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MODEL FOR CARBON METABOLISM IN BIOLOGICAL PHOSPHORUS REMOVAL PROCESSES BASED ON *IN VIVO* ^{13}C-NMR LABELLING EXPERIMENTS

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Abstract—*In vivo* ^{13}C-NMR and ^{31}P-NMR techniques were applied to study phosphorus and carbon metabolism in activated sludge during both the anaerobic and the aerobic stages. By supplying a ^{13}C label on the methyl group of acetate at the beginning of the anaerobic stage, the fate of the label through the subsequent aerobic/anaerobic stages was traced *in vivo*. It was possible to follow the flux of label from acetate to hydroxybutyrate/hydroxyvalerate co-polymer in the first anaerobic stage, then to monitor the conversion of these units into glycogen in a subsequent aerobic stage, and afterwards, by submitting the same sludge to a second anaerobic stage, to observe the flux of labelled carbon from glycogen to the hydroxyvalerate and hydroxybutyrate units. The uptake/release of inorganic phosphate and the extracellular pH were monitored by ^{31}P-NMR in the same experiments. The data provide an unequivocal demonstration of the involvement of glycogen in the biological phosphorus removal process. On the basis of these ^{13}C labelling data, a biochemical model for the synthesis of polyhydroxyalkanoates from acetate and glycogen was elaborated in which the tricarboxylic acid cycle is proposed as an additional source of reduction equivalents. According to this study, from 1 C-mol acetate, 1.48 C-mol P(HB/HV) are synthesized and 0.70 C-mol glycogen are degraded anaerobically, while 0.16 P-mol phosphate is released. In the aerobic stage, 1 C-mol of P(HB/HV) is converted to 0.44 C-mol glycogen. Copyright © 1996 Published by Elsevier Science Ltd

Key words—biochemical model, biological phosphorus removal, activated sludge, anaerobic/aerobic cycle, *in vivo* ^{31}P- and ^{13}C-NMR, metabolism, polyhydroxyalkanoates, glycogen

INTRODUCTION

Control of eutrophication, a worldwide water pollution problem, requires removal of phosphorus from effluents by chemical and/or biological means. Activated sludge systems, modified for enhanced excess phosphorus removal, are at present operating in several countries and show considerable advantages over chemical methods (Yeoman *et al.*, 1988; Toerien *et al.*, 1990).

During the last 30 yr, a number of studies have been performed aiming to understand and to improve the performance of the processes of biological phosphorus removal by activated sludge (Arun *et al.*, 1989). Despite all the effort invested, these processes are not yet well understood and experimental evidence is needed to corroborate several proposed pathways (Wentzel *et al.*, 1991).

Conventional wastewater plants for biological phosphorus removal work on the basis of anaerobic/aerobic cycles. During the anaerobic stage, inorganic phosphate is released by the biomass and carbon substrates (mainly short-chain fatty acids) are taken up and stored as carbon reserves such as polyhydroxyalkanoates. In the aerobic stage, carbon reserves are utilized while inorganic phosphate is taken up and intracellularly accumulated as polyphosphate (Fuhs and Chen, 1975). Enrichment of activated sludge in polyphosphate accumulating microorganisms is only possible due to this metabolic coupling of polyphosphate hydrolysis with the synthesis of carbon reserves under anaerobic conditions. The elucidation of this coupling mechanism is an essential step for the understanding of the full process of biological phosphorus removal.

So far, there are no reports on isolated organisms able to perform phosphorus removal as activated sludge does. Although *Acinetobacter*, *Pseudomonas* and *Aeromonas* spp. were pointed out as major groups of organisms playing a relevant role in phosphorus removal by activated sludge (Brodisch and Joyner, 1983; Cloete and Steyn, 1988; Streichan

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et al., 1990; Hiraishi and Morishima, 1990) this process seems to depend critically on obscure interactions among different organisms present in the sludge population; therefore, these studies must be carried out with sludge and this requirement has been a major drawback in the elucidation of biological phosphorus removal.

The ability to accumulate carbon as different types of reserve materials, allowing survival for long starvation periods and during adverse growth conditions, is widespread among microorganisms (Dawes and Senior, 1973; Santos *et al.*, 1993). Poly-3-hydroxybutyrate (PHB) is an important and well-known microbial carbon reserve (Lafferty *et al.*, 1988; Anderson and Dawes, 1990), and this polymer is the major carbon reserve built up by activated sludge cells when acetate is the carbon source (Comeau *et al.*, 1986); in some cases 3-hydroxyvalerate (HV) units were also found in a co-polymer of HB and HV with variable proportions (Arun *et al.*, 1989; Matsuo *et al.*, 1992; Satoh *et al.*, 1992; Smolders *et al.*, 1994a).

Various models have been proposed to describe activated sludge behaviour (Comeau *et al.*, 1986; Wentzel *et al.*, 1986; Mino *et al.*, 1987). It is generally accepted that during the anaerobic stage, acetate is activated to acetyl-CoA at the expense of ATP and then metabolized via acetoacetyl-CoA to synthesize poly-3-hydroxybutyrate (PHB). The ATP required for the activation of acetate is obtained from polyphosphate hydrolysis and the enzymatic equipment required to perform this process has been identified in *Acinetobacter* species (van Groenestijn, 1988). However, different hypotheses have been advanced to explain the supply of reducing power which is required to convert acetate into PHB. In the models proposed by Comeau *et al.* (1986) and Wentzel *et al.* (1986), reducing power is derived from the metabolism of acetyl-CoA in the tricarboxylic acid (TCA) cycle operating under anaerobic conditions. According to this proposal 9 mol of acetate are needed to produce 4 mol of HB monomers and 9 mol of inorganic phosphate are released, leading to a molar ratio of phosphate release to acetate uptake of 1:1.

The model described by Mino *et al.* (1987) takes into account the observation of a decrease in intracellular carbohydrate (identified as glycogen) during the anaerobic phase, followed by an increase during the aerobic stage, and proposes that reducing power is brought about by metabolism of glycogen under anaerobic conditions. In this model, 6 mol acetate are required to produce 4 mol HB monomers with the concomitant release of 3 mol inorganic phosphate. Thus, a ratio of phosphate release to acetate uptake of 0.5:1 is predicted. Glycogen metabolism was first suggested to proceed via the Embden–Meyerhof–Parnas glycolytic pathway, but this proposal was criticized (Wentzel *et al.*, 1991) on the basis of the finding that this pathway is not operating in *Acinetobacter*, a major group of microorganisms present in activated sludge. Afterwards, a modified Mino model was proposed assuming the Entner–Doudoroff pathway for glycolysis; in both hypotheses the stoichiometry predicted for glycogen degraded:acetate utilized:PHB synthesized is 1:6:4 but the phosphate:acetate molar ratio in the modified model is 2:3. Replenishing of the carbohydrate reserve during the aerobic phase is proposed to occur via the glyoxylate cycle and gluconeogenesis with PHB as carbon source.

Recent compilation and critical evaluation of these models are available (Wentzel *et al.*, 1991; Matsuo *et al.*, 1992 and Satoh *et al.*, 1992).

In vivo NMR has proven to be a very useful technique to study cellular physiology due to its non-destructive and non-invasive characteristics (Lundberg *et al.*, 1990; Santos *et al.*, 1994). With the aim of contributing to the understanding of the biological phosphorus removal process, *in vivo* ^{31}P- and ^{13}C-NMR was applied to investigate, in a non-invasive way, carbon and phosphorus metabolism in activated sludge. The use of isotopically labelled acetate, coupled to ^{13}C-NMR, allowed direct tracing of the label through the P(HB/HV) and glycogen pools on-line, whereas phosphate uptake and release could be monitored in the same sample by ^{31}P-NMR. These unique capabilities of *in vivo* NMR make it an ideal technique for studying biological phosphorus removal.

MATERIALS AND METHODS

Seed sludge

The inoculum was obtained from an activated sludge treatment plant at Beirolas, Lisbon. In the laboratory it was cultivated in mineral medium, containing acetate as carbon source.

Medium

The medium composition was as follows: $NaCH_3CO_2.3H_2O$, 2.04 g; $MgSO_4.7H_2O$, 0.60 g; NH_4Cl, 0.32 g; K_2HPO_4, 0.19 g; KH_2PO_4, 0.09 g; EDTA, 0.10 g; $CaCl_2.2H_2O$, 0.07 g, and 2 ml of trace elements mixture in 1 l of distilled water. The trace elements mixture consisted of $FeCl_3.6H_2O$, 1.50 g; H_3BO_3, 0.15 g; $CoCl_2.6H_2O$, 0.15 g; $MnCl_2.4H_2O$, 0.12 g; $ZnSO_4.7H_2O$, 0.12 g; $Na_2MoO_4.2H_2O$, 0.06 g; $CuSO_4.5H_2O$, 0.03 g; KI, 0.03 g in 1 l of distilled water.

Sequence batch reactor (SBR)

The laboratory reactor consisted of a 2 l vessel with a working volume of 1.5 l, operating at a temperature of 25°C, with agitation and pH 7.0; the pH was controlled by the addition of 0.5 M NaOH or 0.5 M HCl. The reactor was operated as a sequenced batch (SBR) (Irvine and Ketchum, 1989) with a cycle of 8 h, comprehending an anaerobic (2 h), an aerobic (4 h) and a settling period (1 h). After settling, one-third of the liquid phase was withdrawn and replaced by fresh mineral medium (1 h), resulting in a hydraulic retention time of one day. At the end of the aerobic cycle, a purge of biomass was made in order to maintain a mean cell retention time of either 10 or 32 days. Anaerobic and aerobic conditions were achieved by bubbling argon or air at 0.3 l.min^{-1} and monitored by measurement of the redox potential. A stirrer speed of 250 rpm was maintained except

for the settling period. For the NMR experiments, sludge from sequenced batch reactors was used.

Quantification of acetate, phosphorus and nitrogen in the SBR

Acetate was determined by high-performance liquid chromatography (HPLC) using a reversed phase column (SHODEX SH1011) coupled to a UV detector set at 210 nm (Merck). Inorganic phosphate and total phosphorus concentration were determined by segmented flow analysis (Skalar Analytical B. V., Breda, The Netherlands) by the colorimetric method based on reduction of the phosphomolybdate complex with ascorbic acid (*Standard Methods*, 1989). Sample digestion with potassium persulfate in H_2SO_4 at 100°C was performed prior to total phosphorus analysis. Nitrite and nitrate were also determined by segmented flow analysis (Skalar Analytical B. V., Breda, The Netherlands). Nitrite detection was based on the colorimetric reaction with *N*-(1-naphthyl)ethylenediamine after diazotization with sulphanilamide (*Standard Methods*, 1989). Nitrate was separately determined as nitrite after reduction in a cadmium column.

Cell mass determination

For dry mass determinations, sludge samples were filtered through a Whatman GF/C glass microfibre filter (1.2 μm pore size). The filter was dried for 24 h at 100°C and weighed. The ash content was determined after calcination at 550°C for 2 h (*Standard Methods*, 1989).

Extraction of polyhydroxyalkanoates

Freeze-dried cells were extracted with 60 ml of chloroform by vigorous stirring for 24 h at 4°C. After centrifugation (25,000 × *g*, 30 min, 4°C), the solvent in the supernatant solutions was removed by rotary evaporation and the residue dissolved in extra pure chloroform (0.6–1.0% ethanol). This solution was filtered through HA Millipore membranes (0.45 μm pore size) and evaporated under a nitrogen flow; the resulting residue was then dissolved in 0.8 ml of deuterated chloroform.

Sample preparation for in vivo *NMR analysis*

A sample of cell suspension (approx. 80 ml) was taken from the reactor at the end of the aerobic stage, concentrated by centrifugation (8000 × *g*, 15 min, 4°C) and suspended in fresh mineral medium supplemented with 10 mM KCl and 2 mM $MgSO_4.7H_2O$, at pH 7.0, without carbon or phosphorus sources. Experiments were performed with 5 ml of cell suspension (approx. 0.07 g dw). Anaerobic or aerobic conditions in the NMR tube were achieved by using an air-lift system (Santos and Turner, 1986) with bubbling of argon or air, respectively (0.13 l.min^{-1}).^2H_2O was added (5% v/v) in order to provide a lock signal. At the end of the first anaerobic stage, the cell suspension was centrifuged (8000 × *g*, 15 min, 4°C) and the pellet resuspended in fresh mineral medium; at the beginning of the following aerobic stage, potassium phosphate (5 mM) was added.

Acquisition of NMR spectra

^{31}P-NMR and ^{13}C-NMR spectra were acquired with a quadruple nuclei probe head (10 mm diameter) in a Bruker AMX-500 spectrometer, operating at 202.45 MHz for phosphorus and 125.76 MHz for carbon. Phosphorus spectra were acquired without proton decoupling with the following parameters: spectral width, 9.2 kHz; repetition delay, 1.9 s; pulse width, 12 μs (corresponding to a flip angle of 45°); data size, 16K. Phosphorus resonances were referenced with respect to external 85% H_3PO_4. Carbon spectra were acquired with the following parameters: spectral width, 31 kHz; repetition delay, 1.5 s; pulse width, 7 μs (corresponding to a flip angle of 45°); data size, 32K. Proton decoupling was continuously applied by using the WALTZ sequence. Resonances were referenced with respect to external methanol (designated at 49.3 ppm). A probe head temperature of 30°C was always used.

^{13}C-NMR spectra of the chloroform extracts were recorded at 125.76 MHz in a Bruker AMX-500 spectrometer and using a selective probe head (5 mm diameter). The following acquisition conditions were used: spectral width, 50 kHz; repetition delay, 60 s; pulse width, 9 μs (corresponding to a flip angle of 90°); data size, 128K; probe temperature, 28°C. Proton broadband decoupling was applied during the acquisition time only (1.3 s). Chemical shifts were referenced with respect to the resonance of deuterated chloroform at 77.5 ppm. Assignment of the carbon resonances due to the co-polymer P(HB/HV) were obtained from the literature (Doi *et al.*, 1986).

The relative intensities of the carbon resonances in Figs 2, 4 and 6 do not reflect true relative concentrations due to the reasonably fast pulse rate conditions used to acquire ^{13}C-NMR spectra of living cells and diverse nuclear Overhauser enhancement factors of different carbon atoms. In order to obtain the actual concentrations of polyhydroxyalkanoates and glycogen, a capillary containing ^{13}C-labelled formate (9.4 mM equivalent concentration) was inserted in the NMR tube containing the cell suspension and a spectrum was run under non-saturating conditions and with suppression of the nuclear Overhauser enhancement. The areas of the resonances were compared with the area of the formate resonance. The estimated error in the area measurements is ± 10%.

Phosphate concentrations were determined by comparison of the intensities of the resonance due to inorganic phosphate with the increase in intensity observed upon addition of a known amount of phosphate.

Chemicals

[2-^{13}C]acetate (sodium salt, with 99% atom ^{13}C enrichment) and ^2H_2O (99.9% atom ^{2}H) were purchased from Sigma Chemical Co., U.S.A. Extra pure chloroform (0.6–1.0% ethanol) and [^{2}H]chloroform (99.5% atom ^{2}H) were obtained from E. Merck, Darmstadt, Germany. All other chemicals were of reagent grade.

EXPERIMENTAL RESULTS

Sequenced batch reactor measurements

A profile typical of an enhanced biological phosphorus removal process was observed after one week of reactor operation. The evolution pattern of phosphorus removal for reactors inoculated with different sludge samples was considerably reproducible. In the anaerobic phase, phosphorus was released and acetate was completely consumed, whereas in the aerobic phase, phosphorus was taken up. Since acetate was the sole carbon source present and was completely exhausted during the anaerobic phase, it is expected that sludge became enriched in organisms capable of utilizing acetate anaerobically. The observation that nitrite and nitrate concentrations in the fermentor medium were negligible and did not change during either the anaerobic or the aerobic periods excludes the presence of anaerobic denitrifyers or aerobic nitrifyers in the reactor.

Sludge for the NMR experiments was drawn when the performance of the reactor reached a steady state, i.e. when acetate was completely consumed at the end of the anaerobic phase and phosphorus removal was approximately 10 mg P_i/g dw. Unless otherwise

stated, sludge from a reactor operating at a mean cell retention time of 10 days was used.

In vivo *NMR measurements: effect of acetate on phosphate release and external pH*

The effect of presence or absence of acetate on phosphate release by activated sludge from a reactor operating at a mean cell retention time of 32 days was probed by NMR under an argon atmosphere. The evolution of external pH was also monitored. This information is important since pH was not controlled during the NMR experiments. Acetate (20 mM) was added at time zero; phosphate release and pH were monitored by ^{31}P-NMR, and acetate consumption was followed by ^{1}H-NMR. The pH value was measured from the chemical shift of the resonance due to inorganic phosphate by using a calibration curve performed with the suspension medium. Results are shown in Fig. 1. The rate of phosphate release (4.3 μmol P_i $min^{-1}.g^{-1}$ dw) was five-fold higher when acetate was provided as compared to a control experiment where no external carbon substrate was added. The molar ratio phosphate release to acetate consumption was 0.75 (average of two independent experiments). The external pH increased from 7.2 to 7.7 during the first 30 min following acetate addition but did not change thereafter. In contrast, in the absence of acetate, pH decreased steadily with time (Fig. 1).

Carbon and phosphorus quantification in anaerobic/aerobic cycles

Anaerobic stage. In a typical NMR experiment cells were first submitted to anaerobiosis in the NMR tube for 2–3 h in the presence of [2-^{13}C]acetate. The consumption of acetate and build-up of P(HB/HV) was monitored *in vivo* by ^{13}C-NMR, while the release of phosphate was followed in the same sample by ^{31}P-NMR (Fig. 2). The numbering of carbon atoms in the monomeric units of P(HB/HV) is as indicated below.

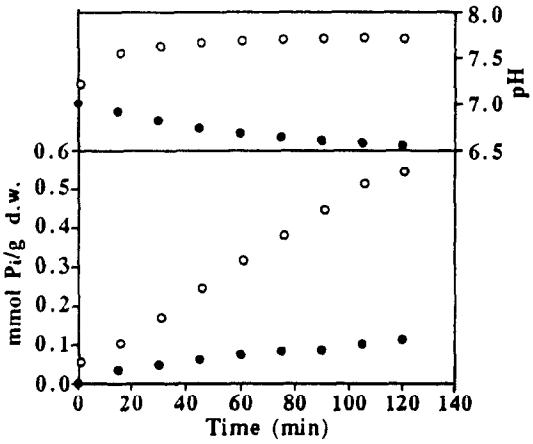
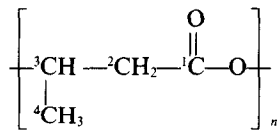
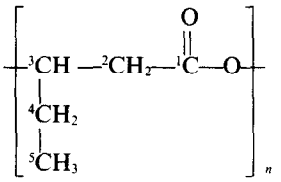


Fig. 1. Effect of the presence of an external carbon substrate (acetate) on phosphate release by activated sludge (from a reactor operating at a mean cell retention time of 32 days), as monitored *in vivo* by ^{31}P-NMR under anaerobic conditions. The time course of external pH is also shown. Open symbols refer to an experiment where acetate was supplied and full symbols refer to a control experiment with no external substrate added.



HB unit



HV unit

Acetate was converted to P(HB/HV) leading to isotopic enrichment on carbons $CH_3(B_4)$ (resonance at 19.7 ppm), and $CH_2(B_2)$ and $CH_2(V_2)$ (resonances overlapping at 40 ppm). This labelling pattern was confirmed in the fully relaxed ^{13}C-NMR spectrum of a chloroform extract of cells submitted to a similar treatment (Fig. 3). The profile of acetate consumption, P(HB/HV) build-up and phosphate release by the sludge is shown in the inset of Fig. 2. The molar ratio of phosphate release to acetate consumption was 0.32. From 1 C-mol labelled acetate consumed, 1.0 C-mol P(HB/HV) was formed. It is important to stress that only the HB units labelled on the methyl groups are considered in this calculation, since the amount of hydroxybutyrate formed was determined from the area of the resonance at 19.7 ppm which is due to the methyl groups of HB units in the co-polymer. The HB/HV ratio in the co-polymer was determined from ^{13}C-NMR spectra of chloroform extracts performed in separate experiments with cells that had been submitted to an anaerobic stage in the presence of [2-^{13}C]acetate (Fig. 3). The ratio of intensities of the ^{13}C-NMR resonances due to $CH_2(B_2)$ in HB (at 41.3 ppm) and $CH_2(V_2)$ in HV (at 39.2 ppm) in spectra run under non-saturating conditions gives directly the HB/HV molar ratio in the polymer. An HB/HV molar ratio of 2.8:1 was found (average of two experiments) and from this a value of 4.26 was calculated for the average number of carbon atoms in a monomeric unit of P(HB/HV). The amounts of HB and HV were added and referred to throughout this article as P(HB/HV).

Aerobic stage. At the end of the anaerobic stage (approximately 3.5 h), the gas atmosphere was changed to air and the catabolism of accumulated labelled P(HB/HV) was followed by NMR for approx. 3.5 h. Selected ^{13}C-and ^{31}P-NMR spectra from this experiment are shown in Fig. 4 with the corresponding profile of glycogen synthesis, P(HB/HV) consumption and phosphate uptake presented in the inset of this figure. The build-up of glycogen concomitant with

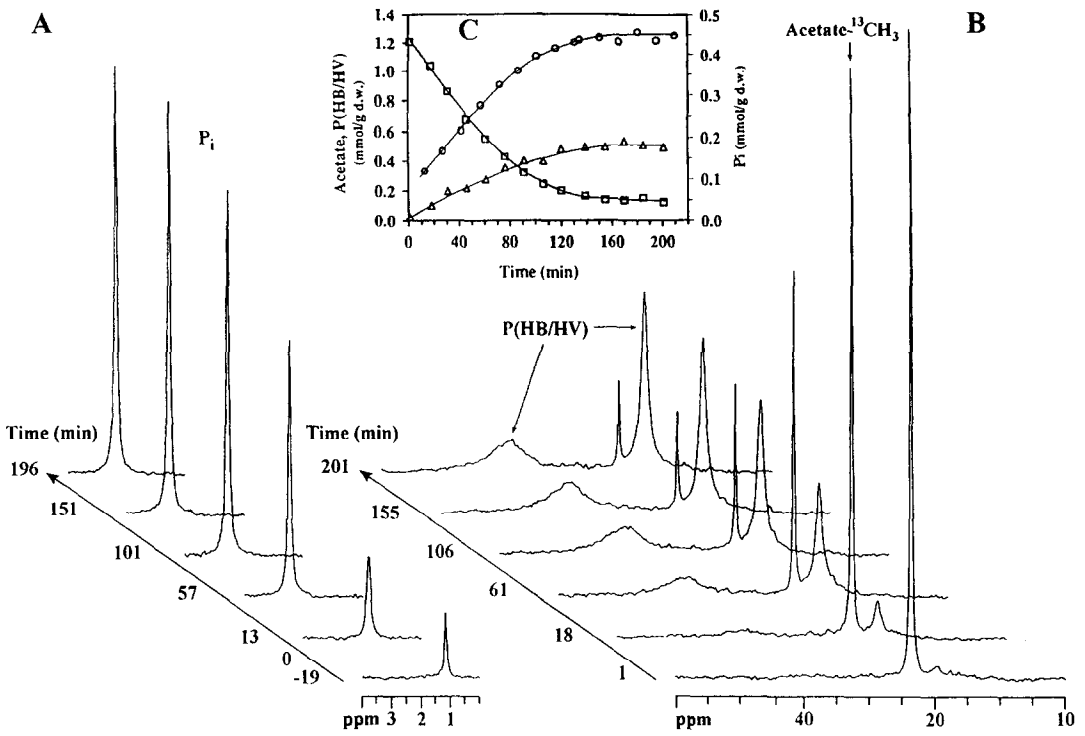


Fig. 2. Time course of phosphate release, acetate consumption and P(HB/HV) formation by activated sludge under anaerobic conditions as monitored by *in vivo* ^{31}P-NMR (spectra **A**) and ^{13}C-NMR (spectra **B**), respectively. The corresponding data are shown in inset **C**. The cell suspension was supplied with [2-^{13}C]acetate at time zero. Spectra were acquired sequentially at the times indicated, alternating between phosphorus and carbon detection. Each spectrum represents 8 min of acquisition for ^{13}C-NMR and 7 min for ^{31}P-NMR. The HB content was determined from the intensities of the resonances due to the methyl group of hydroxybutyrate at 19.7 ppm, after correction for signal saturation; HV was calculated from the measured HB/HV molar ratio of 2.8. Symbols: inorganic phosphate (○); acetate (□); P(HB/HV) (△). Resonance assignments: $CH_3(B_4)$ in P(HB/HV), 19.7 ppm; $CH_2(B_2, V_2)$ in P(HB/HV), 40 ppm; methyl group in [2-^{13}C]acetate, 23.9 ppm.

decrease of P(HB/HV) is clearly observed. Glycogen becomes isotopically enriched only at positions 1, 2, 5 and 6.

From 1 C-mol P(HB/HV) consumed, 0.44 C-mol glycogen were synthesized as calculated from the data illustrated in Fig. 4 and taking into account that 40% of the glycogen pool was not detected by NMR (see below). The molar ratio of P(HB/HV) consumption to phosphate uptake was 1.92 and from 1 C-mol acetate consumed in the anaerobic stage, 0.36 C-mol glycogen were produced in the following aerobic stage.

Polyphosphate was not observed in any of the ^{31}P-NMR spectra of the living cells; the reason for this is the high degree of immobilization of polyphosphate due to long chains and complexation with cations such as Mg^{2+} and K^+ (Roberts, 1987). However, when sludge obtained at the end of the aerobic phase was treated with NaOH, polyphosphate became readily detectable by NMR (Fig. 5).

Second anaerobic stage

In some *in vivo* NMR experiments, cells were submitted to a second anaerobic stage where non-labelled acetate was supplied. The carbon profile observed during this second anaerobic period is illustrated in Fig. 6 for an experiment where 20 mM non-labelled acetate was provided at time zero, when the gas atmosphere was changed from air back to argon. This type of experiment allowed direct monitoring of both the glycogen consumption and the synthesis of labelled compounds derived from glycogen which became labelled during the preceding aerobic stage. This information was not available in the first anaerobic stage since then glycogen was not isotopically enriched and the concentration accumulated was not high enough to allow detection of ^{13}C in natural abundance.

The build-up of HB and HV units synthesized from labelled glycogen was evaluated from the increase in the resonances at 19.7 and 9.5 ppm which are due to the respective methyl groups (Fig. 6); the time course for the utilization of glycogen and synthesis of HB and HV units is plotted in the same figure. The resonance at 161 ppm is due to labelled bicarbonate that is also produced in the anaerobic stage.

Determination of NMR detectability of P(HB/HV) and glycogen

When dealing with quantification of intracellular metabolites *in vivo*, it is essential to take into account

the possibility for partial NMR invisibility of the intracellular compounds under study; this phenomenon is caused by restricted mobility of compounds, for instance, in granules. The NMR detectability of P(HB/HV) was evaluated from the quantitation of ^{13}C-label recovered in a chloroform extract of cells previously submitted to an anaerobic stage where a known amount of [2-^{13}C]acetate was supplied. The label recovery in P(HB/HV) in this extract was similar to that found *in vivo* in experiments as that described above (see Fig. 2) and therefore it was considered that P(HB/HV) was fully detected by NMR under the conditions used here. A similar result was obtained by Barnard and Sanders (1989) in an NMR study on the mobility of polyhydroxyalkanoates from different organisms.

In contrast, glycogen in the activated sludge was not fully detected by NMR: from the data obtained in the experiment illustrated in Fig. 6, it was determined that 5 μmol glucose were converted to 4.0 μmol HB and 6.5 μmol HV. Therefore, at least 14.5 μmol of label were recovered in these monomers, whereas only 10 μmol label present in suitable positions of glucose (C_1 or C_6) were detected in the starting labelled glycogen, meaning that at least 30% of label in glycogen was not detected by NMR. The partial detection of glycogen is not a severe drawback here, since the set of experimental data is enough to define the ratio of glycogen consumed to HV formed, and consequently to calculate glycogen detectability (see below).

Biochemical model for carbon metabolism in the anaerobic stage

In the labelling experiments described above, the molar ratio of HB and HV units derived from glycogen was 4.0:6.5 (data in Fig. 6); furthermore, the bulk polyhydroxyalkanoate has an HB/HV ratio of 2.8:1 and is fully detected by NMR. Since no HV units labelled on the methyl group (resonance at 9.5 ppm) are observed during the first anaerobic stage where acetate is the only source of label (Fig. 2), but this resonance is clearly detected during the second anaerobic stage, where glycogen is the sole source of label, it is concluded that the propionyl moiety in HV is derived exclusively from glycogen. A simple calculation based on these experimental data leads to a severe redox unbalance: since for each 65 mol HV labelled on the methyl group, and 40 mol HB units are observed with label on the methyl group, a total of 182 mol HB units must be formed in order to satisfy the experimental (total HB)/(total HV) ratio of 2.8:1. Therefore, since only 40 mol of HB are labelled, the percentage labelling of the methyl group in the acetyl-CoA pool is 22%, the remaining 78% being derived from unlabelled acetate. In addition to the 80 mol of labelled acetyl-CoA required to form 40 mol labelled HB, 14.3 mol are needed to synthesize the labelled acetyl moieties in 65 mol HV units. Since glycogen is the sole source of label, 159.3 mol of labelled pyruvate, and consequently 79.65 mol glucose, must be catabolized to produce P(HB/HV). These values represent a deficit of 123.4 reducing

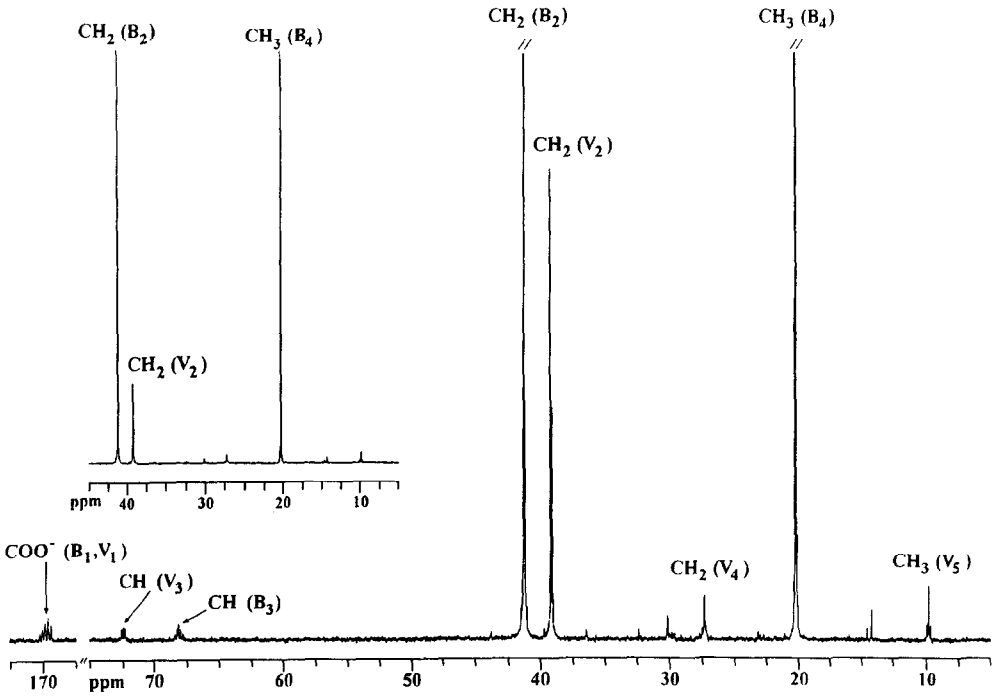


Fig. 3. ^{13}C-NMR spectrum of the co-polymer of hydroxybutyrate and hydroxyvalerate extracted from activated sludge that had been supplied with [2-^{13}C]acetate under anaerobic conditions. The inset represents part of the spectrum plotted with reduction of the vertical scale in order to make evident the intensity ratio of strong peaks.

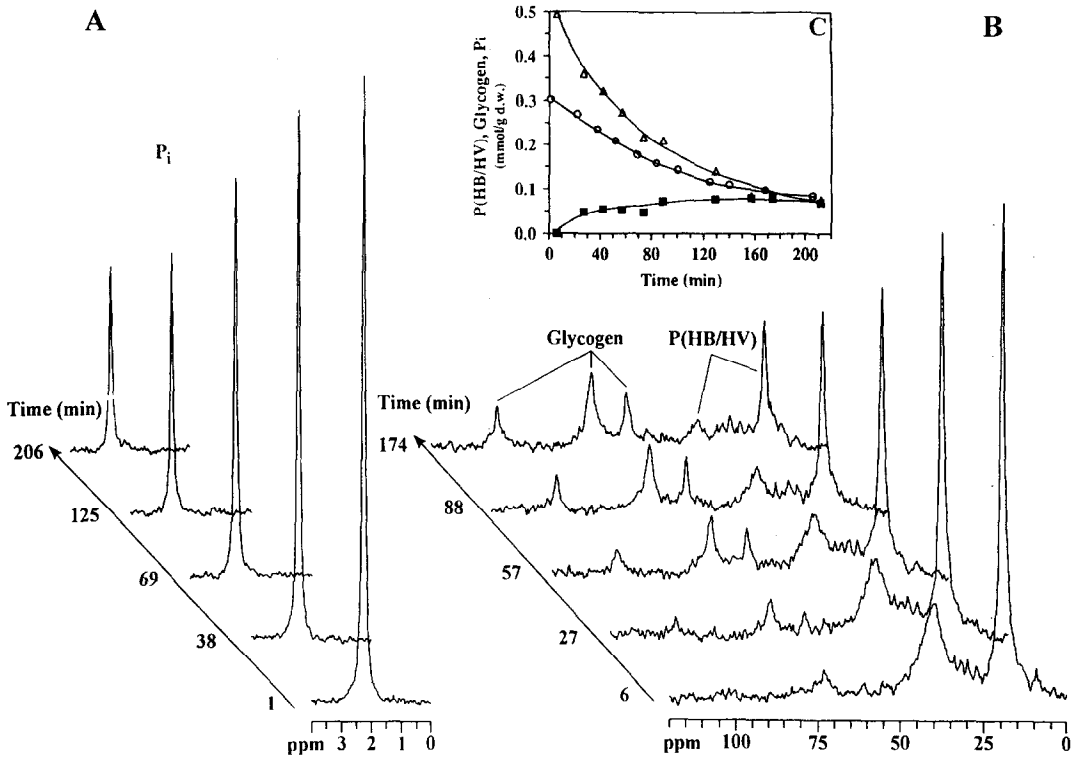


Fig. 4. Time course for the aerobic uptake of phosphate (**A**) and degradation of P(HB/HV) (**B**), by activated sludge under aerobic conditions, as monitored *in vivo* by ^{31}P-NMR and ^{13}C-NMR, respectively. Following the experiment shown in Fig. 2, oxygen was provided at time zero and spectra were acquired sequentially at the times indicated, alternating between phosphorus and carbon detection. Each spectrum represents 8 min of acquisition for ^{13}C-NMR and 7 min for ^{31}P-NMR. The decrease of resonances due to P(HB/HV) at 19.7 and 40 ppm and the build-up of glycogen (C_1 at 100.2 ppm, $C_2 + C_5$ at 71.7 ppm and C_6 at 61.1 ppm) are clearly observed in the ^{13}C-NMR spectra. The corresponding concentration plots are shown in the inset (**C**). Symbols: inorganic phosphate (○); P(HB/HV) (△); glycogen (■). The values represented for glycogen refer to the amounts directly detected by NMR; in order to calculate the total amount the NMR detectability of glycogen (60%) should be taken into account.

equivalents (NADH). In conclusion, it is impossible to fulfil the redox balance with the obtained experimental data unless a source of reducing power, in addition to glycolysis, is considered.

The model proposed in Fig. 7 considers that the required additional reducing equivalents are derived from the TCA cycle through degradation of acetyl-CoA; evidence for operation of the TCA cycle is obtained from the observation of labelled bicarbonate formed not only during the aerobic stage as expected, but also during both anaerobic stages (Fig. 6, resonance at 161 ppm); in the first anaerobic stage the amount of labelled bicarbonate produced is significantly lower, since the corresponding resonance could be detected only when bubbling of argon through the cell sample was stopped (data not shown). The detection of that compound in the first anaerobic stage is particularly relevant here, since in this case, the only source of label is acetyl-CoA and no decarboxylation of this metabolite occurs in the anabolic process leading to the formation P(HB/HV) (Dawes and Senior, 1973). A similar type of argument would apply to the second anaerobic stage in the case where glycogen would be catabolized via the Embden–Meyerhof pathway (pyruvate, succinate or oxaloacetate derived from glycogen are labelled only on C_2 and C_3). However, the possible contribution of the Entner–Doudoroff pathway would lead to production of labelled CO_2 in the decarboxylation step from pyruvate to acetyl-CoA, since half of the labelled pyruvate molecules would be enriched in the C_1 position (Gottschalk, 1988).

Our model assumes that the synthesis of HB and HV proceeds via the metabolic pathways previously proposed to occur in similar phosphorus removing systems (Arun *et al.*, 1989; Matsuo *et al.*, 1992; Satoh *et al.*, 1992). The carbon fluxes indicated in Fig. 7 were calculated on the assumption that reducing power equivalent to 4 NADH molecules is formed in each turn of the TCA cycle. According to this experimental model, 82.7 mol glucose and 356.4 mol acetate are utilized in order to produce 182 mol HB and 65 mol HV. The conversion ratios involving glycogen can finally be calculated: 0.47 C-mol glucose and 0.68 C-mol acetate are used to synthesize 1 C-mol P(HB/HV). Furthermore, according to this model, 1 C-mol non-labelled acetate is utilized in order to synthesize 1.15 C-mol of P(HB/HV), where HB refers only to the units with non-labelled methyl group i.e. 78% of the total HB

pool; this value compares well with the equivalent experimental ratio of 1 C-mol labelled acetate: 1 C-mol P(HB/HV) directly measured in the first anaerobic stage in which labelled acetate was supplied (Fig. 2). This agreement increases the confidence level in our model.

From these calculations, it is concluded that approximately 40% of the glycogen pool in the cells is not detected by *in vivo* ^{13}C-NMR under the experimental conditions used here. Values of up to 70% have been previously reported for the NMR invisibility of glycogen in rat liver (Künnecke and Seelig, 1991).

DISCUSSION

The labelling experiments described above definitely demonstrate the involvement and the role of glycogen in the biological phosphorus removal process. By supplying the ^{13}C label on the methyl group of acetate at the beginning of the anaerobic stage, it was possible to trace by ^{13}C-NMR *in vivo* the fate of the label through the subsequent aerobic/anaerobic stages. This capability of the method derives from the fact that only labelled materials are detected under the short time acquisitions used here. Thus, it was possible to follow the flux of label from acetate to P(HB/HV) in the first anaerobic stage (Fig. 2), then to monitor the conversion of the polymer into glycogen in a subsequent aerobic stage (Fig. 4), and afterwards, by submitting the same sludge to a second anaerobic stage, to observe the flux of labelled carbon from glycogen to HV and HB units of P(HB/HV) (Fig. 6).

The labelling pattern of glycogen on positions C_1, C_2, C_5 and C_6 is in accordance with synthesis from [2-^{13}C]acetyl-CoA via the glyoxylic acid pathway to form oxaloacetate (labelled on C_2 and C_3), and further gluconeogenesis. The fact that the propionyl moiety of HV does not become labelled directly from labelled acetyl-CoA in the first anaerobic stage, but labelling occurs during the second anaerobic stage, shows that the propionyl moiety is directly derived from glycogen which is the sole source of label at that stage (Fig. 6). These results indicate either that the glyoxylic acid cycle does not operate during anaerobiosis or its contribution is negligible.

The quantitative data obtained in these experiments cannot be fitted to a model that considers glycogen as the only source of reduction power for the synthesis of P(HB/HV); a metabolic model which fits our experimental data and includes the operation of the TCA cycle under anaerobic conditions was elaborated (Fig. 7). Early in 1986, the Comeau–Wentzel model assumed the involvement of the TCA cycle in the anaerobic phase; this proposal was weakened by later studies suggesting the involvement of glycogen in support of the Mino model. The TCA cycle involving model was initially challenged on the argument that this cycle would not operate in the absence of oxygen (Mino *et al.*, 1987), but an increasing volume of strong contradictory evidence to this dogma has accumulated in recent years (see Thauer (1988) for a review). A complete TCA cycle has been found to operate, for instance, in the anaerobic eubacteria *Desulfobacter postgatei* (Brandis-Heep *et al.*, 1983; Gebhardt *et al.*, 1983), *Desulfuromonas acetoxidans* (Gebhardt *et al.*, 1985; Schmitz *et al.*, 1990) and *Desulfurella acetivorans* (Schmitz *et al.*, 1990), and more recently also in the anaerobic archae *Thermoproteus tenax* (Selig and Schönheit, 1994) and *Pyrobaculum islandicum* (Selig and Schönheit, 1994).

Our data show that the reducing equivalents required for the synthesis of P(HB/HV) are derived both from glycogen catabolism and from the TCA cycle. In the models available in the literature the involvement of glycogen has been invoked as an essential supply of NADH. Since the TCA cycle is a powerful source of reducing equivalents, one might wonder about the physiological meaning of the aerobic conversion of P(HB/HV) to glycogen. But indeed, this interconversion of carbon reserves represents a good investment in metabolic terms, since glycogen fermentation can supply the cell not only with reducing power in the anaerobic phase, but also with ATP and biosynthetic precursors. According to our model, the TCA cycle generates approximately 30% of the reducing equivalents required for the

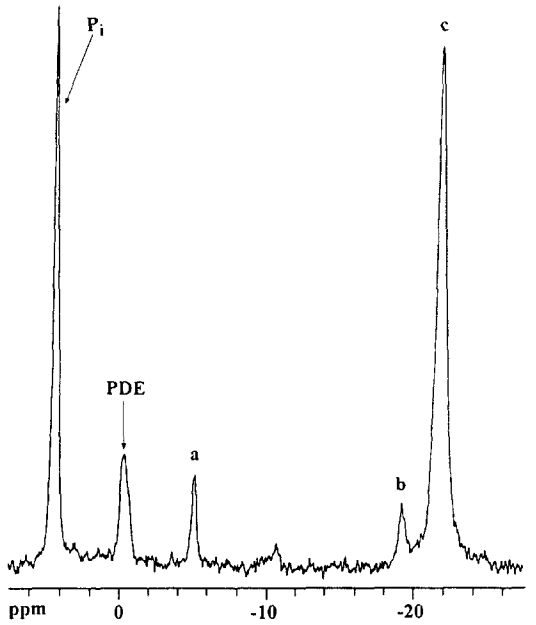


Fig. 5. ^{31}P-NMR spectrum of activated sludge removed from the reactor at the end of the aerobic stage. The pH of the cell suspension was adjusted to 12. Resonances due to polyphosphate were detected only at alkaline pH and not under physiological conditions. Resonance assignments: terminal phosphate group of polyphosphate (a), penultimate phosphate group of polyphosphate (b) and inner phosphate group of polyphosphate (c). The resonances due to membrane phosphodiesters and inorganic phosphate are labelled PDE and P_i, respectively.

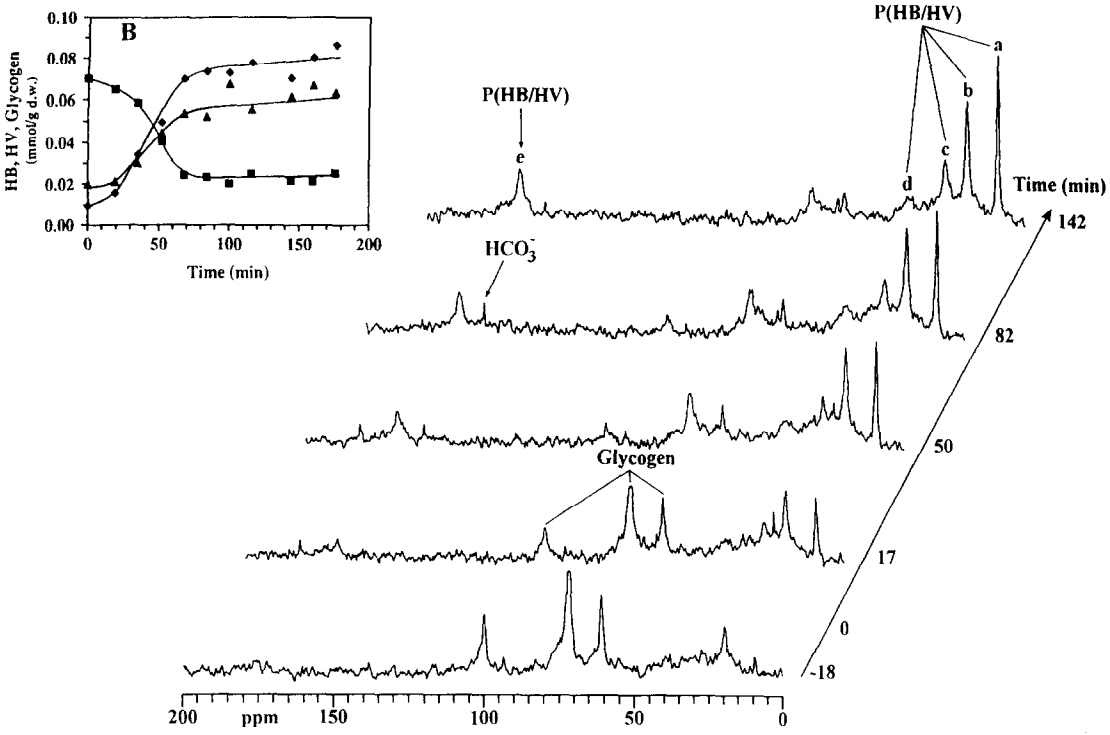


Fig. 6. Time course for the consumption of glycogen and biosynthesis of P(HB/HV), by activated sludge, as monitored *in vivo* by ^{13}C-NMR under anaerobic conditions (A). Following the experiment illustrated in Fig. 4, the gas atmosphere was changed to argon, and at time zero 20 mM unlabelled acetate was added. Each spectrum represents 8 min of acquisition for ^{13}C-NMR. Resonance assignments: P(HB/HV), (a) $CH_3(V_5)$ at 9.5 ppm, (b) $CH_3(B_4)$ at 19.7 ppm, (c) $CH_2(V_4)$ at 27.1 ppm, (d) $CH_2(B_2, V_2)$ at 40 ppm and (e) $COO^-(B_1, V_1)$ at 169.3 ppm (see chemical structure for group reference). Glycogen C_1 at 100.2 ppm, $C_2 + C_5$ at 71.7 ppm and C_6 at 61.1 ppm, and bicarbonate at 161 ppm are clearly observed in the ^{13}C-NMR spectra. (B): plot of the time courses of glycogen (■) consumption and synthesis of labelled HB units (▲) and HV units (◆). Glycogen values refer to the amounts directly detected by NMR; the total amount can be calculated taking into account that only 60% of the glycogen pool is detected.

synthesis of P(HB/HV) and the remaining 70% are supplied by glycogen degradation.

There is an open debate on which glycolytic pathway is utilized by the phosphorus removing organisms. In several studies, glycolysis was assumed to proceed via the Entner–Doudoroff pathway since this has been demonstrated to occur in *Acinetobacter* species (Juni, 1978), organisms populating activated sludge systems (in our case the population of *Acinetobacter* species is only 9%). Unfortunately, the present study cannot contribute to the elucidation of this point, since the labelling pattern of glycogen is such that both the Embden–Meyerhof and the Entner–Doudoroff pathways would lead to identical labelling in the final products, HV and HB.

The value for the HV content reported here is approximately 26%. Few studies in the literature report on the HV content of the polyhydroxyalkanoate polymer in activated sludge, and many assume that only PHB is formed. Values around 10% have been reported in cells supplied with acetate (Satoh *et al.*, 1992; Matsuo *et al.*, 1992) and higher values were found only when substrates such as lactate or propionate were used as carbon sources (Satoh *et al.*, 1992).

As previously pointed out by other authors, a wide range of values for the phosphate:acetate molar ratio is found in the literature, ranging from 0.29 (Mino *et al.*, 1987) to 0.87 (Satoh *et al.*, 1992). In our experiments, a value of 0.75 was found with cells derived from reactors operating with a biomass retention time of 32 days (Fig. 1), but lower ratios (0.32) were observed in all the labelling experiments reported here which were performed with sludge from reactors with 10 days biomass retention time. Recently, a proposal based on the pH dependence of the energy required to transport acetate to the intracellular space was put forward to explain the observed variance in the ratio (Smolders *et al.*, 1994a). This argument is insufficient to explain the low values obtained in our study, since the external pH was always higher than 7.0.

The presence of non-polyphosphate accumulating bacteria that are able to use glycogen both as a source of reducing power for the synthesis of polyhydroxyalkanoates, and of ATP for the activation of acetate (Cech and Hartman, 1993; Satoh *et al.*, 1992) would necessarily lead to deterioration of the phosphate:acetate ratio. Although the presence of this kind of bacteria can not be ruled out completely, the fact that polyphosphate was unequivocally identified in the biomass (Fig. 5) provides clear evidence for the presence of polyphosphate accumulating bacteria.

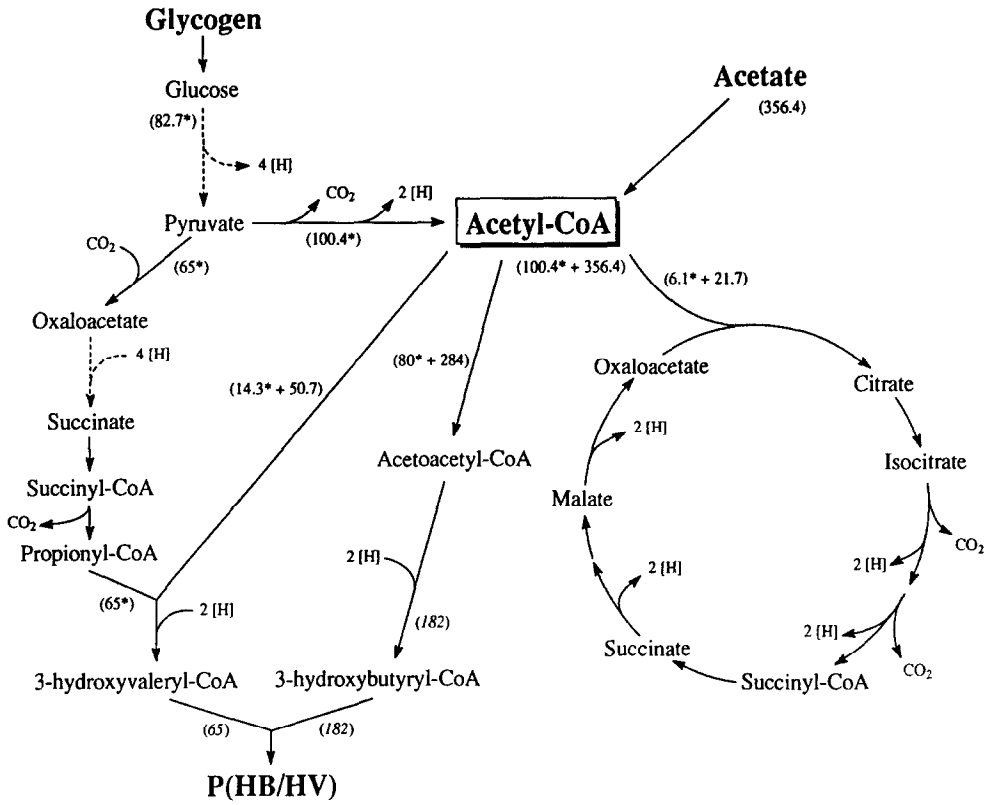


Fig. 7. Metabolic model for conversion of acetate and glycogen to P(HB/HV) by activated sludge under anaerobic conditions; metabolic fluxes were calculated from the NMR labelling experiments described in the text and are indicated in brackets. The values indicated with an asterisk refer to the pools of labelled metabolites derived from labelled glycogen.

Furthermore, the five-fold increase in the release of phosphate observed when acetate is supplied (Fig. 1) is a result in line with the occurrence of coupling between acetate uptake and phosphate release in this system.

According to our model, the overall conversions during anaerobic and aerobic stages are as follows: with 1 C-mol acetate, 1.48 C-mol P(HB/HV) are synthesized and 0.70 C-mol glycogen are degraded anaerobically while 0.16 P-mol phosphate is released. Production of CO_2 (or bicarbonate) was not measured, but this model predicts the formation of 0.22 C-mol per C-mol acetate used (Fig. 7). In the aerobic stage, 1 C-mol of P(HB/HV) is converted to 0.44 C-mol glycogen. These values can be directly compared with those found in the literature. A detailed study on anaerobic and aerobic stoichiometries has been done recently (Smolders *et al.*, 1994a, b) and the agreement with our data is reasonably good. The major difference refers to the amount of glycogen mobilized which is higher in our study (but similar to those reported by Satoh *et al.* (1992) and Matsuo *et al.* (1992)), and to the amount of phosphate released which is lower. This trend is actually in accordance with the fact that the polyhydroxyalkanoate synthesized by our system is significantly richer in HV units and the phosphorus content in sludge is lower (2–3%).

In conclusion, the results reported here clearly demonstrate the involvement of glycogen in the biological phosphorus removal process; furthermore, the data corresponding to the anaerobic phase are explained on the basis of a biochemical model that considers the TCA cycle as an additional source of reduction power for the synthesis of polyhydroxyalkanoates.

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