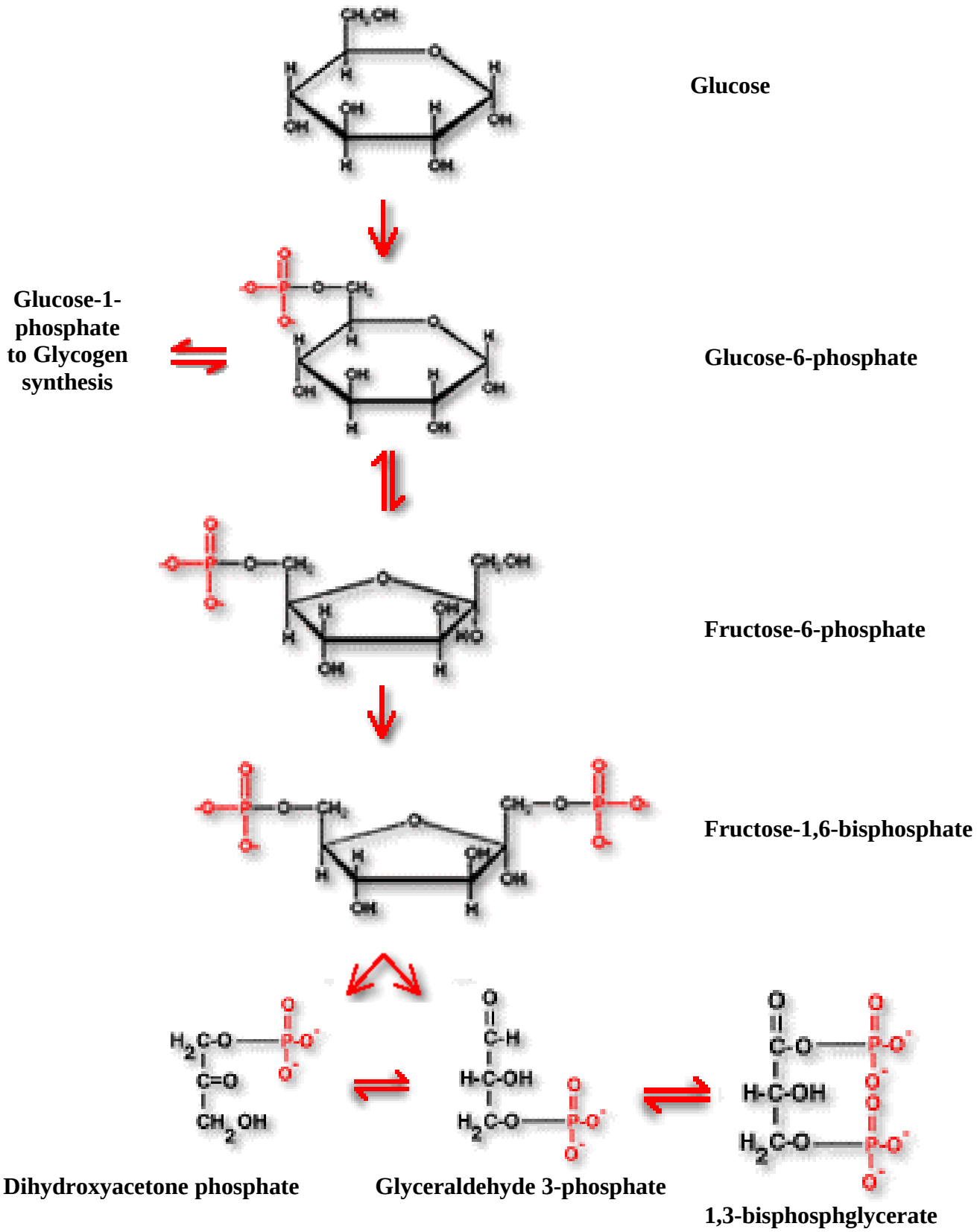


Figure 1. Glycolysis pathway (White, 1995)

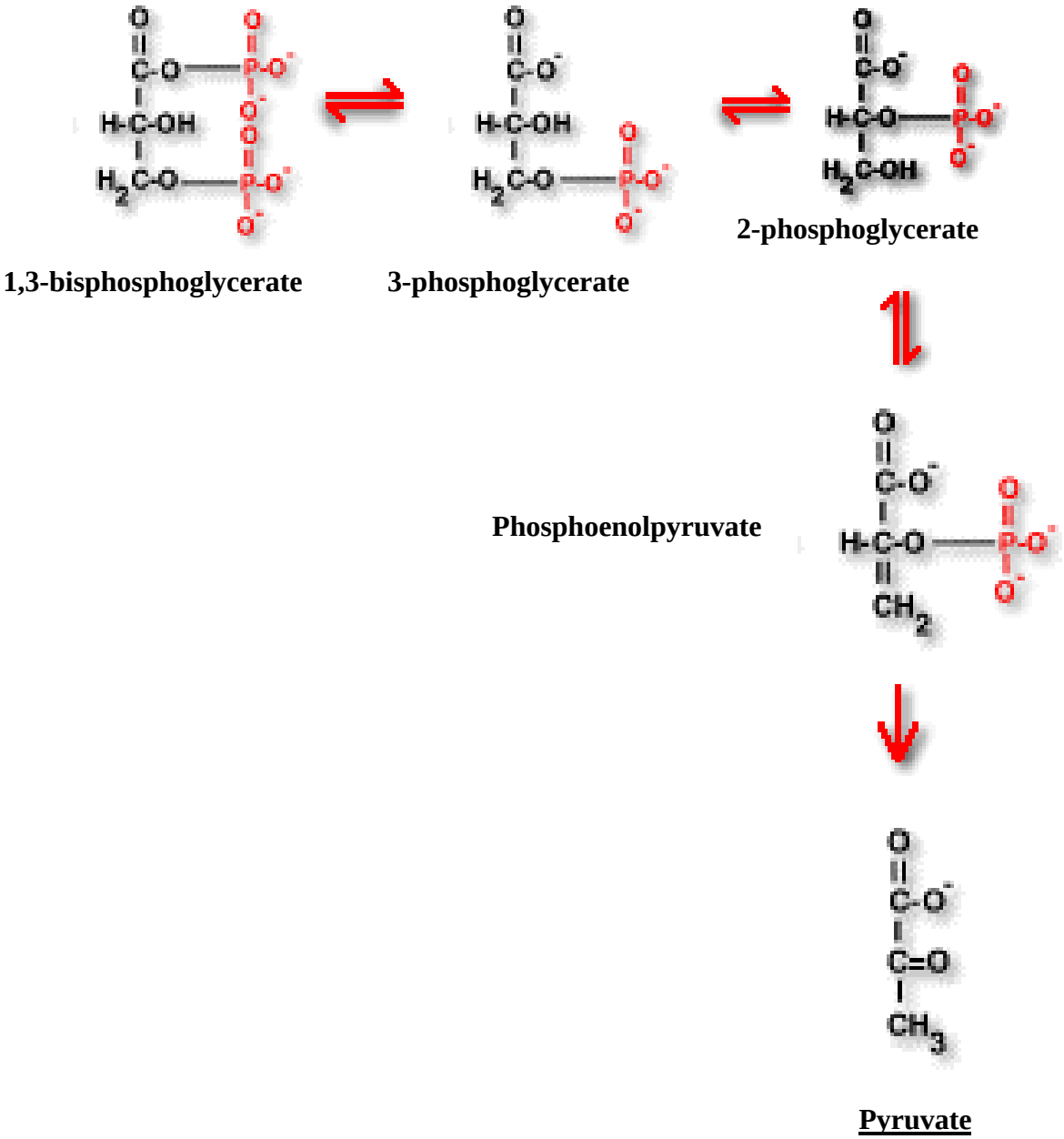


Figure 1. Continued.

Complete and Branched Forms of Tricarboxylic Acid Cycle (TCA)

The TCA cycle, also known as the Krebs cycle, is presented in Figure 2, and it works as a machinery that generates reducing equivalents for different purposes and intermediates for biosynthetic routes such as amino acid production. It was shown to be present in mammals, yeast and a large number of bacterial species (Voet and Voet, 1995). The reactions start with the condensation of acetyl-CoA and oxaloacetate to form a C6 compound, citrate, which will then be taken to oxaloacetate in a cyclic manner to keep the machinery going. According to Moat and Foster (1995) malate is at a pivotal point of the cycle, since it can participate in several reaction such as its oxidation to oxaloacetate via malate dehydrogenase (MDH), and being decarboxylated to pyruvate by malic enzyme for subsequent pyruvate carboxylation to oxaloacetate for reclaiming CO_2. This latter metabolism is used by a few organisms that lack MDH.

α-Ketoglutarate dehydrogenase (α-KGDH) enzyme complex consisting of three enzyme components and succinate dehydrogenase (SDH) are the key points of the TCA cycle, as their activities are strongly controlled by the NAD^+/NADH levels in the cell. Based on studies performed on individual organisms, it has been thought that the membrane bound SDH is inoperative under the absence of oxygen, FADH generated during the SDH activity could not be re-oxidized back to FAD^+ without a terminal electron acceptor. Furthermore, taking *Escherichia coli* as a model organism, Moat and Foster (1995) state that a large number of enzymes that are active under aerobic conditions are repressed under anaerobic conditions. However, for the case of the microbial community operating in a BPR system where the sludge is recycled between anaerobic and aerobic conditions above statements may not be valid. Especially due to the rapid regeneration of NAD^+ pool through utilization of NADH for production of PHA units, the repression of the TCA enzymes may not be as strong. One other option, again based on *E. coli* metabolism is the activation of the branched TCA operation. Rather then operating in a cyclic manner, TCA cycle separates into two branches, one of which is reductive (OAA to succinyl-CoA) and the other is oxidative (citrate to α-ketoglutarate).

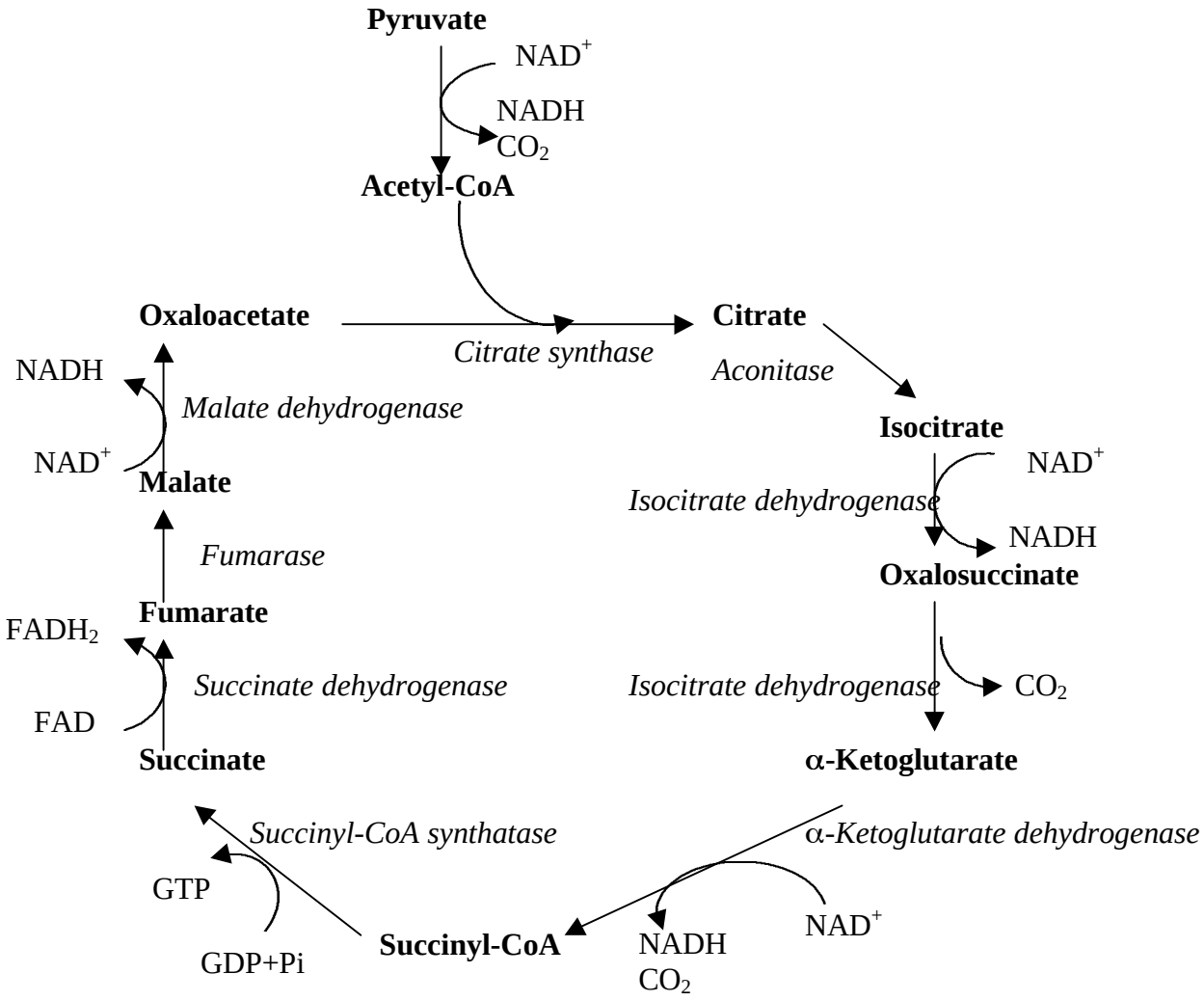


Figure 2. Tricarboxylic acid cycle (Voet and Voet, 1995).

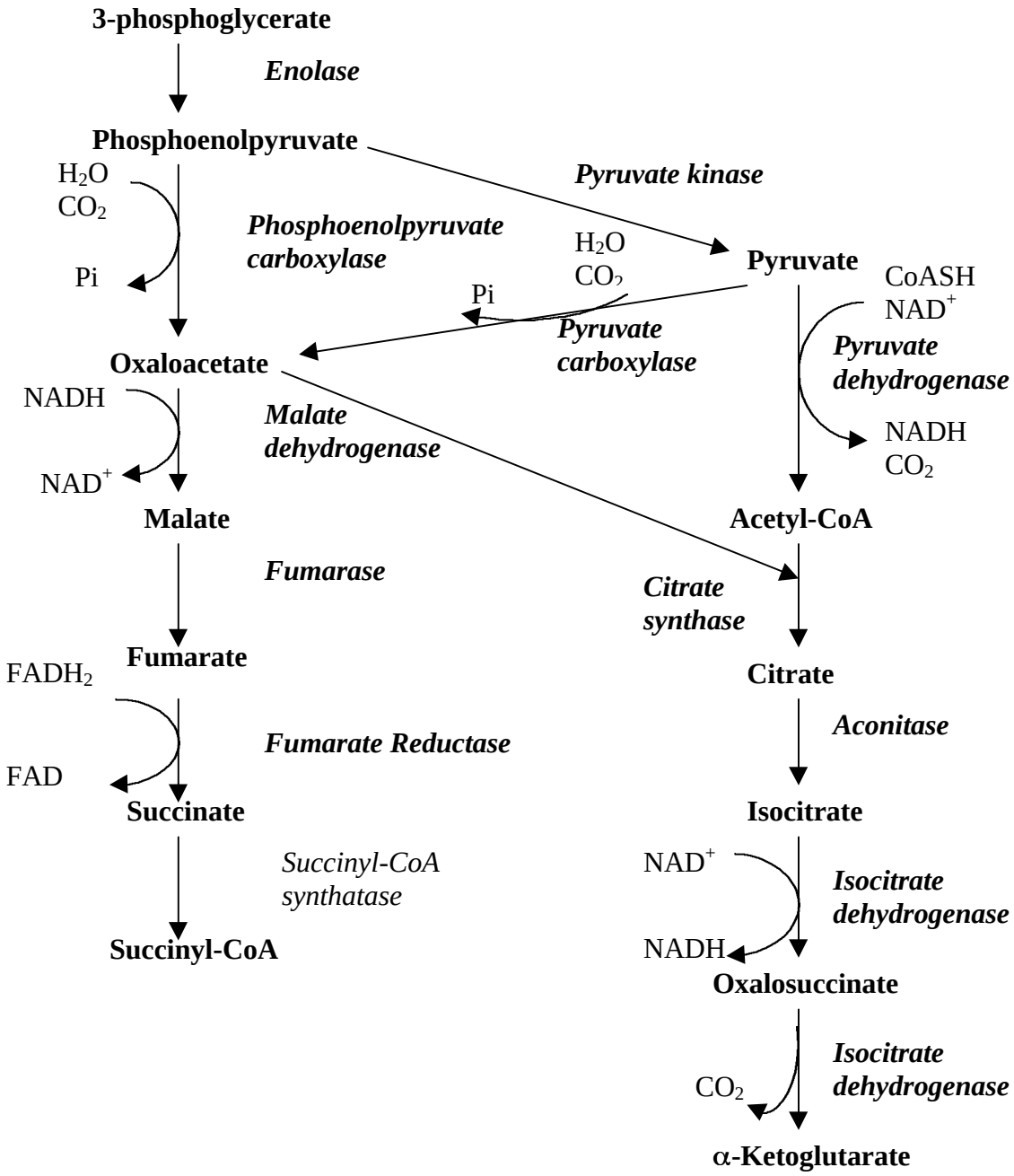


Figure 3. Branched TCA cycle (White, 1995)

The TCA cycle is at the center of the cellular metabolism due to its amphibolic nature. Thus it carries out a number of crucial functions in the cell such as gluconeogenesis, lipid biosynthesis, amino acid biosynthesis, and porphyrin biosynthesis (Voet and Voet, 1995). However, for the cyclic activity of the TCA cycle to function unceasingly, the cycle intermediates must also be replenished. Main replenishment point of the intermediates is at oxaloacetate, which can be produced through the action of pyruvate carboxylase. Besides this, oxidation of odd-chain fatty acids leading to the production of succinyl-CoA, breakdown of amino acids (Table 1), transamination and deamination of amino acids leading to the production of α-ketoglutarate and oxaloacetate also keep the TCA cycle going.

Table 1. Interactions between the TCA cycle intermediates and the amino acids that can be used for their replenishment (Voet and Voet, 1995)

Intermediary compound	**Related Amino Acids**
Pyruvate	Alanine, Cysteine, Glycine, Serine, Threonine, Tryptophan
Acetyl-CoA	Isoleucine, Leucine, Threonine
Acetyl-CoA via acetoacetate	Leucine, Lysine, Phenylalanine, Tryptophan, Tyrosine
α-Ketoglutarate	Arginine, Glutamate, Glutamine, Histidine, Proline
Succinyl-CoA	Isoleucine, Methionine, Valine
Fumarate	Aspartate, Phenylalanine, Tyrosine
Oxaloacetate	Aspargine, Aspartate Via pyruvate carboxylase To glucose synthesis

Glyoxylate Shunt

As presented in Figure 4, glyoxylate shunt is a means to bypass the CO_2 generating reactions of the TCA cycle. Bacteria and some species of higher plants express the enzymes of this bypass. This allows the conversion of acetyl-CoA to result in net increase in malate or oxaloacetate, which is not possible with the TCA cycle alone. Two acetyl-CoA are input per cycle with no loss of CO_2, making the net synthesis of a C4 product possible. The two additional enzymes of the glyoxylate cycle are isocitrate lyase and malate synthase. Isocitrate lyase splits isocitrate into glyoxylate and succinate. The reaction is not particularly favorable (G = +8.7 kJ/mol), so the concentration of glyoxylate product will be low. The malate synthase reaction closely parallels the citrate synthase reaction, incorporating a second acetyl CoA to produce another malate independently of the succinate produced by isocitrate lyase. The reaction is energetically very favorable (G = -48.8 kJ/mol), and proceeds even with low concentrations of glyoxylate. For each oxaloacetate consumed by citrate synthase, two malates can be produced. One goes on to replace the oxaloacetate originally used, and the other represents a net gain that can be made available for gluconeogenesis, or simply to boost the rate of citrate synthesis

Succinate-Propionate Pathway

Propionate production as a result of fermentation reaction of this pathway is presented in Figure 5 (Moat and Foster, 1995). *Propionibacterium* is one of the organisms known to utilize the succinate-propionate pathway. Notice that the reactions going from pyruvate to succinyl-CoA are identical to the reductive reactions of the branched TCA cycle, also utilizing the same enzymes. Thus, it would not be surprising to discover that a combined metabolism of branched TCA and succinate-propionate pathways is operative in EBPR sludges. This would require the reversed actions of the TCA cycle enzymes, and fumarate reductase must also be present. Thus, when *Propionibacterium* converts

pyruvate or phophoenolpyruvate to succinate, it can benefit from three factors: 1) use of fumarate as an electron sink to oxidize NADH; 2) membrane-bound fumarate reductase serving as a coupling site (generating Δp through electron transfer to a quinone which then can be used for ATP generation, solute transport, or for sparing an ATP that would be used to maintain Δp); 3) succinate can be taken to propionyl-CoA or be used for biosynthesis of amino acids and other anabolic precursors (White, 1995).

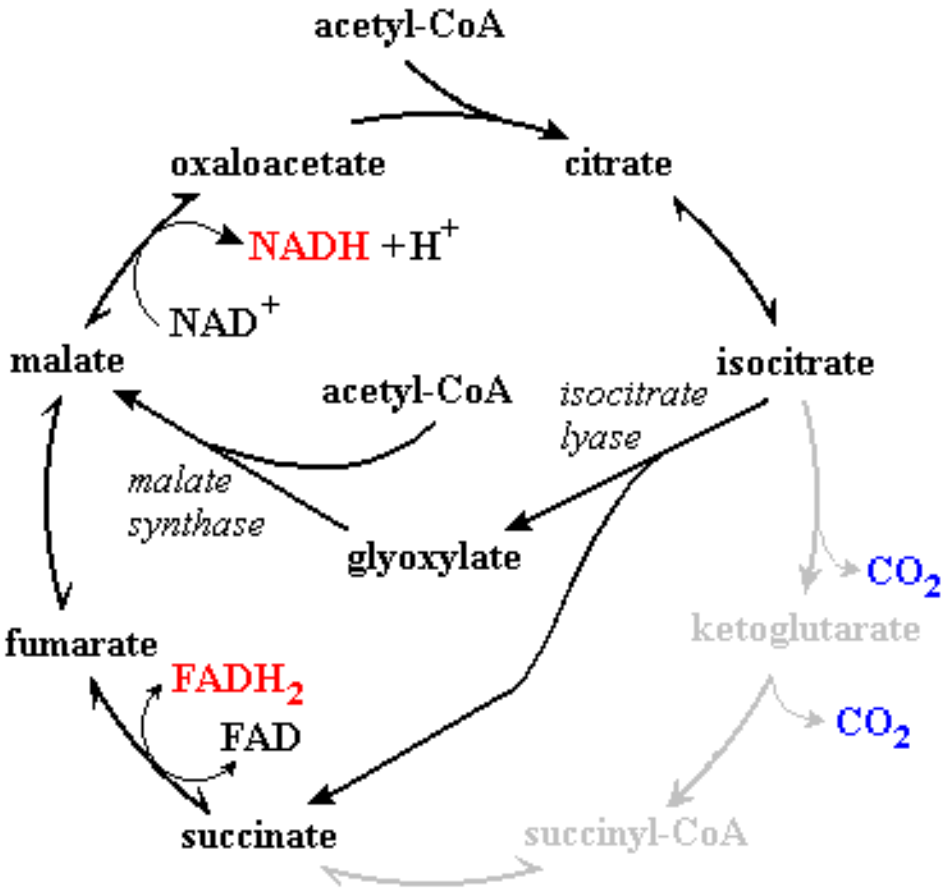


Figure 4. Glyoxylate shunt (Moat and Foster, 1995).

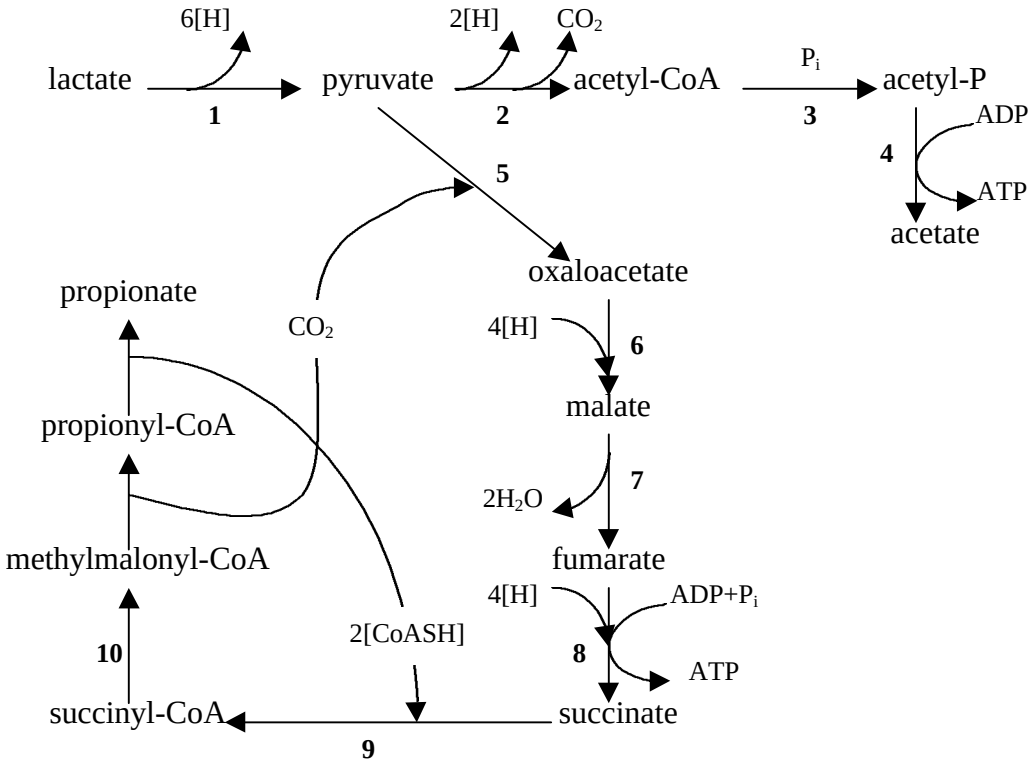


Figure 5. Succinate-Propionate Pathway. (1) lactate dehydrogenase; (2) pyruvate dehydrogenase; (3) phosphotransacetylase; (4) acetate kinase; (5) methylmalonyl-CoA transcarboxylase; (6) malate dehydrogenase; (7) fumarase; (8) fumarate reductase; (9) CoA transferase; (10) methylmalonyl-CoA racemase. (Moat and Foster, 1995)

One interesting point made in Moat and Foster (1995) is related to the participation of poly-P and PPi in phosphorylation reactions. The mass balance for Figure 5 is

3glucose + 4PPi + 11ADP → 4propionate + 2acetate + $2CO_2$ + 11ATP + 4Pi

Direct utilization of the energy present in poly-P and PPi instead of its waste during hydrolysis explains the high cell yields observed in *Propionibacteria*. This may have also apply to EBPR metabolism, as poly-P is abundant in sludge. Such energy saving mechanisms may be providing poly-P removing population with tools that will give them advantages over other heterotrophs.

Figure 6 shows the route for propionyl-CoA production under circumstances where the whole succinate-propionate pathway does not operate, but succinyl-CoA is available. According to Bermudez *et al*. (1998) *Saccharopolyspora erythraea* CA340 utilizes the shown steps for production of erythromycin, and the succinyl-CoA is driven from the TCA cycle. Propionyl-CoA, also replenished from breakdown of odd-numbered fatty acids or amino acids Methionine, Threonine and Valine serves as the prerequisite of erythromycin.

Glycogen as a Storage Product

Glycogen (Figure 7) is one of the major storage carbohydrates ubiquitous in prokaryotes, eukaryotes and mammals. It serves numerous purposes in microorganisms and animals, the major one being its function as carbon and energy reserve. Through the opposition of the breakdown and the synthesis processes undertaken by two different enzymes, i.e. glycogen phosphorylase and glycogen synthetase, respectively, the metabolism is driven in the direction of either production or degradation of glycogen stores (Voet and Voet, 1995).

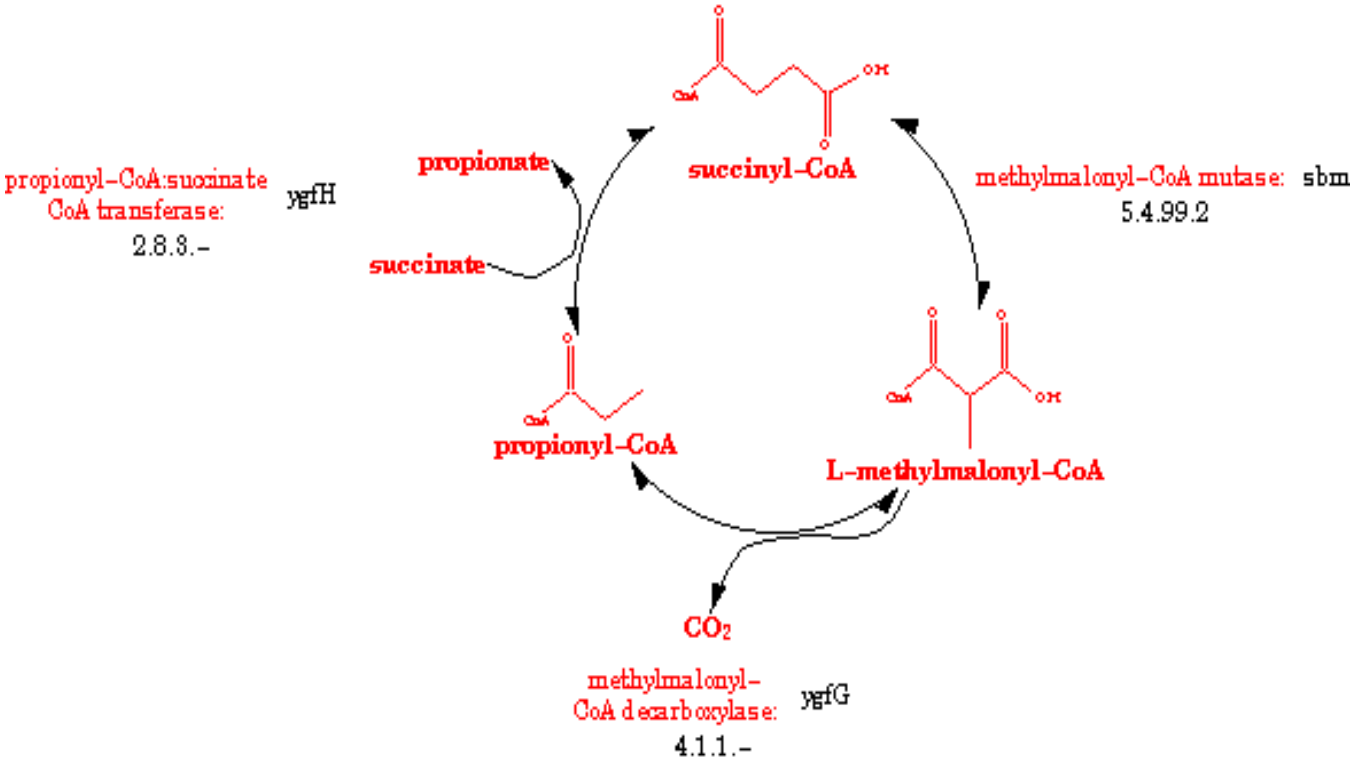


Figure 6. Propionyl-CoA production from succinyl-CoA via methylmalonyl-CoA (Bermudez *et al*., 1998).

Besides its use as an energy or carbon storage product in mammals and plants, over fifty bacterial species are also known to store a glycogen-type product or contain the enzymes involved in the biosynthesis of glycogen. It has been shown that its accumulation occurs following a limitation on growth and in the presence of an excess source of carbon. The growth limitations can include the absence of nitrogen, sulfur or phosphate, or presence of suboptimal pH conditions (Preiss *et al*., 1983). In addition to its role as an energy source for growth, it is also known to provide energy of maintenance under non-growing conditions. Energy of maintenance includes the energy needed for RNA and protein turnover, maintenance of mobility, osmotic regulation, intracellular pH and chemotrophic

response. Thus, the organisms with the ability to store and utilize glycogen reserves can survive better than the ones that lack this ability.

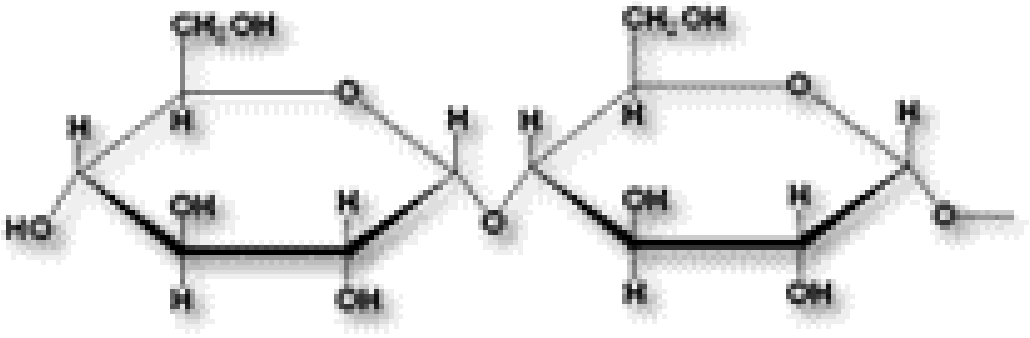


Figure 7. Molecular formula of glycogen (Voet and Voet, 1995)

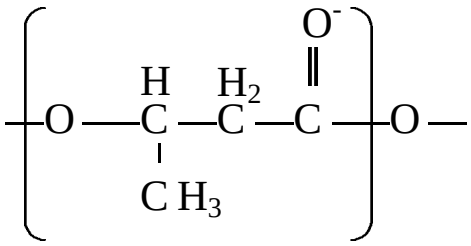
PHAs as Carbon and Energy Storage Product

Polyhydroxyalkanoate polymers are produced by a variety of microorganisms mainly to serve as carbon and energy storage under different stress conditions. Among such conditions, especially the lack of nutritional elements those are essential for growth (i.e. carbon, nitrogen, sulfur, phosphorus or oxygen) alter the normal operation of cellular metabolism. Thus, accumulation of storage products is one of the survival tactics employed by the stressed cells. Phosphorus storage in the form of poly-P, and carbon and energy storage in the form of PHA polymers and glycogen are essential for survival, and since these products are stored as granules with low solubility in water, they do not impact the osmotic balance in the cell (Lee and Choi, 1999). PHA storage also occurs when the external supply of the substrate exceeds the immediate requirements of the cells. These stores can be utilized for growth when the specific limitation causing the

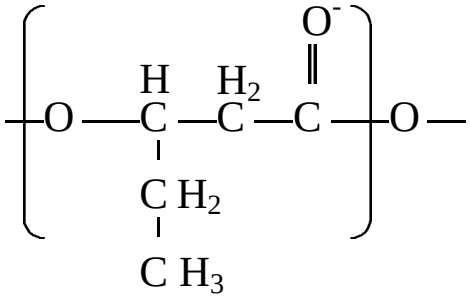
storage is removed, or when the external substrate is not sufficient for growth and maintenance.

Figure 8 illustrates the structures of PHAs (Satoh *et al*., 1992). Lee and Choi (1999) group PHAs into two: short-chain-length (SCL-PHAs) consisting of three to five carbons (e.g. hydroxybutyrate and hydroxyvalerate), and medium-chain-length (MCL-PHAs) consisting of six to fourteen carbons (e.g. hydroxyhexanoate, hydroxyoctanoate and hydroxydecanoate). They summarize the biosynthetic pathways for PHA production that are active in microorganisms with four examples. The pathway found in *Ralstonia eutropha* (formerly named as *Alcaligenes eutrophus*) is representative of most of the short chain length PHAs. Acetyl-CoA is converted to poly-3HB in three steps: -ketothiolase catalyzes the formation of a carbon-carbon bond through condensation of two acetyl-CoA units. Reduction of the acetoacetyl-CoA units to 3-hydroxybutryl-CoA is achieved by NADPH-dependent acetoacetyl-CoA reductase. The last step catalyzed by PHA synthase is linking of the 3-hydroxybutryl-CoA units to the growing poly-3HB chain. Pseudomonads like *Pseudomonas oleovorans*, *Pseudomonas aeruginosa* and *Pseudomonas putida* produce medium chain length PHAs (e.g. 3-hydroxyoctanoate, 3-hydroxyhexanoate and 3-hydroxydecanoate) by incorporating intermediates from -oxidation pathways and PHA precursors from de novo fatty acid synthesis pathways.

Going through the published research dealing with PHA synthesis by numerous microbial species under different conditions reveals that PHA synthesis and regulation take place in relation to a number of constraints. Working with *R. eutropha*, Oeding and Schlegel (1973) and Lee *et al*. (1995) showed that under aerobic conditions and absence of nitrogen, NAD(P)H/$NAD(P)^+$ ratio increases, inhibiting citrate synthase and isocitrate dehydrogenase enzymes of the TCA cycle. Consequently, acetyl-CoA is forced to be converted to acetoacetyl-CoA by -ketothiolase. Other research revealed that P and O limitations also lead to significant PHA storage in different formes depending on the redox state of the cells (Senior and Dawes, 1973; Jackson and Dawes, 1976; Brandl *et al*., 1988; Page and Knosp, 1989; and Anderson and Dawes, 1990; Lee and Chang, 1995; Steinbuchel and Valentin, 1995).



(a)



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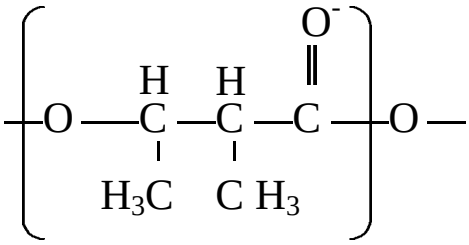

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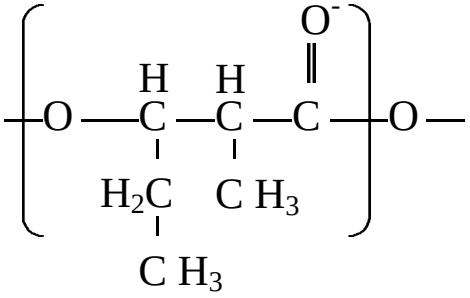

(d)

Figure 8. Molecular formulae of PHA units: (a) hydroxybutyrate; (b) hydroxyvalerate; (c) hydroxymethylbutyrate; (d) hydroxymethylvalerate (Lee and Choi, 1999).

Punrattanasin (2001) also gives a detailed summary of the microbial species that have been determined to store PHA under different growth conditions and a number of metabolic pathways leading to PHA synthesis by these species. Going from isolated microbial species to activated sludge, PHAs accumulate in the anaerobic zones of an EBPR system in response to the presence of substrate in excess of metabolic requirements.

Enhanced Biological Phosphorus Removal and the Role of Storage Products

Biological phosphorus removal is one of the intensively researched topics of the environmental engineering area, and related research goes far back to when Levine and Shapiro (1965) demonstrated that an activated sludge sample could store phosphate under aerobic conditions accompanied by an increase of phosphate (Pi) concentration under low dissolved oxygen levels. Since 1965, different aspects of enhanced biological phosphorus removal (EBPR) have been uncovered; however, there is still an ongoing debate in regards to the prevailing biochemical mechanisms and active microbial populations of such systems.

Wentzel *et al.* (1986) gives a very detailed history of EBPR research starting with Fuhs and Chen (1975) which emphasizes *Acinetobacter* spp. as the major group of organisms mediating the phosphorus removal through accumulation of poly-3-hydroxybutyrate (3HB) from short chain volatile fatty acids (SCVFA). Barnard (1976) added to Fuhs and Chen (1975) research by pointing out that presence of an anaerobic zone followed by an aerobic one is essential for biological phosphorus removal (BPR). Nicholls and Osborn (1979), Rensink (1981) and Marais et al (1983) and Wentzel *et al*. (1985) proposed biochemical explanations of the observed patterns in phosphorus removing sludges. The results of the preceding research lead to the development of the first conceptual biochemical model describing BPR: Comeau *et al.* (1986) proposed that TCA cycle was the source of the reducing equivalents necessary to reduce acetyl-CoA to PHB. They also

suggested that acetate was taken up in its undissociated form (HAc) dissipating the proton motive force (pmf) across the cell membrane, and that the cells had to consume energy from their polyphosphate (poly-P) reserves to maintain a desired pmf by expelling protons. Cations (K^+, Mg^{++}, and Ca^{++}) were shown to be expelled with each Pi at a molar ratio of 0.27, 0.28, and 0.02 mole cation per mole Pi released, respectively. Wentzel *et al.* (1986) performed analysis of the EBPR phenomena based on physiological capabilities of *Acinetobacter* spp. and took the Comeau model a step forward by adding a qualitative dimension to it which was lacking in the original model.

Lotter and Dubery (1987) supported the Comeau-Wentzel model for EBPR through an enzymalogical study exploring the metabolic control mechanisms in poly-P bacteria. Like Wentzel *et al*. (1986) they also based their study on *Acinetobacter* spp. activity. They concluded that metabolic regulation was mainly by feedback control of active enzymes. In other words, the levels of NAD/NADH and AMP/ATP, and acetyl-CoA control 3HB synthesis and degradation under anaerobic and aerobic conditions, respectively.

Comeau *et al*. (1987) investigated the effects of different substrates (acetate, propionate, formate, butyrate, valerate, lactate fermented primary sludge) on EBPR and storage polymers, namely PHAs consisting of 3HB and poly-3-hydroxyvalerate (3HV). Possible glycogen formation for storage purposes was disregarded based on an argument that fermentative reactions would be more favorable compared to glycogen formation and that the bulk solution needed to contain a high concentration of sugars for the glycogen storage to occur, with or without PHA synthesis. For this reason they monitored 3HB and 3HV, and observed that each substrate brought about a different amount of PHA storage with varying HV to HB ratios. Mino *et al*. (1987) studied the effect of poly-P accumulation on acetate metabolism using a sludge acclimated to a feed containing acetate, glucose, and propionate at a range of P/C (mg P/mg C) ratios (0.028, 0.036, 0.064, and 0.084). Also monitoring total carbohydrate (TCH) besides 3HB and Pi, they observed that TCH was consumed in the anaerobic phase and resynthesized in the aerobic phase. Hence, they concluded that the intracellular carbohydrate consumption, reflected

in the decrease in TCH levels supplies carbon for 3HB synthesis. Besides these observations, batch tests with sludge containing higher phosphate (Px) exhibited higher Pi release and acetate uptake, accompanied with higher 3HB storage and TCH consumption. Based on these observations and the widely accepted point that the TCA cycle did not work under anaerobic conditions, they postulated that intracellular glycogen (measured as a part of TCH) served as the source of reducing equivalents and supplemental carbon required for PHA synthesis, and that low Px sludges had a different mechanism for energy production other than poly-P degradation.

Wentzel *et al*. (1991) evaluated the existing biochemical models and suggested a new one called an "Adapted Mino Model". They proposed that the glucose degradation following glycogen breakdown proceeded through the Entner-Doudoroff (ED) pathway, rather than the Embden-Meyerhoff-Parnas (EMP) pathway as originally suggested by Mino and his co-workers. This modification was based on the Juni (1978) research reporting that EMP pathway did not operate in *Acinetobacter* spp. These three initial models are given in Figure 9.

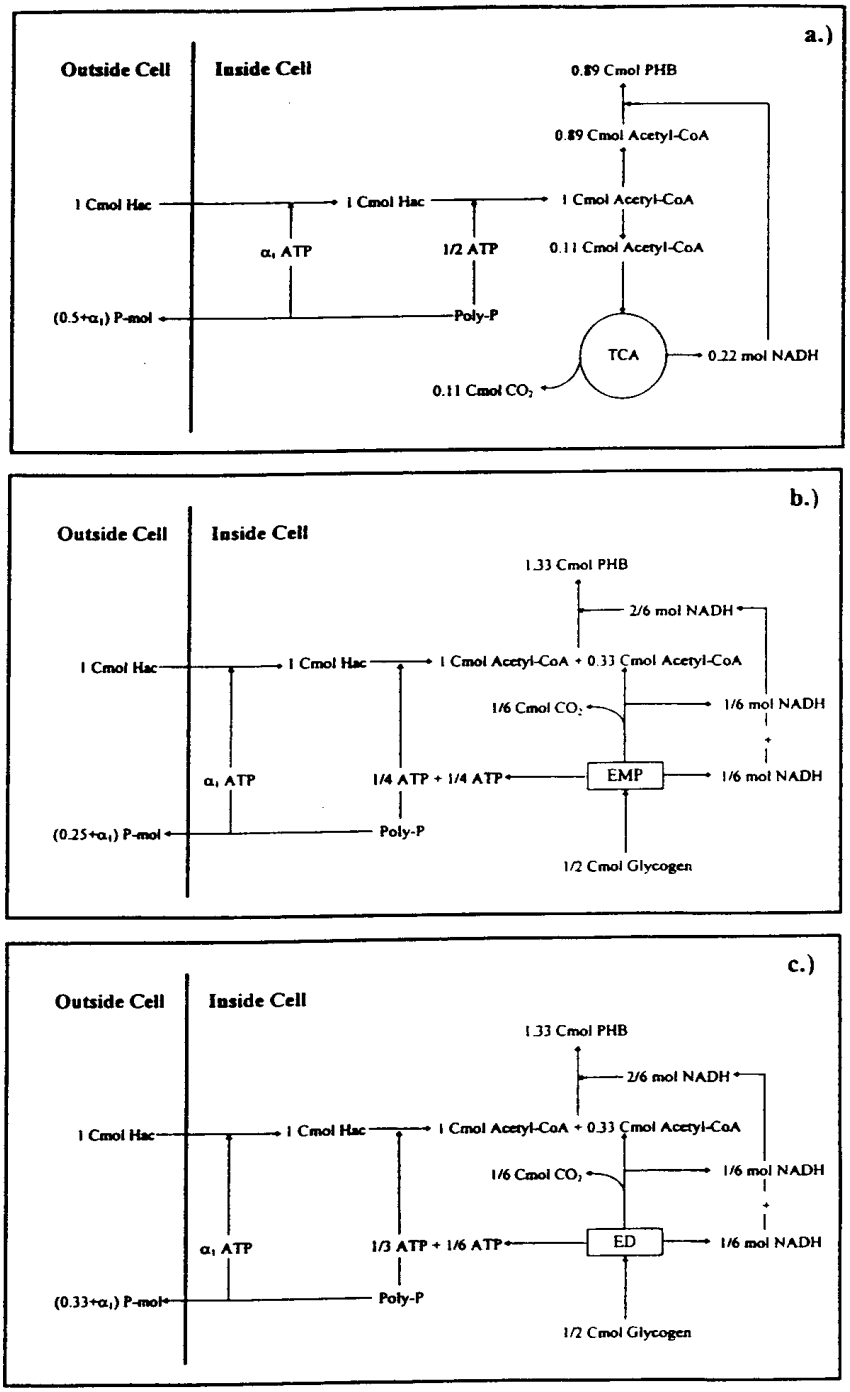

Figure 9. Schematic representation of the three biochemical models describing the carbon flow in EBPR sludge: (a) Comeau Model; (b) Mino Model; (c) Adapted Mino Model (Filipe and Daigger, 1998).

Analysis of the Research That Followed the Establishment of Initial Models

The bulk of the research efforts that followed the proposal of the above presented models aimed at explaining the underlying interactions between the transformations which the substrate and the phosphate go through in the anaerobic zones of EBPR systems. Different researchers operated systems, either continuous or SBR with different influent substrate and phosphorus values. The common parameters monitored by different procedures in these studies were the sludge PHA content and its composition, Px, Pi release and uptake, TCH or glycogen degradation and synthesis, and SCFA uptake. Table 2 gives a summary of the operating conditions imposed on the EBPR systems under study and the corresponding results obtained. Only a portion of the extensive EBPR research will be presented here. However, the studies that will be presented are selected to represent similar research performed by different research groups.

Table 2. Summary of the operating conditions imposed on the EBPR systems under study and the corresponding results. (P-release, PHA production and glycogen consumption are expressed per unit of acetate taken up.)

Author (Yr)	Feed	%P	pH	Temp	HV/HB	System	P-Release	PHA Prod.	Glyc Cons.
Liu et al.(1994)		<2.0				Cont.	0	-	1.08 - 1.47
Mino et al. (1987)		3.3				Cont.	0.145	0.48	0.99
Comeau et al. (1987)		4.1				Cont.	0.70 - 0.80	1.26 - 1.40	-
Arun et al. (1988)		4.3-4.5				Cont.	0.21 - 0.39	0.32 - 0.74	0.06 - 0.48
Mino et al. (1987)		6.5				Cont.	0.39	0.55	1.02
Satoh et al. (1992)		6.3-7.1				Cont.	0.44	1.36	0.6
Smolders et al. (1994)	Acetate	7.0-7.5	5.5-8.5			SBR	0.26 - 0.76	1.26 - 1.36	-
Pereira et al. (1996)		2.0-3.0	7	25	0.35	Batch	0.16	1.48	0.7
Christensson et al. (1998)		9.5	7	20	0.18		0.5	1.28	0.64
Lie et al. (1997)			7				0.49	1.36	
Louie et al. (2000)	Domestic WW					Batch		1.8	
Romanski et al. (1997)	Acetate	0.16				Batch	0.85		
"	Acetate	0.125				Batch	0.66		
"	Acetate	0.083				Batch	0.34		
Liu et al.(1997)		1.5	7.0-8.0			Batch	0.022	2	1.39
		5	7.0-8.0			Batch	0.28	1.7	0.9
		6	7.0-8.0			Batch	0.45	1.49	0.77
		8	7.0-8.0			Batch	0.47	1.47	0.65
		9.1	7.0-8.0			Batch	0.54	1.19	0.4
		10	7.0-8.0			Batch	0.51	1.41	0.58
		11	7.0-8.0			Batch	0.61		0.62
		12.1	7.0-8.0			Batch	0.66	1.37	0.42
Schuler and Jenkins (2000)	Acetate	2-36	7.15-7.25	23.5	~0.20	Batch	0.10 - 0.70	1.6 - 0.80	1.0 - 0.35
Kisoglu et al. (2000)	Acetate		7.0-8.0	20		Cont.	0.81	1.06	0.43
	Acetate		7.0-8.0	20		Cont.	0.93	0.95	0.36
	Acetate		7.0-8.0	20		Cont.	0.8	0.83	0.31
Comeau (1987)								0.89	0
Mino (1987)								1.33	0.5
Adapted Mino (1991)								1.33	0.5

Impact of Different Substrates on EBPR Performance:

In order to resolve the biochemistry of EBPR mechanisms and uncover the pathways leading to different storage products with different carbon sources, a substantial amount of research has been focused on EBPR response achieved with different organic compounds. Some of these studies dealt with the argument made in Comeau *et al.* (1987) regarding the production of 3HB from substrates with an even number of carbon atoms and the production of 3HV from substrates with odd number of carbon atoms. Somiya *et al.* (1988) ran batch tests on an anaerobic-aerobic sequencing batch reactor (SBR) sludge using glucose and acetate as the carbon source. They showed that there is a linear relationship between Pi release and dissolved TOC uptake, which is well documented today. The results of the batch tests showed different storage patterns for glucose and acetate fed sludge. With glucose they mainly had carbohydrate storage and some 3HB accumulation. However, with acetate major storage product was 3HB and carbohydrates were utilized in contrast to what was observed with glucose feed, and it was accompanied with more Pi release per TOC removed. They also observed aerobic 3HB storage when bulk solution contained leftover substrate from the anaerobic period. Abu-ghararah and Randall (1991) studied the response of poly-P bacteria to various SCVFAs present in fermented domestic wastewater (i.e. formate, butyrate, isobutyrate, acetate, propionate, valerate, and isovalerate). They found a good increasing trend between P-release and P-uptake considering the test results obtained with each SCVFA, formate giving the smallest ratio and acetate yielding the largest value, with a very strong correlation (r^2=0.99) and a slope value of 1.22. They also reported that the Comeau-Wentzel model did not accurately predict P release and uptake.

Satoh *et al.* (1992) conducted anaerobic batch tests on sludge taken from a continuous system, to observe the impact of acetate, propionate and lactate feeding on P release and uptake patterns. They identified the presence of 3HB, 3HV and 3-hydroxy-2-methylbutyrate (3H2MB) and 3-hydroxy-2-methylvalerate (3H2MV) by ^{13}C NMR and GC-MS analyses. They postulated a pathway that explained the carbohydrate patterns observed in EBPR research, considering glycogen as a reserve, which supplied both

energy, and reducing power for anaerobic metabolism. The glycogen model presented in Satoh *et al.* (1992), however, is constructed without the inclusion of fed acetate in the reactions leading to PHA storage from glycogen. Net reducing power production from the glycogen model was zero. Thus, the reactions that couple the uptake of carbon substrate (acetate, propionate, etc.) and its conversion to PHAs, which would require additional reducing power, were not included in the mass balance calculations. Although they would change all the reducing equivalent and ATP/ADP balance presented in, they were assumed to be taking place as additional reactions leading to additional glycogen consumption to satisfy the reducing power requirement, also producing energy at the same time. A more established model is presented in Satoh *et al.* (1993). Nonetheless, while attempting to explain possible role of glycogen in phosphorus removal, their results lead them to propose the presence of glycogen dependent organisms that can survive without poly-P stores. The results of this study were considered as major steps towards the distinction between the phosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), although the study did not provide any enzymatic proofs to support the activity of the proposed pathways. GAO's ability to store and utilize glycogen as an energy source makes them competitors of poly-P storing organisms, and based on these they were thought to be the cause of deterioration in EBPR performance observed in a number of cases (Cech and Hartman, 1990; Cech and Hartman, 1993).

As a continuation to Satoh *et al.* (1992), Matsuo *et al.* (1992) presented possible pathways leading to 3HB, 3HV, 3H2MB and 3H2MV storage from acetate, propionate, and glucose. A number of possible reactions were presented and then disregarded based on the reasoning that reducing power accumulation would occur and, as a result, the metabolic activity would cease. The authors thought that this way of reasoning and isolating the reactions, although it simplifies the model construction, might cause some of the pathways to be overlooked. Hitherto, these were the most comprehensive biochemical explanations provided based on experimental work.

Working on the impact of glucose and starch on EBPR metabolism, Randall *et al*. (1994) tested system response to numerous carboxylic acids via batch tests. Besides also observing the deterioration of phosphorus removal with glucose, they determined that uptake of different substrates did not effect the location (cytosol, cell wall, cellular membrane and periplasmic space) and form of the phosphorus storage, and poly-P degradation occurred through zero-order kinetics at all locations once aeration was stopped. Hill *et al*. (1989) also determined the presence of immobilized poly-P outside the cytoplasmic membrane complexed with metal cations through ^{31}P-NMR studies. Both studies and Randall (1993) implied that poly-P degradation was mediated by membrane bound enzymes. Regarding the enzymes responsible for poly-P degradation, Ohtake *et al*. (1994) ran genetic studies on *Klebsiella aerogenes* and *Escherichia coli* with P storage. Through genetic manipulations with four different recombinant plasmids carrying poly-P kinase (*ppk)* (PPK enzyme is responsible from poly-P synthesis and it polymerizes the terminal phosphate of ATP into poly-P in a reversible reaction.), acetate kinase (*ack*) and phosphate-specific transport (*pst*) genes, they managed to obtain a *K. aerogenes* strain with P accumulation capabilities. Under phosphorus limited conditions they observed different responses from *K. aerogenes* and *E. coli,* the former one storing far more P then the latter although both contained a PPK enzyme with 93% similarity in amino acid residues. They have also found that transport mechanisms might be limiting *E. coli's* phosphate uptake. Manipulating *E. coli's pst* and *ppk* genes, they have achieved a P storage of up to 16 % of dry cell weight. These results show that recombinant gene technology can be a useful tool to investigate certain aspects of EBPR, and these findings should have implications in model development.

In the light of these, the best way to investigate the anaerobic metabolism of phosphorus removal appears to be considering paths that can keep the microbial activity going by balancing the NAD^+/NADH and ADP/ATP ratios, major regulators of most biochemical activity (Voet and Voet, 1995). Hence, working with different substrates helped develop an understanding of the overall mechanism of EBPR in that determination of possible reactions to balance reducing power production besides the need for energy for phosphate accumulating and non-phosphate accumulating populations has become important.

Consequent implication of this is that the storage of PHAs and glycogen is the most efficient way to achieve the required balance. The varying composition of PHAs, and storage and consumption patterns of glycogen observed with different substrates are merely the consequences of the fulfillment of this requirement by different groups of organisms.

Competing microbial groups in EBPR systems:

The issue of the competition between the PAOs and GAOs became an integral part of the research on EBPR performance with different organic substrates. This competition was investigated by Satoh *et al*. (1993) on biomass (1.5-2.0 %P; 7%VSS) fed either with acetate or propionate. They did not observe any phosphorus release although PHA storage and carbohydrate degradation occurred under acetate and propionate feed. Major storage products were 3HB and 3HV with acetate feed, and 3HV and 3H2MV with propionate. Carucci *et al*. (1995) performed a similar study with sludge acclimated to glucose, and they had periods of good EBPR and periods of no EBPR at all. According to the batch test results, neither glucose nor acetate feed yielded any difference in PHB and glycogen patterns for these periods.

These contradictory results obtained in a number of other studies led the researchers to try to manipulate the population dynamics within EBPR sludge. Hence, they tried to manipulate the ratio of PAOs to GAOs by changing the feed phosphorus to carbon (P/C) ratio, or in other words the feed chemical oxygen demand (COD) to total phosphorus (TP) (COD/TP) ratio. This concept was inherently present in earlier studies in the form of biomass phosphorus content (Px; %P in dry solids). For a biomass to have a high phosphorus content, the feed phosphate concentration must be sufficiently high and thus PAOs must dominate (Punrattanasin and Randall, 1998). In order to study the competition between PAOs and GAOs, Liu *et al*. (1996) cultivated a biomass with hindered EBPR by using a feed P/TOC ratio of 2/100. They achieved peculiar results with the series of batch tests run with numerous carbon substrates. Mainly with glucose

and sucrose, glycogen accumulation in the anaerobic period was observed, with significantly high 3HV production (87 and 90 %, respectively). However, feeding acetate and glucose simultaneously resulted in significant glycogen degradation but it was less then the amount observed with acetate only. They did not observe any Neisser positive cells (i.e. poly-P storing organisms).

More recent research dealt with a possible concerted action of the TCA cycle and the glycogen degradation to provide the required reducing equivalents necessary for the synthesis of PHA. Based on an *in vivo* NMR study performed with labeled acetate, Pereira and her co-workers (1996) suggested that the TCA cycle might be operating during the anaerobic stage of phosphorus removal. Their deduction about the TCA cycle was based on the detection of labeled bicarbonate under anaerobic conditions that was reasoned to have been produced in the TCA cycle. Their suggested model presented in Figure 10 assumes the partitioning of carbons between the TCA (30% contribution) cycle and PHB/HV production. Thus, succinate must be taking part both in the TCA cycle and in the production of the HV units which necessitates a concerted action between succinate dehydrogenase and succinate thiokinase. The phosphorus content of their sludge was 2 to 3 %, and the HV fraction of their harvested PHB/HV co-polymer was 26%. The low %P and high %HV numbers indicate that their sludge was not significantly enriched by a poly-P storing community. This may be one of the reasons why their quantitative data did not fit any one of the two major models. Thus, the fraction of the P-removers in the sludge determines the acetate-PHA-glycogen balance that is connected to the P-release and P-uptake patterns observed with a specific sludge. In a similar study, Maurer *et al*. (1997) used solid state NMR as a tool to study the carbon flow in a functioning EBPR biomass fed with domestic sewage. They further confirmed the interaction between the substrate, PHA and glycogen. However, they do not comment on the anaerobic operation of the TCA cycle, and they suggest that the glycogen breakdown proceeds through ED pathway. Louie *et al*. (2000) add another dimension to the debate by suggesting that the glyoxylate pathway is active under anaerobic conditions to provide reducing equivalents and to maintain a stable NAD^+/NADH balance (Figure 11). They also suggest that “unknown” endogenous

nutrients otherwise unavailable for PHA synthesis are utilized especially yielding the HV units on the co-polymer. They mention glycogen and volatile fatty acids produced by either endogenous fermentation or oxidation of longer chain fatty acids as the possible sources of these endogenous nutrients. Their suggestion is that these cryptic nutrients are not available until exogenous nutrients such as acetate are supplied to metabolically balance the PHA precursors and NADH produced by the oxidation of the fatty acids.

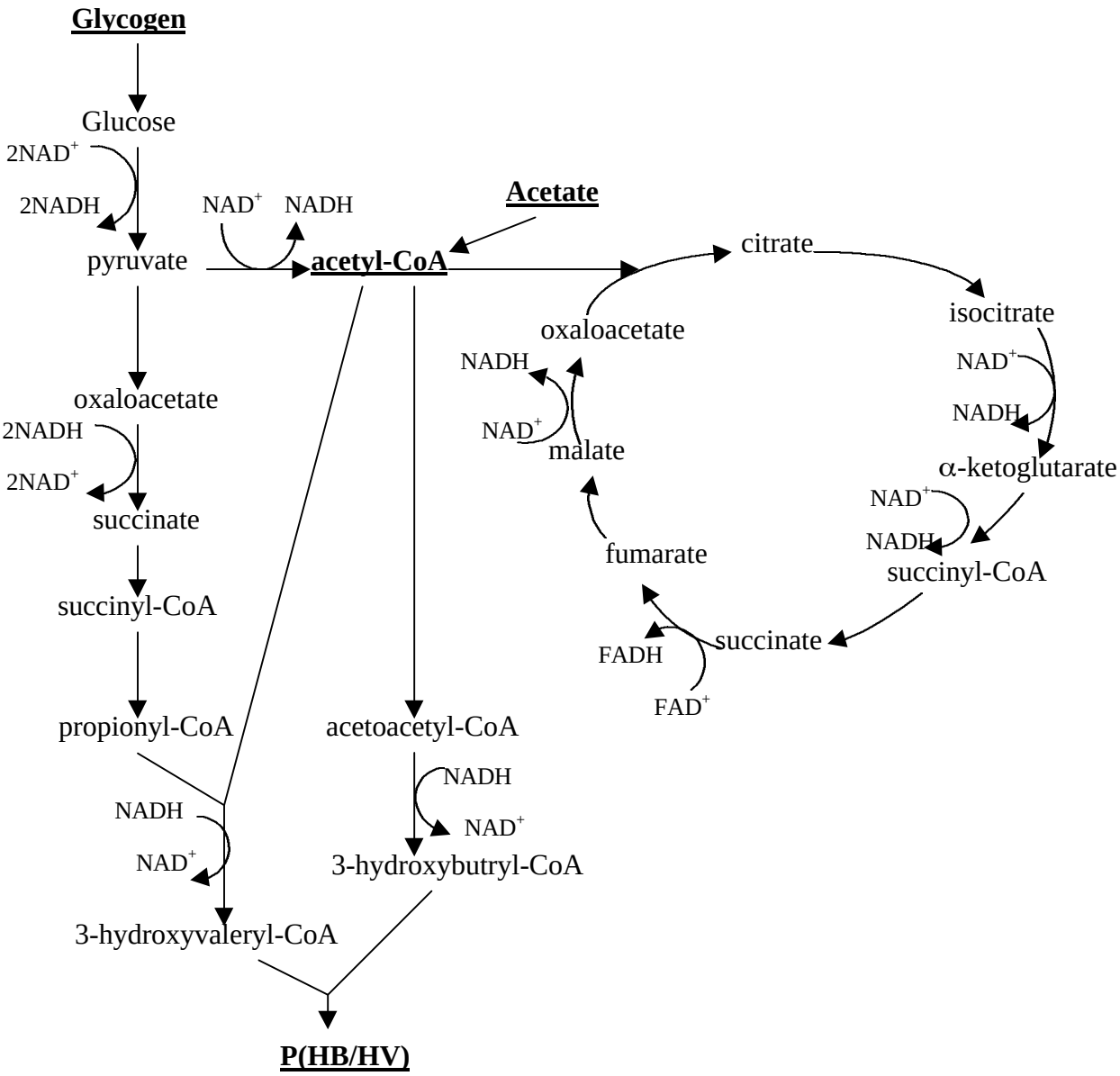

Figure 10. EBPR model developed by Pereira and her co-workers (1996).

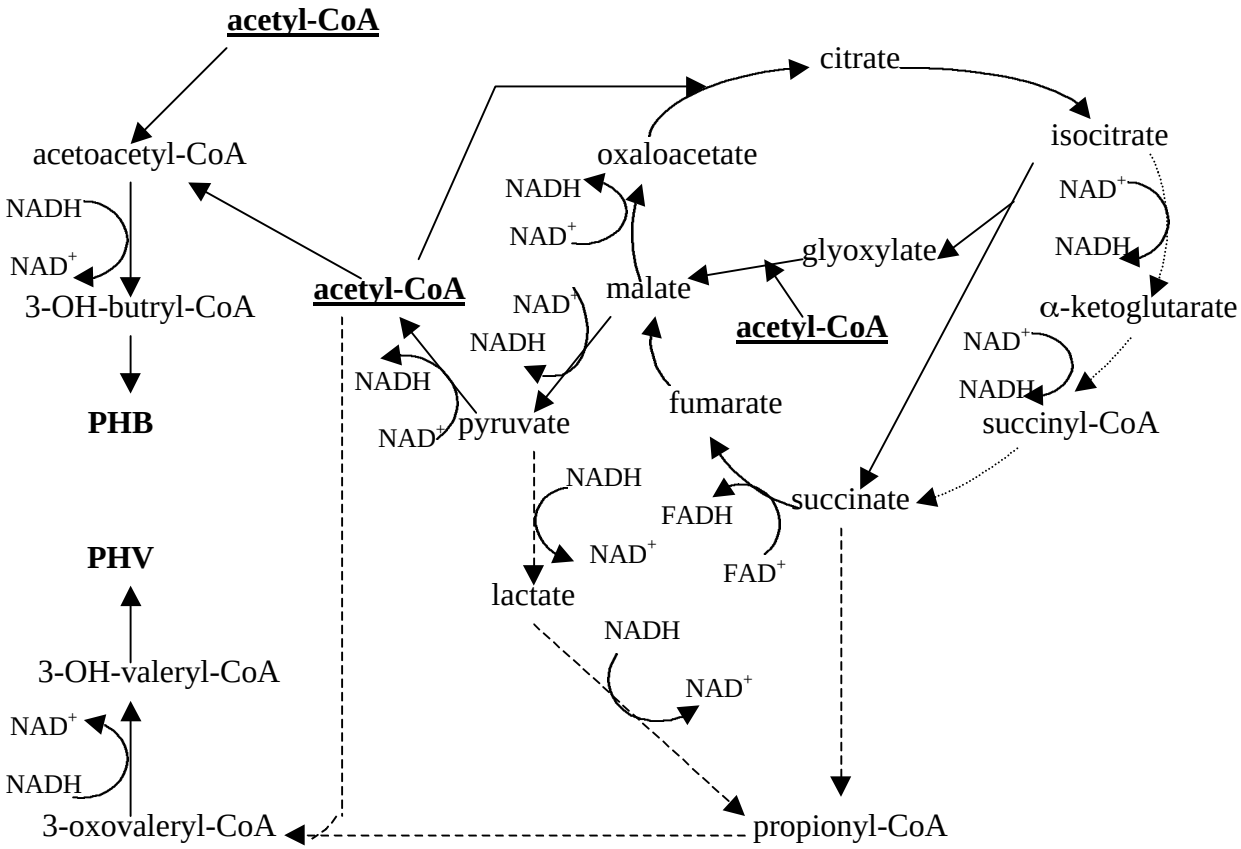

Figure 11. Schematic representation of the involvement of the glyoxylate bypass as suggested by Louie *et al.* (2000)

Glycogen Metabolism

Possibly owing to its widespread utilization in cell functioning, glycogen metabolism is strictly regulated by a complex control mechanism. Especially in mammals, the control of glycogen metabolism is under different control mechanisms such as changes in Ca^{2+} signals, hormonal control (glucagon in liver and insulin in muscles), and energy requirements of brain, liver and muscle cells. However, the main control mechanism observed in all glycogen bearing organisms is allosteric regulation of the operating enzymes, mainly by AMP, ATP, and G-6-P. These enzymes are

- *Glycogen synthesis:*
  1. Glycogen synthase
  2. Phosphorylase kinase
  3. Protein kinase C and protein kinase-3 in higher animals, and others in humans.
  4. Phosphorylase phosphatase
  5. Branching enzyme

- *Glycogen degradation:*
  1. Glycogen phosphorylase
  2. Phosphorylase kinase
  3. cAMP-dependent protein kinase
  4. Phosphoprotein phosphatase
  5. Debranching enzyme

The enzymes active in glycogen metabolism act either in favor of or against glucose-1-phosphate (G1P) production. Thus, there are other points in gluconeogenesis or glycolysis where G1P production or degradation can be further regulated for the control of the glycogen metabolism. For example, glycogen break down can be required to satisfy the energy needs of the cell. Hence, high ADP/ATP or NAD+/NADH levels in the cell will signal the enzymes for glycogen degradation. The need for intermediates for cell growth also will require glycogen breakdown. 6-Phosphofructo-1-kinase (PFK) and

fructose-1,6-bisphosphatase (FBPase) are ideal points for such control to direct the metabolites either in glycolysis or in gluconeogenesis direction, respectively. These enzymes interconvert fructose-6-phosphate (F6P) and fructose-1,6-bisphosphate (F1,6BP). This, thus, controls the partitioning of carbohydrate between hexose phosphate and triose phosphate pools, because FBP is readily equilibrated with the triose phosphates, glyceraldehyde-3-phosphate (GADP) and dihydroxyacetone phosphate (DHAP). As a consequence, control of these enzymes also controls the glycogen pool. For example, FBPase is controlled by four major means (Muise and Storey, 1997): (1) FBPase/PFK activity ratio; (2) low/high levels of allosteric inhibitors such as F2,6BP and AMP; (3) low/high temperatures that alter the enzyme substrate affinity, and enzyme sensitivity for allosteric regulators (Holden and Storey, 1995); (4) reversible protein phosphorylation by the combined action of protein kinases and protein phosphatases.

Glycogen Synthesis

As with the production of any biopolymer, the synthesis of glycogen involves initiation, elongation and conclusion steps. The initiation step is carried out by a designated protein, "glycogenin", which has the capability of autoglucosylating activity which leads to the production of a short α(1,4)-glucosyl chain from UDP-glucose (UDP-G) (Francois and Parrou, 2001) and the chain is covalently attached to a tyrosine residue. Following this, glycogen synthase catalyzes the formation of α(1,4)-glucosidic bonds from UDP-G to the non-reducing end of linear α(1,4)-chains of glycogen. In contrast to the mechanism in animals, the glucosyl-donor in plants and bacteria is ADP-glucose produced from ATP and G-1-P by the action of the ADP-glucose pyrophosphorylase. The rest of the enzymatic reactions are very similar (Preiss *et al*., 1983).

G6P is one of the main allosteric stimulators of the enzyme and it undergoes reversible covalent phosphorylation as a regulation mechanism. At active state, the enzyme is non-phosphorylated and it is almost insensitive to the sugar phosphate. Thus, the ratio of the activities assayed in the absence and in the presence of G6P can provide a good estimate

of the phosphorylation state of the enzyme (Francois and Hers, 1988). The protein kinases active in the regulation of the glycogen synthase phosphorylation depend on the host organism. cAMP, Ca^{2+} and calmodulin are the main regulators of the protein kinase (PKA) in general. Protein phosphatases play a critical role in glycogen synthesis. In yeast, for example, there are four different clases of Ser/Thr protein phosphatases. In summary, the activation site of glycogen synthase is ultimately dependent on the relative activity of protein kinases and protein phosphatases. This phosphorylation–dephosphorylation equilibrium is controlled by the levels of G6P and cAMP, which are affected by external stimuli. The major role of G6P is to act as a stimulator of the dephosphorylation and an inhibitor of the phosphorylation process. After this point the linear α(1,4)-glucosyl chains are ramified by the action of amylo (1,4)$\rightarrow$(1,6)-transglucosidase (branching enzyme). The branching enzyme transfers a block of 6 to 8 residues from the end of a linear chain to create an α(1,6)-linkage to an internal glucosyl unit on an adjacent chain. The degree of branching depends on the organism. In yeast it is estimated that the glycogen particles contain 7 to 10 % of α(1,6)-linkages. The branched structure of glycogen can be classified to be high or low based on the colorimetric reaction of the cells to iodine crystals vapor: green-purple with low branching, brown with high branching.

Although the glycogen synthesis steps are the same, bacteria and plants lack a high level of complicated regulation. It has been shown that the regulatory step in glycogen synthesis was the ADP-G pyrophosphorylase step. Controlling the synthesis at the initial step also saves the cells energy in the form of ATP. Besides, the glycolytic intermediates are known to be activators for ADP-G synthesis, while AMP, ADP and Pi act as inhibitors. This strongly indicates that glycogen synthase is impacted from the energy state of the cells, and the presence of glycolytic intermediates possibly indicate the presence of carbon excess. However, the activator specificity of ADP-G pyrophosphorylase varies between groups of microorganisms. Preiss *et al*. (1983) lists seven groups of photosynthetic and non-photosynthetic bacteria known to store glycogen, grouped according to the major activator of the enzyme of each. They attribute this variation to the carbon assimilation pathway dominant or significantly present in the

microorganism or plant cell. One generalization made based on their list can be that when the bacteria predominantly obtain their energy through utilization of sugars via glycolytic pathway, ADP-G pyrophosphorylase is activated mainly by fructose-1,6-bis-phosphate (F1,6P).

Glycogen Degradation

The degradation of glycogen is as elaborately regulated as its synthesis. It can be achieved either through amylolysis by α-glucosidases producing glucose, or through sequential reactions involving phosphorolysis and debranching activities producing G1P and glucose (Francois and Parrou, 2001). Glycogen phosphorylase releases G1P from the linear glycogen molecule α(1,4)-bonds starting from the non-reducing ends of the outer branches. This shortens the molecule to limit dextrin, and the debranching enzyme transfers one maltosyl or maltotriosyl unit to an adjacent linear α(1,4)-chain, and releases glucose by cleaving the remaining α(1,6)-linkage. Thus, phosphorylase continues its action after the debranching enzyme clears the way. Disruption of either enzyme system results in the failure of the glycogen degradation.

Glycogen Phosphorylase: Based on the three-dimensional structure, and positions of regulatory and active site residues, the enzyme monomer can be divided into two domains: N-terminal and C-terminal domains. N-terminal domain, extending from amino acid residue 1 to 482 is referred to as the regulatory domain since it contains the majority of the ligand binding residues. It also contains all but one of the residues that form contact with residues in the other subunit. These residues are required for transmitting conformational changes across subunits upon ligand binding. There also exists an "activation subdomain" which has been shown to undergo complex tertiary and quaternary changes on ligand binding. The C-terminal domain, extending from amino acid residue 483 to 842 is referred to as the "catalytic domain" since it contains the active site and pyridoxal phosphate binding residues. The active site cleft is located between the N- and C-terminal domains (Hudson *et al*., 1993). AMP, glycogen, glucose and

purine nucleosides bind to distinc sites on the enzyme: AMP activation site, glycogen storage site, active site, and purine nucleotide inhibitor site.

Glycogen phosphorylase is a dimeric enzyme that can be found in two metabolic states: a and b. The contact with a second monomer stabilizes the enzyme structure through hydrophobic forces. This contact can be weakened by deforming agents and by the chemical modification of slow-reacting SH groups such as Cys 108 and Cys 142. Liganding of the nucleotide site of phosphorylase *b* with AMP or G-6-P, and phosphorylation of Ser 14 residue stabilizes the contacts between the monomers, resulting in the formation of *a* state. Hence its regulation is strongly based on allosteric transitions. For example, when the glucose was replaced by a G-1-P analogue, significant structural changes can be observed. Ligands binding to the nucleotide site enhance the affinity of substrates to the active site even if they are inhibitors like ATP or G-6-P. Besides, the results of cooperative interactions of ligands can be different from those of individual ligand binding. Consequently, the ligand binding reactions control the enzyme-catalyzed reactions acting on phosphorylase, such as the phosphorylation and dephosphorylation of the enzyme by phosphorylase kinase and phosphatase. Phosphorylase kinase is activated by glycogen and inactivated by G6P, ATP and glucose. AMP on the other hand is an activator of the enzyme by removing G6P inhibition.

When glycogen phosphorylase enzymes from different sources, i.e. bacteria, yeast, slime mold, plants, insects, fish, amphibians and mammals are compared, it is found that all phosphorylases require the cofactor, pyridoxal phosphate, for activity, but they differ in their affinity and specifity for polysaccharides, their modes of regulation and their physiological roles (Hudson *et al*., 1993). According to Hudson *et al*. (1993) in lower organisms phosphorylases vary dramatically in their ability to respond to allosteric control mechanisms. Bacterial and plant phosphorylases are active in the absence of phosphorylation and AMP (Newgard *et al*., 1989). The yeast enzyme requires phosphorylation for maximal activity, but it is insensitive to activation by AMP. Non-mammalian phosphorylases also exhibit wide differences in their polysaccharide affinities and specificities. In *E. coli* there is a true glycogen phosphorylase and there is a

maltodextrin phosphorylase which shows specificity for low molecular mass, unbranched α(1,4)-polyglucoses. They are encoded by distinctly different genes, *glg*P and *mal*P. Also, according to Schinzel and Nidetzky (1999) glycogen phosphorylase encoding genes found in *E. coli, Streptococcus salivarius, B.subtilis,* and *B. stearothermophilius* are located among the glycogen biosynthetic genes. In spite of the genetic and catalytic similarities, phosphorylases isolated from these organisms showed differences in regulatory mechanisms. Thus, this suggests the presence of a common ancestor from which different phosphorylase enzymes specific for different polysaccharides have evolved. It also was shown that although the number of amino acid residues changed proportional to the hierarchy of the cells studied, the internal structure of the phosphorylase enzyme, and the active site and cofactor binding residues are highly conserved from one cell to the other. The differences at other parts of the enzyme (phosphorylation and AMP binding sites, and dimer contact sites) put it under a much stricter allosteric control for mammalian cells compared to non-mammalian cells.

Conditions Leading to Synthesis and Degradation of Glycogen: Its Role in the Cell

Changes in environmental conditions will lead to cellular adaptations. Nutrient availability and stress conditions that can be simulated with high temperatures, sodium chloride, hydrogen peroxide, copper sulfate, or weak organic acids result in glycogen and trehalose production in *Saccharomyces cerevisiae* (Baker's yeast). Two extreme nutritional conditions that result in changes in glycogen and trehalose content in yeast cells are (1) the lack of one essential nutrient, i.e. nutrient starvation which results in "growth arrest" and leads to an increase in the storage; and (2) the abundance of all essential nutrients, i.e. nutrient sufficiency which leads to maximal growth with low storage of carbohydrates. Absence of nitrogen, carbon, phosphorus or sulfur would lead to nutrient deficiency. Glycogen accumulation agrees with the concept that a reserve carbohydrate accumulates when nutrients are still abundant, and is mobilized during the growth starvation period of nutrient deficient conditions. Based on the study by Enjalbert *et al*. (2000) on respiration-deficient yeast cells, it can be stated that the major function of

glycogen is to provide carbon and energy for maintenance of cellular activities when nutrients are depleted. They have shown that respiration-deficient cells accumulate more glycogen during fermentative growth on glucose, and they mobilize it much faster than the wild-type cells at the onset of glucose depletion. Since the respiratory defects cannot use the by-products of the glucose fermentation, they consume the available G6P immediately following glucose depletion, which follows with inactivation of glycogen synthase and activation of glycogen phosphorylase, and leads to an immediate degradation of available glycogen. The disaccharide trehalose on the other hand has been shown to be a good stress protectant, one of the stress conditions being cold temperatures (Eroglu *et al.*, 2000; Guo *et al.*, 2000).

Other Functions of the Glycogen Stores:

Cryoprotection: Like other cryoprotectants, carbohydrate driven ones also operate by depressing the freezing point and supercooling point. Research on insect cold hardiness was the origination point of the research on their cryoprotecting properties. Since 1957, glycerol and other polyols have been researched in freeze tolerant and freeze avoiding insects. Although glycerol is the most common cryoprotectant, sorbitol, mannitol, ribitol, erythitol, and ethylene glycol are also frequently observed. The advantages of the polyols as cryoprotectants are that they are highly soluble in aqueous solutions, nontoxic, have few effects on enzymatic or metabolic processes, freely penetrate across the cell membranes, and, most importantly, they stabilize the native state of proteins to prevent denaturation at freezing temparatures. The key point that ties the cryoprotection to EBPR is that they are derived from the glycogen pool. Figure 12 summarizes the pathways of synthesis for glycerol, trehalose and sorbitol. Glycerol is derived from the dihydroxyacetone phosphate (DHAP) (glycerol-3-phosphate) pool of glycolysis. Glycerol can also be produced from glyceraldehyde-3-phosphate and glyceraldehyde. Glycerol synthesis requires reducing equivalents for the conversion of DHAP to glycerol-3-phosphate. ATP is required for the PFK reaction. However, in a system where glycolysis is already operative for other purposes, this requirement is negligible. In

EBPR systems ATP generation via breakdown of the poly-P bonds under anaerobic conditions is possible.

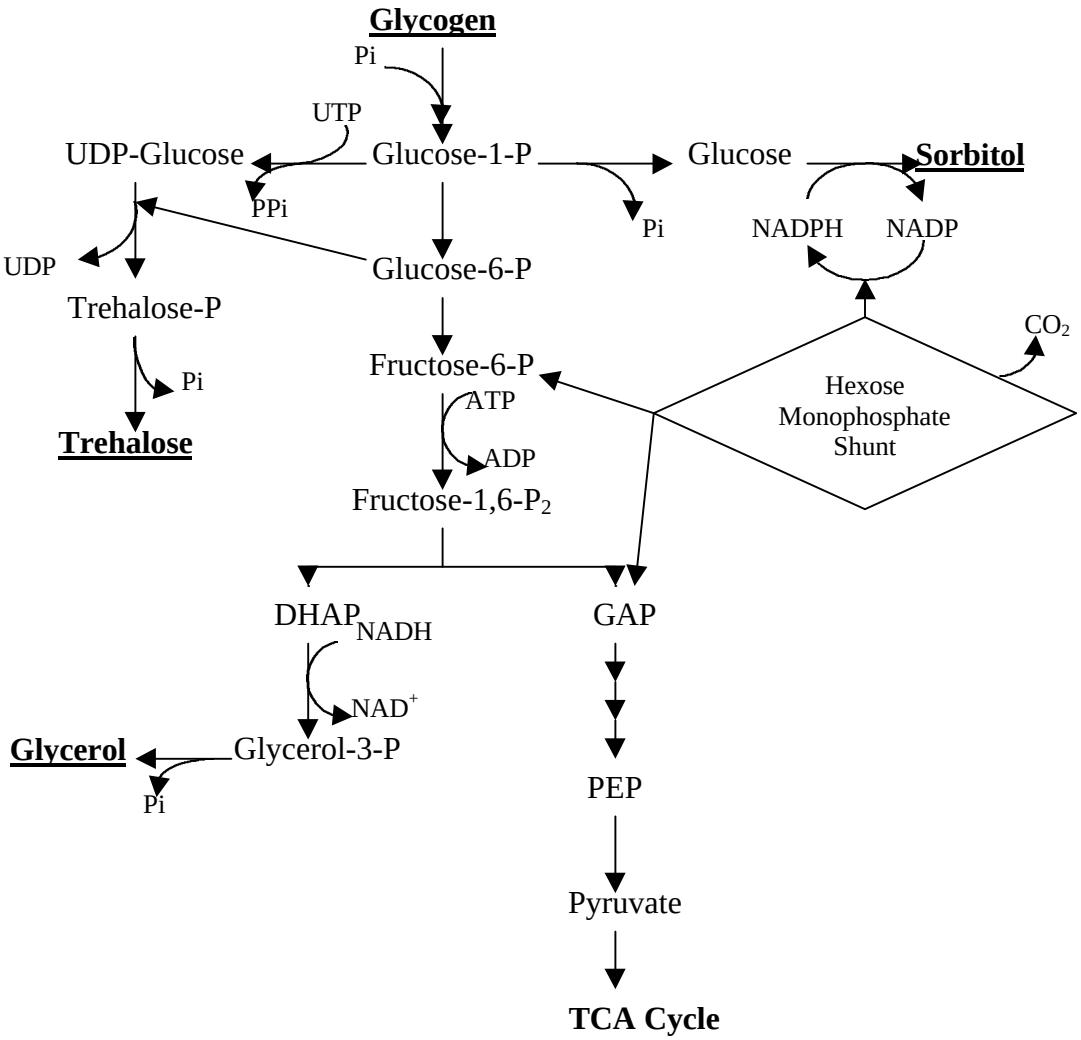

Figure 12. Metabolic pathways for glycerol, sorbitol and trehalose biosynthesis (Storey and Storey, 1990)

Being dependent on the glycogen breakdown, glycerol production is regulated by the actions of glycogen phosphorylase to some extent. According to Storey and Storey (1990) for the case of insect cold hardiness, glycogen phosphorylase activation (i.e.

conversion of *b* to *a* form of the enzyme) is triggered by exposure to low temperatures between 0° and 5°C. The basis of this reaction is that phosphorylase phosphatase is inactivated by cold, whereas phosporylase kinase is only slowed, letting the latter one dominate over the other. Thus, it is a basic cold shock response to release carbohydrate energy under stress conditions. When the system is exposed to warmer temperatures, the cryoprotectant pool is reconverted to glycogen, or redirected to the glycolysis route, which would eventually result in production of CO_2 and acetyl-CoA.

Balancing the Reducing Equivalents: Glycerol is one of the sinks or regeneration points for NADH produced during the catabolic reactions under anaerobic conditions (Flores *et al.*, 2000). Glycerol can then be utilized as a carbon source under aerobic conditions. The transport of glycerol to the outside of the cell is dependent on the osmotic pressure of the medium. Glycerol being a good osmoregulator, it will remain in the cell under hyperosmotic conditions. Under normal conditions the release takes place through a channel protein, and its latter uptake is either through passive diffusion or active transport under osmotic stress.

Anaerobic COD Stabilization

The existence of anaerobic stabilization by EBPR processes has been empirically demonstrated by Randall *et al*. (1992b) and Wable and Randall (1994), and further confirmed by Barker and Dold (1995 & 1996a&b). The data indicate that anaerobic stabilization can result in large savings (10 to 30%) in aeration energy costs during BNR wastewater treatment, and it needs to be seriously considered during design for optimum economy of operation. The existence of anaerobic stabilization has been shown through experimental work as shown in Table 3 (Randall *et al*., 1992b). The data resulted from a side-by-side comparison of conventional activated sludge (CAS) and BNR with EBPR systems. The same analytical and computational techniques were used for the determination of the total oxygen requirements of the conventional and BPR systems, yet the differences in oxygen consumption are striking.

Table 3. COD/oxygen utilization mass balance results (Randall *et al*., 1992b)

Phase	System	$TOR_{predicted}$, g/day	TOR_{actual}, g/day	AnS/COD_s, %
IA	Conventional	23.9	25.0	
	BPR	20.2	16.5	18.3
IB	Conventional	31.9	27.4	
	BPR	19.9	18.2	8.4
IC	Conventional	43.3	42.4	
	BPR	36.2	32.6	10.0
IIA	Conventional	26.1	25.6	
	BPR	22.0	19.4	11.6
IIB	Conventional	34.9	34.1	
	BPR	28.7	21.7	24.6
IIC	Conventional	33.8	34.2	
	BPR	29.5	21.4	27.3

AnS = Anaerobic Stabilization of COD; TOR = Total Oxygen Requirement

Also, evaluation of operational data from existing plants (Barker and Dold, 1995 and 1996a) has indicated the definite presence of anaerobic stabilization at plants that include anaerobic zones as part of their operation. By exploring the biochemical reactions taking place in EBPR processes, particularly those controlling the storage mechanisms, i.e., PHA, poly-P and glycogen storage, the basic mechanisms resulting in the anaerobic stabilization of COD can be sought. Reactions leading to the production, consumption and maintenance of the EBPR storage products have the potential to explain all or a major part of the stabilized COD, as the carbon source (i.e. VFAs) entering the anaerobic zone of the EBPR plants passes through a series of modifications before reaching a zone where an electron acceptor (nitrate or oxygen) is available. In order to be able to attribute the losses to individual mechanisms, the active biochemical pathways need to be determined because none of the currently accepted biochemical models are sufficient to explain the microbial metabolism under different conditions.

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