J O H A N N E S B U R G C I T Y C O U N C I L

CITY HEALTH AND CITY ENGINEER'S
DEPARTMENTS

REPORT TO THE WATER RESEARCH COMMISSION ON A TWO YEAR STUDY ON THE ENHANCEMENT OF BIOLOGICAL PHOSPHATE REMOVAL BY ALTERING PROCESS FEED COMPOSITION (METABOLIC CONTROL MECHANISMS)

BY

L H LÖTTER

MARCH 1989

ISBN 0 947447 21 0

N O T I C E

The mention of trade names of commercial products in this publication, is for illustration purposes and does not constitute endorsement or recommendation for use by the City Council of Johannesburg, or the Water Research Commission. The opinions expressed in this report are those of the author and do not necessarily reflect the views of the City Council of Johannesburg or the Water Research Commission.

Copies of this report are obtainable from the Water Research Commission, P O Box 824, PRETORIA, 0001 Tel : (012) 330-0340 or Fax : (012) 70-5925.

This report was submitted to the Rand Afrikaans University, as a thesis in partial fulfilment of the requirements for the PhD degreed in Biochemistry and was translated by the author.

Further information may be obtained from the author at Johannesburg City Health Department, P O Box 1477, Johannesburg, 2000. Tel : (011) 728-7373 or Fax : (011) 728-7373.

REPORT BY THE JOHANNESBURG CITY COUNCIL ON A TWO YEAR STUDY ON THE ENHANCEMENT OF BIOLOGICAL PHOSPHATE REMOVAL BY ALTERING PROCESS FEED COMPOSITION (METABOLIC CONTROL MECHANISMS)

EXECUTIVE SUMMARY

1 PROJECT AIMS

During the two year extension of the Contract, the aim of the work described in this report was to confirm postulates contained in the biochemical model of biological phosphorus removal. The model requires that control occurs by feedback mechanisms. If it is accepted that metabolic control occurs by means of feedback inhibition, then the arguments of the model can be summarised as follows :-

- Anaerobic conditions

  Uptake of acetate causes phosphate release, acetate is converted to polyhydroxybutyrate, acetate is oxidised by the tricarboxylic acid cycle to provide NADH for PHB synthesis. Initial high levels of NADH stimulate PHB synthesis. Low ATP levels stimulate polyphosphate degradation to provide the cell with energy.

- Aerobic conditions

  Phosphate is taken up and high ATP levels stimulate conversion to polyphosphate. The low NADH levels stimulate degradation of PHB. Lack of external carbon leads to low acetyl co-enzyme levels which stimulate PHB degradation.

The aim of this study was therefore to investigate the following :-

- Nature of metabolic control which is maintained in Acinetobacter under activated sludge conditions.
- Uptake and metabolism of acetate under anaerobic conditions.
- Dependance of phosphate release on acetate uptake.
- Polyphosphate metabolism under anaerobic and aerobic conditions.
- Polyhydroxybutyrate metabolism under anaerobic and aerobic conditions.
- Tricarboxylic acid and glyoxylic acid cycle activity under anaerobic conditions.

2 RESEARCH WORK

The dominant phosphate removing bacteria were isolated from an activated sludge plant and tested for their ability to accumulate polyphosphate and polyhydroxybutyrate. One of these isolates was selected for further study.

Key enzymes in polyphosphate and polyhydroxybutyrate metabolism were studied under different environmental conditions. Studies were also carried out to ascertain the type of metabolic control mechanisms prevailing in the phosphate removing bacteria.

3 CONCLUSIONS

Polyphosphate Metabolism

The oxygen level and the nature of the carbon source, both had an effect on polyphosphate synthesis and degradation. Polyphosphate was degraded in the presence of substrate in the absence of oxygen and accumulated under aerobic conditions.

The most important synthesis enzyme, polyphosphate kinase, was observed in the polyphosphate accumulating bacterium studied.

The modification of enzyme activity by the carbon growth source which was observed, emphasizes the relationship between polyphosphate metabolism and other intracellular processes.

Carbon Metabolism

Acetate uptake under anaerobic conditions was observed in the Acinetobacter calcoaceticus var lwoffi isolate. Under anaerobic conditions polyhydroxybutyrate was accumulated, but during the subsequent aeration, the polyhydroxybutyrate was degraded. The same synthesis and degradation pattern was observed under sequential anaerobic and aerobic conditions in activated sludge plants.

Several metabolites had a modifying effect on β-hydroxybutyrate and isocitrate dehydrogenase, both of which are key enzymes in carbon metabolism.

Metabolic Control

The sequential exposure of the Acinetobacter cells to anaerobic and aerobic conditions, did not exert a direct modifying effect on enzyme activity. In other words, enzyme synthesis was not altered and enzymes were not phosphorylated.

The intracellular environment in respect of nicotinamide adenine dinucleotides and adenosine phosphates, is directly dependent on the external oxygen level. The fluctuation in the levels of these metabolites is responsible for the fine metabolic control which is practised by this organism, to survive sequential anaerobic/aerobic conditions.

Biochemical Model

The model hypothesizes that the aerobe, Acinetobacter, takes up

acetate under anaerobic conditions and converts it to polyhydroxybutyrate. Energy for activation of the acetate is derived from polyphosphate degradation. The tricarboxylic acid cycle is sequentially stimulated and inhibited under these conditions by the alteration in the NADH/NAD ratio.

These studies have shown that a positive relationship exists between polyphosphate degradation and acetate uptake under anaerobic conditions. These studies have also shown that the anaerobic phase does not result in chemical modification of the enzymes or alteration in their synthesis, thus confirming the model hypothesis of enzyme regulation by metabolic feedback.

These studies confirmed the synthesis of polyphosphate under aerobic conditions after anaerobic substrate uptake. Polyhydroxybutyrate degradation under aerobic conditions was also confirmed. Under these conditions, the nicotinamide adenine dinucleotide is oxidised, which leads to the cessation of the inhibition by NADH. β - hydroxybutyrate dehydrogenase is also inhibited by acetyl CoA and oxaloacetate. Acetyl CoA enters the tricarbxylic acid cycle by a condensation reaction with oxaloacetate. The inhibitory effect of these two metabolites on PHB degradation indicates that if the tricarboxylic acid cycle is operating maximally, the degradation of polyhydroxybutyrate is not necessary.

The results of this study also provide fundamental reasons for operational observations which are already well-known.

The presence of nitrate in the anaerobic zone provides certain organisms with a terminal electron acceptor. Acetate will thus be used for denitrification and will not be converted to polyhydroxybutyrate. Synthesis of this accumulation product requires acetyl CoA and high NADH levels, both of which will not be available under denitrifying conditions. The absence of accumulated carbon and thus, energy source, reduces the organism's ability as a result of the lack of an energy source to take up phosphate and accumulated polyphosphate under aerobic conditions.

Polyphosphate accumulation is also dependent on stimulation of polyphosphate kinase, which occurs by simultaneous phosphate release and substrate uptake under anaerobic conditions.

The correct aeration of the aerobic zone is fundamental to the success of this process. Without sufficient oxygen, the electron transfer path and oxidative phosphorylation to form ATP, will not function satisfactorily. Polyhydroxybutyrate will not be degraded and the anaerobic process of acetate uptake will be deleteriously affected. Excess ATP conversion to polyphosphate will also not occur.

4 RECOMMENDATIONS FOR FUTURE WORK

Although considerable progress has been made in understanding the underlying processes and full-scale success of biological phosphate removal has been achieved, further research is essential in this area, in order to maintain wastewater treatment in South Africa on the level which is essential for the future expansion of the country's industries :-

- Data obtained from theoretical studies must be consolidated into operational protocols.

- Operational observations under new design configurations should be incorporated in the biochemical and mathematical models to ensure that these models are maintained at the forefront of technological advances in this field.

TABLE OF CONTENTS

CHAPTER THREE

EXPERIMENTAL PROCEDURES

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

CHAPTER 6

ACKNOWLEDGEMENTS

The research and investigational work described in this report was carried out during the period 23 August 1983 to 31 December 1988, and was financed by the Water Research Commission, in partnership with the Johannesburg City Council. The project was guided by a Steering Committee constituted as follows :-

Dr H N S Wiechers (Chairman)	
Replaced by Mr J A McGlashan	Water Research Commission
Mr J Goodman	Johannesburg City Council
Mr D W Osborn	Johannesburg City Council
Professor G v R Marais and	
Prof G Ekama	University of Cape Town
Mr A Gerber	Division of Water Technology (CSIR).

This work could not have been carried out without the constant support and encouragement of the Deputy Town Clerk:Technical, the City Engineer and the Medical Officer of Health, whose Departments were involved in the execution of this Contract.

Particular thanks are due to the following :

- E van der Merwe, for special technique development and analyses.
- Professor J C Schabort and Dr I A Dubery of RAU Biochemistry Department, for their guidance in respect of enzymatic studies.
- M Fourie, for microbiological work.
- M Annandale and G van der Merwe for the efficient control of the Contract funds.
- G v R Marais, G Ekama, P Dold, R E Loewenthal and M Wentzel of the University of Cape Town, for stimulating discussion and advice.
- P W Weideman and P E Marais for secretarial services to the Steering Committee.
- E de Beer, for assistance in the preparation of many of the diagrams which appear in this report, and typing.
- Professor Hilliard S Hurwitz, Medical Officer of Health, Johannesburg, and the Water Research Commission for permission to submit the research work for degree purposes.

ABBREVIATIONS AND SYMBOLS

α	Alpha
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
anaerobic	no oxygen - no nitrate present
aerobic	oxygen present
anoxic	oxygen present - nitrate present
β	Beta
CoA	Co-enzyme A
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid disodium salt
FAD	Flavin adenine dinucleotide
g	Gram
γ	Gamma
h	hour
H	Hydrogen
K	Potassium
K_m	Michaelis constant

M	Molar
mg	milligram
Mg	Magnesium
mℓ	millilitre
mM	milliMolar
Mℓ	Megalitre
MM	Molecular mass
mRNA	Messenger ribonucleic acid
N	Nitrogen
Na	Sodium
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
O	Oxygen
P	Phosphorus
PHB	Polyhydroxybutyrate
Poly P	Polyphosphate
RNA	Ribonucleic acid
S	Sulphur
STEMEDAX	Scanning transmission electron microscopy coupled to energy dispersive analysis of X-Rays
TKN	Total Kjeldahl Nitrogen
Tris	Tris-(hydroxymethyl) amino methane
Y	Growth yield
µg	microgram
µℓ	microlitre
µM	microMolar

CHAPTER ONE

Introduction

Rapid population growth and industrial development, spurred on by modern exploitation of natural resources, can pose serious threats for the environment.

By considering a total raw sewage flow of 4 700 Mℓ/d, Wiechers (1987) calculated that the pollution load in respect of chemical oxygen demand, phosphorus and nitrogen on the water environment in 1982 was already 271 t COD/d, 25 t P/d and 125 t N/d respectively.

This pollution load will rise until the limited water sources can no longer assimilate it satisfactorily. It is already expected that future demands for water in the Republic of South Africa will exceed the economically viable sources within 2 to 3 decades (Hart and Allanson, 1984).

Water remains essential for the future socio-economic development of the Republic. Prevention of further deterioration of water sources and even improvement of existing water quality, is essential for continued economic development. Surveys on the condition of storage dams in South Africa have emphasized the increasing eutrophication of the systems (Toerien et al., 1975; Foundation for Research and Development, 1985). One of the reasons for the increasing eutrophication is that South African legislation requires that effluents be returned to the rivers of origin after treatment (Government Gazette, 1956). This requirement was supposed to encourage optimal utilisation of water, but the lack of standards for nitrogen and phosphorus levels in the effluents caused serious eutrophication problems in South African dams.

Eutrophication can be defined as excess plant growth as a result of enrichment of the water by nutrients (particularly nitrogen and phosphorus)(Hart and Allanson 1984).

In a survey of 98 South African dams it was found that 11 % showed signs of serious eutrophication. The hypertrophic Hartbeespoort Dam suffers from a number of aesthetic and water quality problems. The blue-green algae Microcystis aeruginosa build up in large floating mats which considerably decreases the recreational value of the dam. The algal yield of this dam is the highest in the world. As a result of the unfavourable environment, the fish population comprises only three species (Foundation for Research and Development, 1985). The lower Vaal River is also experiencing problems with Eichornia crassipes and large algal populations which, as in the case of dams, has a deleterious effect on the recreation potential (Wiechers and Best, 1985).

A further problem is the additional treatment cost which eutrophication has created at a number of water treatment plants (Viljoen, 1984; Taylor et al., 1984).

In most South African water systems, phosphate is the limiting nutrient (Toerien et al., 1975). Removal of phosphate should therefore, control eutrophication and the resultant excess plant growth.

In 1980 the Water Act was amended to make provision for an orthophosphate concentration of 1mg P/ℓ in effluents in certain catchment areas (Government Gazette, 1980). The implementation of this limit was postponed until August 1985 to give local authorities more time to implement the technology of phosphorus removal (Wiechers and Best, 1985).

Phosphate can be removed from wastewater in two ways, namely, through chemical precipitation or biological activity. Osborn et al., (1986) showed that the additional cost of phosphorus removal by chemical precipitation ran to 2,47 c/kℓ, in comparison with 1,93 c/kℓ for the biological method.

Although the phosphate removal capacity of the approximately thirty plants which were specially designed for nutrient removal is disappointing, (only one removes phosphate to below the limit on a continuous basis), (Wiechers et al., 1984), Pitman (1983) showed that biological phosphate removal is possible with the activated sludge process.

Johannesburg finds itself in an unenviable position in that it is situated in the middle of two drainage systems, namely, one flowing southwards to the Vaal River and the second northwards, to the Hartbeespoort Dam (see Figure 1.1).

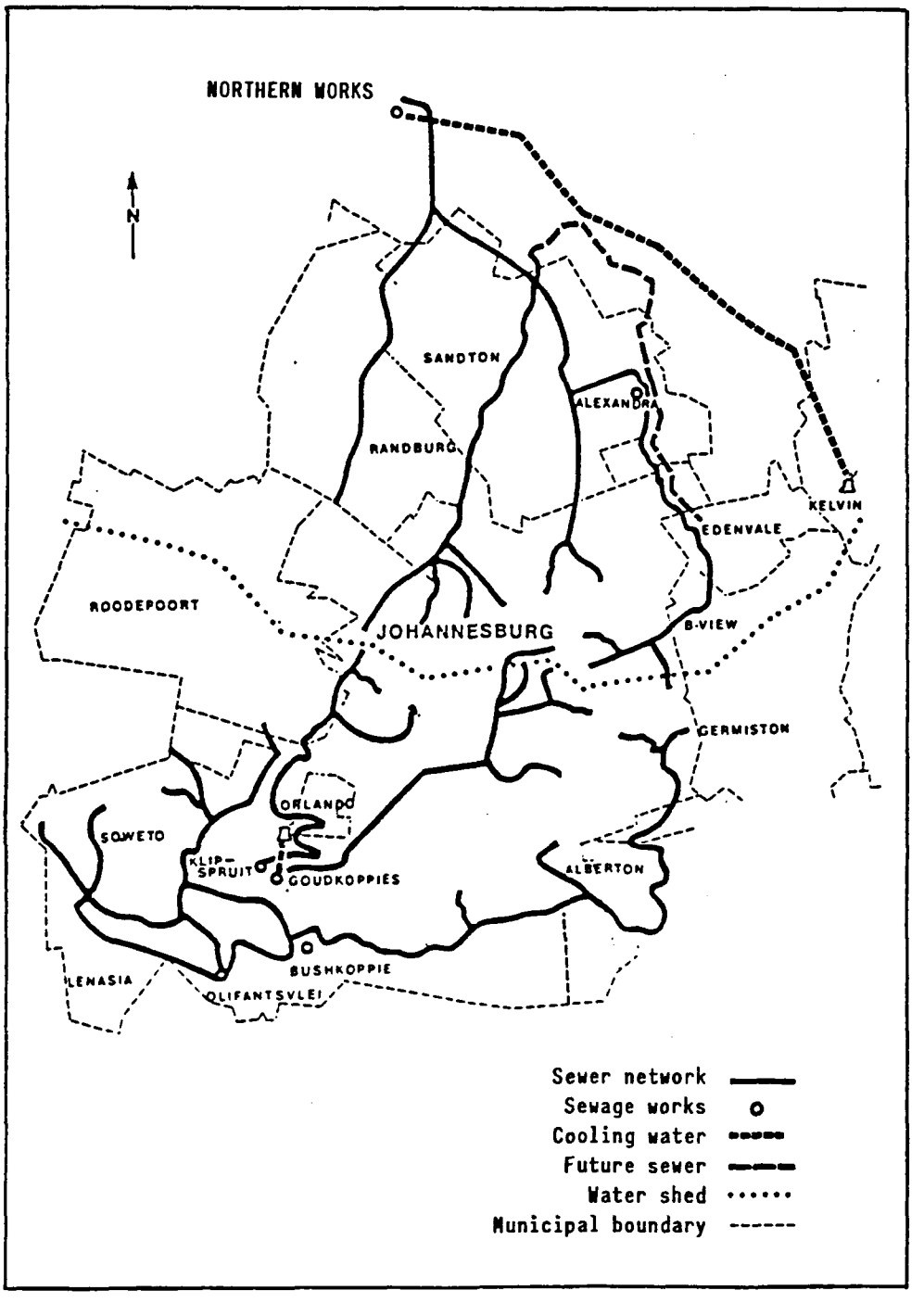


Figure 1.1 : Johannesburg's drainage system

Both are sensitive catchment areas where the strict 1 mg P/ℓ limit is mandatory (Government Gazette, 1980). Grobler and Silberbauer (1984) predicted that to prevent serious nuisance conditions at Hartbeespoort Dam in the future, a limit of 0,5 mg P/ℓ will be necessary. It is thus essential that Johannesburg applies phosphate removal technology in the most cost-effective manner.

Approximately 400 Mℓ of sewage is treated daily by the Johannesburg City Council. Only 200 Mℓ of this is treated by nutrient removal plants. In order to keep the cost of sewage purification as low as possible, it is essential that biological phosphate removal be applied successfully to this 200 Mℓ.

The aim of this study wass to gain more information on the metabolic control mechanisms which underlie the process. This knowledge can then be applied to the more efficient operation of plants.

At the same time, plant and laboratory scale investigations were undertaken. The results of the latter studies have been reported by Osborn et al, (1989).

The problems which are currently being experienced with metabolic control mechanisms in the activated sludge process, are discussed in detail in Chapter 2.

CHAPTER TWO

Literature survey

2.1 Introduction

The aim of any biological water treatment process is to purify the wastewater by using the organic and inorganic nutrient components of the wastewater as a food source for micro-organisms. In order to achieve this goal the process must provide the micro-organisms with a well-balanced diet as well as a suitable environment for the utilisation of the nutrients.

The activated sludge process was originally designed in England, in 1914, so-called because the process involves the production of an activated mass of organisms. Originally, organic waste was added to a reactor which contained a bacterial suspension under aerobic conditions (Metcalf and Eddy, 1979). The original design has since undergone a number of changes, among others, the inclusion of anaerobic (no oxygen or nitrate), and anoxic (no oxygen, but nitrate) zones. See Figure 2.1 for a schematic representation of the three-phase Bardenpho Process.

The original application of the activated sludge process was to remove carbon compounds. Later, nitrification and denitrification was also accomplished using this process. In an attempt to remove nitrogen successfully, Barnard suggested a four-phase process in 1973. This process, which is known as the Bardenpho Process, was later patented.

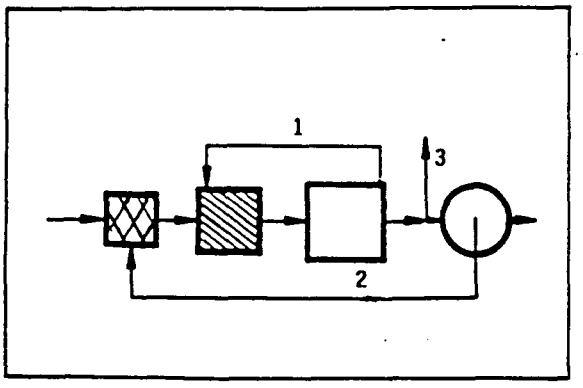


Figure 2.1 : A schematic representation of the three-phase Bardenpho process Anaerobic ▨ ; anoxic ▨ ; aerobic □ ; 1: return mixed liquor; 2: return activated sludge; 3: waste

This process can also be operated as a five-phase system. Increased phosphate removal with this configuration was later reported by Barnard (1975).

2.2 The Bardenpho Process

In this process sludge organisms are sequentially subjected to anaerobic, anoxic and aerobic conditions to promote maximum uptake of carbon, nitrogen and phosphorus. The optimum relationship of carbon (in terms of COD), to nitrogen and phosphorus (COD : N : P) for microbial growth is given as 100 : 5 : 1 (Langley et al., 1968), and 100 : 2 : 0,24 (Simpson and Hemens, 1973). The annual average COD : N : P ratios for the Johannesburg Goudkoppies and Northern Works were 79 : 6 : 1 and 40 : 4 : 1 respectively, in 1984 (Chief Director : Technical Services, 1985). By comparing these figures with the optimal ratio for bacterial growth, it is clear that all the nitrogen and phosphorus cannot be incorporated in the biomass by normal aerobic metabolism.

In addition to the shortage of carbon relative to nitrogen and phosphorus, the assimilability or availability of these nutrients must be taken into account. If the COD, nitrogen and phosphate levels in the final effluent of the Johannesburg Goudkoppies and Northern Works plants are studied (see Table 2.1), it is clear that

differences exist in the assimilability of carbon in these two plants, and that the efficiency of phosphate and nitrogen removal differs significantly.

TABLE 2.1
FINAL EFFLUENT COMPOSITION

Plant	COD as O		mg/ℓ Ammonia as N		Nitrate as N		Orthophosphate as P	
	min	max	min	max	min	max	min	max
Northern Works Module 3	29	140	2,4	13	0	11	0	9,4
Goudkoppies	36	190	3,2	13	0	7,1	0	1,1

Although this study is limited to the interactions between carbon and phosphate metabolism, which will be discussed later, it is useful to consider certain aspects of nitrogen metabolism at this stage.

Nitrogen occurs in a variety of inorganic forms in nature. The most common being ammonia (NH_3) nitrate (NO_3), nitrite (NO_2), and nitrogen gas (N_2). Specific enzyme systems are required for the utilisation of these different forms of nitrogen. As in the case of carbon, nitrogen compounds in sewage are eventually converted to cell material and gas. A series of reactions is responsible for these conversions. Organic nitrogen is converted to ammonia (nitrification), which subsequently undergoes denitrification (Focht and Chang, 1975; Winkler and Thomas, 1978). Nitrification takes place in two steps and is accomplished by two distinct bacterial types. In the first step, ammonia is oxidised to nitrite by Nitrosomonas spp (Focht and Chang, 1975; Winkler and Thomas, 1978). The nitrite is then oxidised to nitrate by Nitrobacter spp.

Oxygen is essential for the growth of these two bacteria, although Nitrobacter is more sensitive to oxygen deficiency (Painter, 1970).

The carbon requirements of these bacteria are satisfied by carbon dioxide (Watson, 1974). As in the case of sewage carbon, only a small portion of the total influent nitrogen (TKN) is available for nitrification as evidenced by the residual TKN in the effluent (van Haandel et al., 1982). In contrast, denitrification is a reduction process where nitrite and nitrate are converted to nitrogen gas.

Denitrification can be considered a respiratory mechanism where molecular oxygen is replaced by nitrate (Winkler and Thomas, 1978). While the concentration of nitrate which is produced in the aerobic zone by nitrification is only related to the influent nitrogen, nitrogen removal by denitrification in the anoxic zone is also dependent on the available COD (van Haandel et al., 1982). Oxidisable substrate is essential for the denitrification reactions (Winkler and Thomas, 1978). A wide variety of heterotrophic bacteria which include Pseudomonas and Aeromonas spp can accomplish denitrification (Brown et al., 1974). While certain organisms can only reduce nitrate to nitrite or nitrite to nitrogen, others can accomplish the complete reduction of nitrate to nitrogen gas (Focht and Chang, 1975).

Easily biodegradable carbon is directly absorbed by the biomass in the anaerobic zone (Siebritz et al., 1983; Wentzel et al., 1985). Volatile fatty acids like acetic acid are specifically absorbed by phosphate removing bacteria and metabolised to polyhydroxybutyrate. The uptake of acetic acid causes a release of phosphate to the external medium (Wentzel et al., 1986). Simultaneously, the slowly biodegradable carbon is caught up in the biomass matrix and so removed from the external medium (Dold et al., 1980).

The effluent from this zone is thus changed in respect of its carbon and phosphate composition. The biodegradable carbon is removed and only non-biodegradable COD remains in the effluent. The phosphate concentration rises, while the nitrogen concentration remains unchanged. The effluent of the anaerobic zone enters the anoxic zone, which is also fed by the nitrate-containing return sludge from the aerobic zone. A portion of the slowly-biodegradable COD which

is caught up in the sludge matrix, undergoes hydrolysis by extracellular enzymes of the denitrifying bacteria. A source of readily biodegradable carbon for denitrification is thus supplied (Lötter, 1987).

A certain amount of phosphate and ammonia can be used for cell synthesis during denitrification. The effluent from this zone will thus contain lower phosphate and ammonia levels. The stored polyhydroxybutyrate is oxidised in the aerobic zone by phosphate removing bacteria, in order to provide carbon for cell material and energy production. Additional carbon is provided by the further extracellular hydrolysis of enmeshed COD (Dold and Marais, 1986). Simultaneously, phosphate is taken up and converted to cell material and polyphosphate. The carbon dioxide which is produced by complete oxidation of intra- and extracellular carbon sources, is used as a carbon source for the nitrifying bacteria which convert ammonia to nitrate.

In the following survey an attempt is made to review phosphorus removal by the activated sludge process and to discuss the microbiological and biochemical aspects of the process.

2.3 Phosphorus Removal by the Activated Sludge Process

After the observations of Srinath et al., (1959) that more phosphorus than is necessary for normal metabolism, is removed from the liquid in an activated sludge system, this phenomenon created great interest. Levin and Shapiro, (1965) studied phosphate uptake in great depth. Their experiments showed that organic carbon stimulated phosphate uptake and further, that while phosphate is taken up under aerobic conditions, release occurred under anaerobic conditions (Levin and Shapiro, 1965). Sekikawa et al., (1966) carried this research further and came to the conclusion that phosphate release in activated sludge occurred as a result of unfavourable conditions, namely, lack of nutrients and oxygen, as well as addition of toxic substances. Shapiro further observed that anoxic conditions induced phosphate release (Shapiro, 1967).

Further investigation of this phenomenon showed that the released phosphate is largely derived from acid soluble intracellular phosphate (Shapiro et al., 1967).

During this period phosphate removal was observed in American plants by a number of researchers (Vacker et al., 1967; Milbury et al., 1971). The biological nature of phosphorus removal as shown by Shapiro and others (Sekikawa et al., 1966; Shapiro et al., 1967), is further supported by the radioactive tracer studies of Yall et al., (1971) where lack of nutrients and oxygen as well as addition of toxic substances stimulated phosphate release, while reversal of these inhibitory conditions resulted in phosphate uptake. There are however, researchers who query the biological nature of this phenomenon.

The simultaneous decrease in calcium and phosphate in the liquid phase of an activated sludge plant, encouraged Menar and Jenkins to conclude that phosphate is removed by a precipitation mechanism (Menar and Jenkins, 1970).

Although this survey only covers biological phosphate removal, it is interesting to put suggestions that biological and chemical removal complement each other in perspective. Lan et al., (1983), claim that calcium phosphate precipitation occurs in the aerobic zone as a result of a pH increase. Low carbon dioxide yield and high carbon dioxide stripping as a result of intensive aeration, can result in an increase in the pH. Arvin (1985) has also reported examples of biologically mediated phosphate precipitation.

Levin et al, (1972), reported phosphate removal in a large plant. Without aeration, intracellular phosphate was released and during subsequent aeration the phosphate was taken up again (Levin et al., 1972).

Barnard suggested in 1974, that phosphate can be removed by manipulation of the Bardenpho Process. By implementing these suggestions, Nicholls considerably improved the phosphorus removal

at the Johannesburg Alexandra extended aeration plant by turning off the aerators for a certain period, so as to create anaerobic conditions (Nicholls, 1975).

In a survey of biological phosphate removal in 1976, Barnard summarised the requirements for successful phosphate removal as follows. The activated sludge must undergo an anaerobic phase which induces the release of phosphate. For successful phosphate removal this anaerobic phase must be strictly anaerobic, in others words, no nitrate may be present.

In his experimental work Barnard observed no significant increase in phosphate removal with increased pH values (Barnard, 1976). These findings confirm the biological nature of the process. A lack of correlation between calcium and phosphate precipitation with increased pH was also observed by Rensink et al., (1981).

In the beginning of the Eighties, researchers started to investigate fundamental aspects of the process in greater depth. Researchers at the University of Cape Town had already postulated a mathematical model of the activated sludge process in 1976 (Marais and Ekama, 1976). This basic model was later extended to include denitrification (van Haandel et al., 1982).

In 1983 the model was further refined to include phosphate removal (Siebritz et al., 1983). During the development of this model, the importance of rapidly biodegradable COD was postulated (Siebritz et al., 1983). The deleterious effect of nitrate in the anaerobic phase which was observed by Nicholls (1975) and Barnard (1976) is explained by Siebritz et al., (1983) by the utilisation of rapidly biodegradable COD for denitrification and not for phosphate release for which it is essential.

The observations of Nicholls were pursued by Venter et al., (1978) on a second extended aeration plant. By creating anaerobic conditions at the inlet of the plant, an ortho-phosphate concentration of 1 mg P/ℓ could be obtained in the effluent. The

incorporation of an anaerobic zone in the Johannesburg Goudkoppies plant where nitrate is restricted to a minimum, also led to good phosphate removal (Osborn and Nicholls, 1978). The efficiency of this plant was not consistent and after 3 years intensive investigation, it was observed that the inconsistency correlated with changes in influent load. Where the influent COD was relatively low, competition appeared to occur between phosphate and nitrogen removal. The possible importance of the nature of the COD was also postulated in this investigation (Pitman et al., 1983).

After these investigations, a shallow gradient relief sewer was commissioned to transport sewage to Goudkoppies. Analysis of the sewage showed high concentrations of rapidly biodegradable COD which was mainly present in the form of volatile fatty acids (Osborn et al., 1986).

Since the commissioning of this sewer, the efficiency of this plant has been very consistent and an effluent orthophosphate concentration of less than 1 mg P/ℓ is maintained.

The dependence of phosphate release on the concentration of organic substrate has been reported by a number of researchers (Hascoet and Florentz, 1985; Wentzel et al., 1985). The effect of different carbon sources on phosphate release has also been investigated.

Potgieter and Evans (1983) compared phosphate release under anaerobic conditions in the presence of volatile fatty acids and sugars. Only the volatile fatty acids induced phosphate release. In separate studies, phosphate release in the presence of methanol was observed. The eventual phosphate concentration was however, approximately 50 % of the levels observed with acetate (Malnou et al., 1984; Gerber et al., 1986).

The dependence of phosphate release on the nature of the carbon source has been confirmed by a number of researchers. By adding substrates to an anoxic zone, Jones et al., (1985) showed that acetic acid, butyric acid, ethanol and methanol enhanced phosphate

release under these conditions, and that subsequent phosphate removal was stimulated. De Vries et al., (1985) observed the same patterns in a pilot plant. Although the results of Gerber et al., (1986) differ quantitatively from the other researchers, the same dependence on substrate type is evident in their work. The stimulatory effect of acetate on phosphate release and subsequent phosphate removal was observed by all these researchers. Attempts to correlate the uptake of organic substrate with phosphate release led to conflicting results.

In contrast with Gerber et al., (1986), Fukase et al., (1982) observed good phosphate release with glucose at a molar relationship of glucose to phosphate of 2 : 1. The molar ratio with acetate was 1 : 1. Arvin et al., (1985) observed different molar ratios, namely, glucose to phosphate, 0,6 to 0,8 and acetate to phosphate, 0,7 while Siebritz et al., (1983) differed even further with a molar ratio of acetate to phosphate of 2 : 1, which was later confirmed by Wentzel et al., (1985). De Vries et al., (1985) also obtained a ratio of 2 : 1 with acetate.

The importance of volatile fatty acids as carbon source, has been recognised by a number of researchers and has been investigated on plants. At the Johannesburg Northern Works, primary sludge was allowed to ferment in a digester, after which the supernatant was transported to the anaerobic zone. Although the phosphate level in the effluent did not reach the required 1 mg P/ℓ level, the phosphate removal was considerably improved by this procedure (Osborn et al., 1986).

In another attempt to improve substrate characteristics the primary sludge was recirculated directly to the anaerobic zone, a procedure which again had an improving effect on phosphate removal (Nicholls et al., 1985). The use of primary sludge fermentation has already been successfully applied in Canada (Rabinowitz and Oldham, 1985).

Experiments with a laboratory-scale fermentation plant, coupled to an activated sludge plant in France, have confirmed that the

external production of suitable substrate is feasible (T'Seyen et al., 1985).

The production of volatile fatty acids in digesters and primary settling tanks was compared at the Johannesburg Northern Works. The mass of volatile fatty acids is considerably greater in the primary settling tanks and this process is recommended (Osborn et al., 1986). The successful production of volatile fatty acids is however, not sufficient and steps must be taken to reserve these substrates exclusively for phosphate removal. In order to achieve this goal, a second anoxic zone was incorporated in front of the anaerobic zone, which accomplished denitrification of return sludge. This procedure resulted in consistent phosphate removal to less than 1 mg P/ℓ (Osborn et al., 1986).

2.3.1 Nutrient Dynamics

In order to evaluate the inter molecular relationships between nutrients in and outside the biomass, it is necessary to summarise relevant observations.

Levin and Shapiro (1965), investigated the beneficial effect of biodegradable carbon on phosphate uptake. These studies also showed the necessity of sufficient oxygen. Phosphate uptake showed strong dependence on oxygen levels. Wells (1969) also emphasized the importance of good aeration and demonstrated differences in the phosphate uptake characteristics of different sludges. Phosphate-acclimatised sludges showed a phosphate uptake rate of 65 mg P/ℓ/h. With both of these groups, phosphate release was observed after extended aeration.

Shapiro already observed phosphate release under anaerobic conditions in 1967. Phosphate release under anaerobic conditions in an activated sludge plant is now generally accepted as essential for efficient phosphate removal (Nicholls and Osborn, 1979; Siebritz et al., 1983; Wentzel et al., 1985).

Although the movement of phosphate in and outside the biomass enjoyed considerable attention in subsequent years, the correlation of this phenomenon with cation movement and substrate uptake, was much later in attracting attention.

Siebritz et al., (1983) observed a direct relationship between easily biodegradable COD uptake and phosphate release under anaerobic conditions. This research was further refined by Wentzel et al., (1985) in that they determined phosphate release at different acetate concentrations. Although the release rate is not affected by acetate concentration, the mass of phosphate which is released, is equivalent to the mass of acetate added in the ratio 1 : 2 and 1 : 5 mg P : mg COD or approximately 0,4 to 1,0 mol P/mol COD as acetate.

Arvin observed a ratio of 0,68 mg P/mg COD for acetate and 0,41 mg P/mg COD for propionate. Arvin simultaneously studied the movement of cations. Under anaerobic conditions phosphate release occurred simultaneously with the release of potassium and magnesium and uptake of calcium (Arvin and Kristensen, 1985). The relevant molar ratios were 0,23 mol/mol for K : P and 0,32 mol/mol for Mg : P. Gerber and Winter, (1985) observed that the uptake and release patterns of phosphate coincided with the patterns for potassium, magnesium and sulphate. The exchange ratios for potassium and magnesium were approximately 0,25 mol/mol P (Gerber et al., 1987); figures which compare well with the figures of Arvin and Kristensen (1985).

The sulphate ratio was 0,09 mol S/mol P (Gerber et al., 1987). In contrast with Arvin, these researchers observed no exchange of calcium. By using the STEMEDAX system, Buchan (1981) showed that the electron-dense polyphosphate granules consisted chiefly of phosphorus and calcium, with traces of magnesium and potassium. During a comparative study of phosphorus compounds in activated sludge, Mino et

al., (1985) observed that the large differences in total phosphorus between different sludges depended greatly on the low molecular mass polyphosphate and metal phosphates. The low molecular weight polyphosphate in particular, contributes to the high phosphorus content of sludges which are produced in anaerobic/aerobic processes. Although low and high molecular mass polyphosphates are accumulated, it is the low molecular mass material which is released and taken up again. These researchers further suggested that the high molecular mass polyphosphate provided phosphate for growth under aerobic conditions (Mino et al., 1985).

2.4 Microbiology of Activated Sludge

Sewage flora, from which activated sludge bacteria are derived, is a heterogeneous mixture. It is expected that bacteria which can process carbon and nitrogen compounds in sewage, will be dominant. In this study, only heterotrophic bacteria will be considered. Shortly after the development of the activated sludge process, researchers started to study the bacteria in the process. In 1944 Allen found that Gram-negative rods dominated the population.

McKinney and Horwood (1952), later identified Escherichia spp, Paracolobactrum spp, Nocardia spp, Bacillus spp and Flavobacterium as floc-forming organisms. The bacteria which were isolated were chiefly obligate aerobes (Jasewicz and Porges, 1956). In the Sixties, techniques for this type of investigation were considerably improved and researchers began to report more quantitative results. Viable plate counts of $1,0 \times 10^8/m\ell$ were obtained (Prakasam and Dondero, 1967). The contradictory results which were obtained in respect of the identity of the heterotrophic bacteria can probably be ascribed to different methods and differences in the nature of the sludges (Allen, 1944; McKinney and Horwood, 1952; Jasewicz and Porges, 1956; Dias and Bhat, 1964; Unz and Dondero, 1967). Comparative studies with activated sludges which were fed on different carbon sources, confirmed the latter. Although the bacterial populations differed significantly, sludge activity

correlated well with population structure (Prakasam and Dondero, 1970).

During a preliminary step in a biological phosphorus removal study, heterotrophic bacteria were identified in a laboratory-scale activated sludge plant. As well as bacteria which had already been described by previous researchers, Acinetobacter spp and Sphaerotiulus spp occurred (Bendict and Carlson, 1971). In 1975, Fuhs and Chen isolated Acinetobacter spp from activated sludge plants and found that they were capable of luxury phosphate uptake in a complete medium. These researchers stated that the luxury uptake which was observed in this type of plant, was accomplished by Acinetobacter spp.

In the Eighties, the microbiology of biological phosphate removal attracted great interest. Although various population studies identified Acinetobacter spp as the dominant heterotrophic bacteria (Hart and Melmed, 1982; Buchan, 1983; Kerdachi and Roberts, 1983; Lötter, 1985; Lötter and Murphy, 1985), other researchers did not find these bacteria dominant (Brodisch and Joyner, 1983; Malnou et al., 1984; Beccari et al., 1985, Meganck et al., 1985; Suresh et al., 1985). The correlation between bacterial population and process efficiency was emphasized by a number of researchers (Prakasam and Dondero, 1970; Cech and Chudoba, 1983; Poole, 1984). The high occurrence of Acinetobacter spp in biological phosphate removing plants stimulated further studies on this organism. Before proceeding with the role of these organisms in phosphate removal, it is necessary to review the physiology of these bacteria.

2.4.1 Acinetobacter spp

Since Beijerinck isolated Acinetobacter anitratus then Micrococcus calcoaceticus in 1911, similar organisms have been isolated by a number of researchers, and different names given to them (Henriksen, 1973). During the Sixties, Acinetobacter isolates were thoroughly studied with the aim of separation into different species. Except for the

separation into two groups on the basis of nutritional characteristics (Baumann et al., 1968), and the separation of clinical and water isolates (Pagel and Seyfried, 1976), a satisfactory separation between species is not yet available. The API 20E test procedure (Analytlab, 1977), makes provision for two sub-species, namely, lwoffi and anitratus on the basis of glucose fermentation. Nevertheless, only two species (Acinetobacter calcoaceticus and Acinetobacter lwoffi) appear in the Approved List of Bacterial Names (Skerman et al., 1980) and only Acinetobacter calcoaceticus is described in Bergey's Manual of Systematic Bacteriology (Juni, 1984).

Schutte divided Acinetobacter isolates into two main groups on the basis of biochemical tests and morphology. One group comprised mainly clinical isolates which were identified as Acinetobacter calcoaceticus var anitratus, while the other, namely, Acinetobacter calcoaceticus var lwoffi, were isolated from anaerobic digesters and activated sludge plants.

Acinetobacter spp occur widely in nature and can easily be isolated from soil, water and sewage samples (Warskow and Juni, 1972; Henriksen, 1973; Juni, 1978). More recently, Acinetobacter has been isolated from several activated sludge plants (Buchan, 1983; Lötter and Murphy, 1985). All Acinetobacter spp are aerobic, Gram-negative, non-motile, catalase positive, oxidase negative (Henriksen, 1973; Henriksen, 1976; Juni, 1978; Fricke et al., 1982), while lines vary in the use of citrate, acid formation and liquefaction of gelatine, as well as hemolytic activity (Henriksen, 1976; Juni, 1978).

Although certain sugars can be utilised, these organisms cannot obtain energy for growth from fermentation (Juni, 1978). The nutritional spectrum for every line appears to be unique (Juni, 1978). Even in Baumann's extended study

of nutritional spectra, nutritional requirements could not be used as a characteristic identification factor (Baumann, et al., 1968).

Acinetobacter grow well on simple mineral media containing one carbon compound as carbon and energy source (Baumann et al., 1968; Warskow and Juni, 1972), and grow equally well on liquid and solid media (Henriksen, 1976; Deinema et al., 1980).

The growth of Acinetobacter calcoaceticus which is isolated from soil, on ethanol is dependent on magnesium and sulphur in the medium. Exhaustion of these compounds leads to intracellular accumulation of acetate and inhibition of ethanol oxidation (Abbott et al., 1973). In media which contains acetate and ethanol, acetate is used preferentially (Abbott, 1973). The same growth patterns were observed with Acinetobacter calcoaceticus which was isolated from the sediment of a fresh water dam (du Preez et al., 1981). The growth of Acinetobacter calcoaceticus on these substrates is used in the manufacture of single cell protein (du Preez et al., 1984). Comparative studies with C_2 - C_5 fatty acids as substrates under aerobic and oxygen-limiting conditions showed that the carbon sources, except for isobutyric acid, were completely utilised under both conditions. The growth rate under oxygen-limiting conditions was considerably slower than under aerobic conditions (Schutte, 1987).

After the observation that Acinetobacter spp from an activated sludge plant accumulated phosphate in the form of polyphosphate (Fuhs and Chen, 1975), this characteristic was investigated by other researchers. Lawson and Tonhazy (1980) observed the same characteristics in a number of activated sludge isolates. Buchan investigated the metabolism of polyphosphate in Acinetobacter spp using an electron microscope (Buchan, 1983).

Buchan's research showed the dependence of polyphosphate metabolism on oxygen levels. Polyphosphate accumulation in Acinetobacter isolates is stimulated specifically by phosphate starvation and the addition of acetate (Murphy and Lötter, 1986). In contrast with Henriksen's findings (1976), Acinetobacter isolates from several activated sludge plants used glucose for growth (Lötter et al., 1986). Approximately half of the 75 isolates which were investigated in this study could accumulate polyphosphate and the carbon storage product, polyhydroxybutyrate (Lötter et al., 1986). The accumulation of polyhydroxybutyrate by these organisms was originally observed by Fuhs and Chen (1975) and later confirmed by Lawson and Tonhazy (1980).

Most Acinetobacter strains are not capable of metabolising nitrate to nitrite in conventional reduction tests (Juni, 1978). An activity similar to hydroxylamine reductase has been observed in extracts of an Acinetobacter strain which was adapted to grow on a medium containing nitrate or nitrite as the sole nitrogen source (Jyssum and Joner, 1966). The strains, which in fact reduced nitrate to nitrite, could not use it as an alternative electron acceptor (Juni, 1978). In contrast with this, Acinetobacter isolates from activated sludge plants can in fact, reduce nitrate to nitrite (Lötter, 1985; van Groenestijn and Deinema, 1985; Lotter et al., 1986) and even in some cases, nitrite to nitrogen (Lötter, 1985; Lötter et al., 1986).

Acinetobacter isolates which can use glucose as carbon and energy source, used the Entner-Doudoroff path for degradation exclusively (Henriksen, 1973; Juni, 1978). It appears as if Acinetobacter spp do not contain the necessary enzymes for direct phosphorylation of hexoses (Juni, 1978). The occurrence or not of glucose dehydrogenase in Acinetobacter spp determines whether the strain can convert hexoses to hexuronic acids under aerobic conditions (Juni, 1978). A cytochrome-linked glucose dehydrogenase which can

use a number of aldoses as substrates has been isolated from Acinetobacter (Hauge and Halberg, 1956). This dehydrogenase is apparently unique because it does not use a nicotinic acid derivative or a flavine as co-enzyme, or prosthetic group (Hauge and Mürer, 1964).

Further research with this prosthetic group identified it as 4,5-dihydro-4,5 dioxo-1 H pyrolo [2,3-f] quinoline-2,7,9 tricarboxylic acid which is known by the trivial name pyrolo-quinoline-kinone. The O-quinone residue appears to be the active portion of the molecule, a characteristic which has also been observed in other dehydrogenase enzymes Duine et al., 1979). Comparative studies of glucose dehydrogenase activity in acid forming and non-acid forming Acinetobacter spp have emphasized the importance of this prosthetic group. The absence of glucose dehydrogenase (GDH) activity in the non-acid forming isolates was not as a result of a lack of the protein itself, but a lack of the prosthetic group (van Schie et al., 1984). Glucose oxidation in Acinetobacter is an energy transduction process, in other words, the oxidation of glucose results in the formation of a proton-motive force which serves as an energy source for the uptake of other metabolites (van Schie et al., 1985). Glucose thus acts as an additional energy source and more biomass is produced from acetate and glucose than in the presence of acetate alone (Muller and Babel, 1986). The ability of acid forming Acinetobacter strains to use glucose as growth substrate depends on their ability to degrade glucuronic acid (Juni, 1978). As can be expected for an obligate aerobe, the tricarboxylic acid cycle is functional in A. calcoaceticus.

Citrate synthase and aconitase activity have been observed (Taylor and Juni, 1961; Weitzman and Jones, 1968). Citrate synthase plays a key role in terminal oxidation in that the enzyme catalyses the condensation of acetyl-CoA with oxaloacetic acid to form citrate and thus controls carbon

entry into the tricarboxylic acid cycle. In a survey of citrate synthases from different bacteria, it was shown that the enzyme from A. calcoaceticus is inhibited by high levels of NADH. This inhibition is reversed by the addition of AMP (Weitzman and Jones, 1968). Competitive product inhibition with citrate and CoA has been observed with various concentrations of acetyl-CoA, while non-competitive product inhibition has been shown with oxalo-acetic acid at different concentrations, in the case of the enzyme from Acinetobacter anitratus. This enzyme is also inhibited by NADH and NADPH while AMP, potassium and α-ketoglutaric acid activate the enzyme (Johnson and Hansen, 1974).

Two NADP-dependant iso-enzymes of isocitrate dehydrogenase have been observed in Acinetobacter lwoffi (Self and Weitzman, 1970). The higher molecular mass iso-enzyme (IDH-11) is significantly stimulated by AMP and ADP (Parker and Weitzman, 1970). The addition of acetate to Acinetobacter calcoaceticus which is grown in a succinate limiting medium causes an increase in the specific activity of isocitrate dehydrogenase and isocitrate lyase (Reeves et al., 1983). Further research in this area has shown that this increase in activity occurs simultaneously with an increase in the relative ratio of IDH-11 to IDH-1 (Reeves et al., 1986). In addition to isocitrate lyase the second enzyme of the glyoxylic acid cycle, malate synthase, has also been observed in Acinetobacter spp (Juni, 1978).

By monitoring the key enzymes α-ketoglutarate dehydrogenase and iso-citrate lyase Sturm and co-workers concluded that Acinetobacter possess a control mechanism which regulates the simultaneous operation of the glyoxylic acid and tricarboxylic acid cycles. Succinate exerts an effect on this regulation (Sturm et al., 1970). They stated further that in the aerobic Acinetobacter the glyoxylic acid cycle is regulated by C_4 intermediates of the tricarboxylic acid cycle, in contrast with the facultative Escherichia coli

which is regulated by C_2 intermediates (Herman and Bell, 1970).

In this organism NADH acts as a negative effector for α-ketoglutarate dehydrogenase as well as for citrate synthase. As in the case of citrate synthase, AMP and ADP act as positive effectors (Weitzman, 1972).

Acetokinase and phosphotransacetylase are used in the utilisation of acetate (Taylor and Juni, 1961). Propionate is converted to pyruvate by a series of reactions. The pyruvate is then oxidised to acetyl-CoA (Hodgson and McGarry, 1968).

The link between the NAD dependant malate enzyme and the NAD-dependant malate dehydrogenase to metabolise oxaloacetic acid to pyruvate has been observed in cell-free extracts of Acinetobacter spp (Dolin and Juni, 1978). This series of reactions makes it possible for oxaloacetic acid to be used as growth source. As a result of the absence of pyruvate kinase in Acinetobacter spp (Taylor and Juni, 1961), this linked reaction with the malate enzyme is essential for the degradation of C_4 dicarboxylic acids.

The degradation of aromatic compounds like benzoate, mandalate, kinate and tryptophan by Acinetobacter spp has been intensively studied. This organism has two parallel pathways for the degradation of benzoate, which both occur via α-keto-adipic enollactone to α-keto-adipic acid and eventually to succinic acid and acetyl-CoA (Juni, 1978).

Protocatechuic acid is an intermediate in the degradation from shikimic acid and quinic acid and is considered the inducer of five of the enzymes involved in the process (Canovas et al., 1968; Ingledew et al., 1971). These five enzymes are controlled by a common inducer and repressor in A. calcoaceticus (Canovas et al., 1968; Ingledew et al.,

1971). A few of the transferase enzymes which are involved in this process have already been isolated from A. calcoaceticus (Canovas and Johnson, 1968). Two isofunctional forms of the enollactone hydrogenase which are both synthesized under different control processes have also been isolated from A. calcoaceticus (Katagiri and Wheelis, 1971). A. calcoaceticus can be induced by 3-chlorobenzoate to oxidise benzoate to catechol. In the presence of succinate or pyruvate, 3-chlorobenzoate itself can be oxidised (Reber and Thierbach, 1980). 4-chlorobenzoate acts like the 3-chloro derivative as an inducer, but the 2-chloro and 2,6 dichlorobenzoate have no inductive effect (Reber, 1982).

L-mandelate is oxidised to benzoate by the sequential action of L-mandelate dehydrogenase, phenol glyoxylate carboxylyase and benzaldehyde dehydrogenase. All three of these enzymes are induced by phenol glyoxylate (Juni, 1978). An unusual D-mandelate dehydrogenase has been isolated from mutant A. calcoaceticus growing on D-mandalate (Hills and Fewson, 1983). This enzyme is controlled in the same manner as the normal mandelate enzyme (Hills and Fewson, 1983).

A. calcoaceticus also contains L-lactate and D-lactate dehydrogenases which are very similar to the mandelate enzymes (Allison et al., 1985). The D-lactate and D-mandelate dehydrogenase have molecular masses of 62 800 and 59 700 respectively, pI values of 5,8 and 5,5 and both contain non-covalently bound FAD as cofactor (Allison et al., 1985).

Many Acinetobacter lines are capable of using hydrocarbons (C_{18}-C_{20}) as carbon and energy sources (Juni, 1978). Organisms which are grown on these compounds are characterised by intracytoplasmic membrane-bound hexadecane inclusions (Scott and Finnerty, 1976; Müller et al., 1983).

In the same manner as other micro-organisms which grow on hydrocarbons, A. calcoaceticus manufactures an extracellular surface active material. Growth is characterised by reduction in the surface active tension of the medium. The bacteria also show adhesion to the hydrocarbon substrate which is lost during growth on soluble substrates (Neufeld et al., 1983; Neufeld and Zajic, 1984). A. calcoaceticus isolates which degrade hydrocarbons, manufacture an assymetric poly-anionic polymer which is known as emulsan (Rubinowitz et al., 1982; Goldman et al., 1982). The adhesion to the hydrocarbon is partially dependant on the polymer and partially dependant on a number of thin fimbriae on the cell surfaces (Rosenberg et al., 1982). This adhesion can be inhibited by the presence of extracellular polysaccharide capsules (Rosenberg et al., 1983). The occurrence of extracellular emulsifiers was originally limited to isolates which grew on hydrocarbons, but more recently bioemulsifiers have been observed in the extracellular fluid of 16 different Acinetobacter isolates after growth on ethanol (Sar and Rosenberg, 1983). Growth on hydrocarbons apparently induces NAD dependant alcohol dehydrogenase and NADP dependant aldehyde dehydrogenase activity (Fischer et al., 1984), which occurs in the cytoplasmic membrane.

The biologically active form of emulsan is closely associated with the cell and cannot be replaced by free emulsan in the medium (Pines and Gutnick, 1986). This form of the polymer consists of a mini-capsular layer on the surfaces of cells in the log-growth phase, and is released into the media by the action of an extracellular esterase when the stationary phase is reached (Shabtai and Gutnick, 1986). The polymer yield and composition depends on the carbon source (Bryan et al., 1986).

Growth on n-alkanes induces the synthesis of cytochrome P-450, which is not observed in A. calcoaceticus during

growth on other media (Asperger et al., 1984). Non-phenobarbital type hepatic cytochrome inducers like biphenol, indene and fenathene also act as inducers of this system (Asperger et al., 1985).

Baumann et al., (1968) showed that 70 out of 106 independant isolates could use 2,3 butanediol as carbon and energy source. Acinetobacter isolates which can use this compound or acetyl methyl carbinol as carbon source, apparently develop a cyclic pathway for conversion to acetate (Juni, 1978).

Studies on the respiratory components of an Acinetobacter isolate indicate the presence of a particular bound cytochrome b_1, cytochrome a, cytochrome a_2 and flavine (Whittaker, 1971). The cytochromes and flavine can either be reduced with NADH or with succinate and oxidised with oxygen, as can be expected if they are components of an electron transfer pathway. Succinate and NADH oxidase activities are totally inhibited by cyanide, which indicates that the cytochrome system is the only terminal oxidase system for these substrates (Whittaker, 1971).

In another Acinetobacter isolate, cytochromes o, a and d are capable of acting as terminal oxidases. Growth on alkane and non-alkane substrates only result in small differences in cytochrome composition and oxidase activity. Oxygen levels on the other hand significantly influence the cytochrome composition (Ensley and Finnerty, 1980). Maximum molar growth yield on oxygen (YO_2 max) is not influenced by the oxygen level in other Acinetobacter isolates (Hardy and Dawes, 1985). Growth yields in the presence of limited oxygen is however, low, apparently as a result of an effective P/O ratio of only 1 (Fewson, 1985).

Glucose is the only substrate of Acinetobacter spp which is degraded to an intermediate of the Embden-Meyerhof pathway.

Other substrates are degraded to acetate, pyruvate or an intermediate of the tricarboxylic acid cycle. Acinetobacter spp are deficient in pyruvate kinase and can therefore not (in the usual manner) form phospho-enol pyruvate from pyruvate. Except for this enzyme, all the other enzymes involved in the reversal of the Embden-Meyerhof pathway have been observed in Acinetobacter spp (Juni, 1978). Acinetobacter spp have in fact, two methods to form phospho-enol pyruvate from pyruvate, namely, by phospho-enol pyruvate synthetase and the ATP-dependant decarboxylation of acetate catalysed by phospho-enol pyruvate carboxykinase (Juni, 1975). One of the key enzymes of this reversal, fructose-1,6-diphosphatase has already been partially purified from Acinetobacter spp (Mukkada and Bell, 1969).

Ammonium salts, nitrate and nitrite serve as nitrogen sources for growth. Glutamate dehydrogenase, glutamate synthetase and glutamic synthetase have been observed in cell-free extracts of Acinetobacter which have been grown on ammonium salts (Jyssum and Joner, 1965; Jyssum and Joner, 1966; Savageau et al., 1972). The enzymes for the synthesis of tryptophan and branched amino acids have also been observed in this organism (Juni, 1978).

2.5 **Bacterial metabolism**

In a phosphate removing activated sludge plant the micro-organisms are exposed to alternating anaerobic, anoxic and aerobic conditions. The group of bacteria responsible for the removal of excess phosphate are heterotrophic organisms which have specific carbon requirements. This review handles only those aspects of phosphate and carbon metabolism at different oxygen levels which could possibly have a bearing on these so-called poly P bacteria.

2.5.1 Polyphosphate metabolism

Inorganic polyphosphates occur widely in micro-organisms and

have been observed in bacteria, algae and fungi (Kulaev and Vagabov, 1982). The biochemistry of these compounds have been the subject of a number of review articles (Harold, 1966; Dawes and Senior, 1973; Kulaev, 1975; Kulaev and Vagabov, 1983).

2.5.1.1 Intracellular localisation

Polyphosphate containing granules (volutin granules) have been demonstrated by a number of researchers, using cytochemical techniques (Voelz et al., 1966; Fuhs and Chen, 1975; Deinema et al., 1980). The use of electron microscopy helped to show that polyphosphate granules in heterotrophic bacteria are associated with DNA fibrils and nucleoplasma (Voelz et al., 1966; Friedberg and Avigad, 1968; Deinema et al., 1980), or with the site of oxidation reduction (Mudd et al., 1958). Electron microscopy was later combined with X-Ray energy dispersion (EDAX) to expose the chemical nature of the granules (Baxter and Jensen, 1980). These researchers showed that considerable quantities of potassium and relatively little calcium and magnesium was present in the polyphosphate granules under normal growth conditions (Baxter and Jensen, 1980). When the medium contained an excess of a specific metal like magnesium, barium, manganese or zinc, it accumulated in large quantities in the polyphosphate granules. By using the same technique Buchan (1983) showed that calcium was the chief cation associated with intracellular polyphosphate in activated sludge bacteria. Van Groenestijn and Deinema (1985) observed the presence of calcium, magnesium and potassium in polyphosphate granules in Acinetobacter and further showed that potassium was essential for polyphosphate synthesis in this organism.

2.5.1.2 Effect of growth conditions on polyphosphate accumulation

In 1958 Mudd et al. showed that a competitive nucleic acid

synthesis and polyphosphate accumulation existed in mycobacteria. Glucose as substrate favoured nucleic acid synthesis, while malate favoured polyphosphate accumulation. These observations show the relationship between carbon and polyphosphate metabolism. In the same study, polyphosphate was used for nucleic acid synthesis. Most of the polyphosphate was in the acid insoluble form (Mudd et al., 1958). This type of polyphosphate is also considered the most important accumulation product in Hydrogenomonas spp. In a complete growth medium polyphosphate is accumulated until the external phosphate is exhausted. Thereafter, the polyphosphate is used for the continued synthesis of organic phosphorus compounds. Under anaerobic conditions no orthophosphate is taken up and no polyphosphate is used (Kaltwasser, 1962). In denitrifying bacteria, orthophosphate is rapidly taken up by phosphate deficient cells under aerobic and anoxic conditions (Kaltwasser, 1962).

The reverse relationship between nucleic acid synthesis and growth, and polyphosphate accumulation which was observed by Mudd, was later confirmed by Harold (1963) in Aerobacter aerogenes. This relationship however, remained a function of the growth medium. Polyphosphate accumulation could be induced or repressed by manipulation of the phosphate and sulphur content of the medium (Harold and Sylvan, 1963). Addition of orthophosphate to Aerobacter aerogenes which have previously been starved in respect of phosphate induced polyphosphate accumulation (Harold, 1964).

In the nitrifying bacteria Nitrosomonas, the steady state concentration of polyphosphate depends on the balance between the rate of ATP generating and ATP utilising reactions (Terry and Hooper, 1970). Nett polyphosphate synthesis is favoured where ammonium oxidation (ATP generating) in the absence of protein synthesis (ATP utilising) occurs. In Nitrobacter, polyphosphate

synthesis begins only after complete oxidation of nitrite (Eigener and Bock, 1972).

Polyphosphate accumulation in Acinetobacter isolates occurs as a function of the growth conditions (Deinema et al., 1980). As in the case of other bacteria, Acinetobacter use accumulated polyphosphate as a phosphate source and show enhanced polyphosphate synthesis after phosphate starvation (Van Groenestijn and Deinema, 1985). Induction of polyphosphate accumulation by anaerobic conditions has been observed in the facultative anaerobe E. coli. The chain lengths of the polyphosphates are dependant on the culture age (Rao et al., 1985).

In contrast with the above, the subjection of the obligate aerobe A. calcoaceticus to anaerobic conditions leads to polyphosphate degradation and release of phosphate to the external medium (Ohtake et al., 184; Murphy and Lötter, 1986).

In contrast with the polyphosphate utilisation pattern, described above, Neisseria gonorrhoeae does not show enhanced polyphosphate synthesis when the organism is placed in a phosphate rich medium, after phosphate starvation. Polyphosphate is also not used as a phosphate source during phosphate starvation (Noegel and Gotschlich, 1983).

2.5.1.3 Enzymatic Biosynthesis and Degradation

Polyphosphate kinase which was originally isolated from E. coli (Kornberg et al., 1956) catalyses the following reaction :

$$ATP + (polyphosphate)_n \rightleftharpoons ADP + (polyphosphate)_{n+1}$$

Subsequently, the enzyme was isolated from a number of organisms which accumulate polyphosphate, including Corynebacterium xerosis (Muhammed, 1961) Azotobacter vinelandii (Zaitseva and Belozerskii, 1960), Salmonella minnesota (Mühlradt, 1971), Arthrobacter atrocyaneus (Levinson et al., 1975), Propionibacterium shermanii (Robinson et al., 1984), Pseudomonas vesicularis (Suresh et al., 1985) and Acinetobacter calcoaceticus (T'Seyen et al., 1985).

In certain procaryotic organisms this enzyme appears to be the key enzyme in polyphosphate metabolism, as shown by the fact that mutants of Aerobacter aerogenes (Harold and Harold, 1963) and Anacystis nidulans which do not contain polyphosphate kinase (Vaillancourt et al., 1978) cannot accumulate polyphosphate. In his original research with this enzyme, Kornberg (Kornberg et al., 1956) found that ADP was a strong inhibitor of the enzyme. Later, he identified the apparent inhibition as the reverse reaction, in other words

$$\text{ADP} + (\text{polyphosphate})_n \rightleftharpoons \text{ATP} + (\text{polyphosphate})_{n-1}$$

In contrast, the reverse reaction could not be shown for the enzyme from C. xerosis (Muhammed et al., 1959). This enzyme had an absolute requirement for magnesium and was inhibited by ADP and AMP. The same inability to catalyse the transfer of a phosphate residue from polyphosphate to ADP was observed in the enzyme from A. vinelandii. ADP inhibits this enzyme. Non-adenine nucleotides cannot replace ATP in the polyphosphate synthesis reactions. The amount of ATP varies very little during synthesis, which indicates a continuous replenishment of the used ATP (Zaitseva and Belozerskii, 1960).

The enzyme from Mycobacterium smegmatis catalyses the reverse reaction and shows an absolute requirement for

magnesium. The reaction is inhibited by AMP, ADP and high concentrations of ATP (Suzuki et al., 1972).

The enzyme from A. atrocyaneus shows the same inhibition pattern but the reaction will occur with either manganese or magnesium (Levinson et al., 1975).

Phosphate starvation stimulates polyphosphate kinase activity in Aerobacter aerogenes which leads to a simultaneous increase in the rate of polyphosphate accumulation as soon as orthophosphate is made available to the starved bacterium (Harold, 1964). A similar rapid increase in polyphosphate kinase activity occurred in A. atrocyaneus when the organisms were aerated in the absence of orthophosphate (Levinson et al., 1975). In contrast with this, the polyphosphate kinase from E. coli shows very little reaction to the absence of orthophosphate in the medium (Nesmeyanova et al., 1973). A partially purified enzyme from E. coli exhibits an absolute requirement for orthophosphate for maximum activity (Li and Brown, 1973). As can be expected from the dependance of polyphosphate accumulation on growth conditions, polyphosphate activity is also influenced by the growth phase of Ps. vesicularis (Suresh et al., 1985).

The polyphosphate which is produced by the reaction of the enzyme from P. shermanii comprises more than two hundred phosphate residues (Robinson et al., 1984). Studies on the mechanism of this enzyme have shown that the elongation reaction occurs without dissociation of intermediate products from the enzyme, with the result that only high molecular mass polyphosphate is synthesized (Robinson and Wood, 1986). A second enzyme which links polyphosphate to adenine nucleotide metabolism has been observed in some micro-organisms (Winder and Denneny, 1957).

This enzyme catalyses the following reaction :

$$AMP + (polyphosphate)_n \rightleftharpoons ADP + (polyphosphate)_{n-1}$$

Recently, another enzyme which links polyphosphate with energy metabolism was observed in species of Acetobacter, Achromobacter, Brevibacterium, Corynebacterium and Micrococcus (Murata et al., 1980).

The enzyme catalyses the following reaction :

$$NAD^+ + (polyphosphate)_n \rightleftharpoons NADP^+ + (polyphosphate)_{n-1}$$

and is known as polyphosphate dependant NAD^+ kinase.

Szymona and Szymona (1979), observed an enzyme of polyphosphate metabolism which is linked to hexose utilisation in mycobacteria, namely, polyphosphate : glucose-6-phosphotransferase (polyphosphate glucokinase) which catalyses the transfer of a phosphate group to glucose

$$Glucose + (polyphosphate)_n \rightleftharpoons Glucose\text{-}6\text{-}phosphate + (polyphosphate)_{n-1}$$

The existence of this enzyme shows that a phosphate residue from polyphosphate can be used directly in phosphorylation reactions. Studies with the partially purified enzyme indicate that ATP and polyphosphate glucokinase activity exists in Mycobacterium phlei (Szymona and Ostrowski, 1964). Attempts to trace polyphosphate glucokinase in Azotobacter vinelandii and Rhodospirillum rubrum were unsuccessful. A number of Nocardia species however, contained both ATP and polyphosphate glucokinase activity (Szymona et al, 1967).

More recent studies on the enzyme in Nocardia minima have shown that three forms of the enzyme exist in this organism (Szymona and Szymona, 1979). Enzyme activity has been

observed in P. shermanii and P. freudenreichii. The rate of glucose phosphorylation is much more rapid with polyphosphate than with ATP (Wood and Goss, 1985). Studies with the partially purified enzyme from P. shermanii indicate that ATP and polyphosphotransferase activities are catalysed by the same protein (Pepin and Wood, 1986).

Another enzyme which catalyses the synthesis of polyphosphate has been observed in certain bacteria (Kulaev et al., 1971) and later in the bacterial parasite Bdellovibrio bacteriovorus (Kulaev and Vagabov, 1983). This enzyme participates in the following reaction :

$$\text{1,3 - diphosphoglycerate} \rightleftharpoons \text{3 - phosphoglycerate} + \underset{n}{(\text{polyphosphate})} + \underset{n-1}{(\text{polyphosphate})}$$

In Micrococcus lysodeikticus, P. shermanii and E. coli, the activity of this enzyme is affected by the culture age. The activity is higher in the stationary phase (Kulaev et al., 1971). This enzyme with polyphosphate kinase, contributes equally to polyphosphate synthesis in E. coli (Nesmeyanova et al., 1973), and similarly, is not controlled to any significant degree by external orthophosphate (Nesmeyanova et al., 1974).

Degradation of polyphosphates occurs through the participation of two enzymes. The enzyme polyphosphate-depolymerase splits the molecule into smaller fragments according to the following reaction :

$$\underset{n}{(\text{polyphosphate})} + H_2O \rightleftharpoons \underset{n-x}{(\text{polyphosphate})} + \underset{x}{(\text{polyphosphate})}$$

Until the present time, this enzyme has only been observed in eucaryotic organisms (Kulaev and Vagabov, 1983).

The polyphosphatases cleave one terminal phosphate residue from the polyphosphate chain :

$$(\text{polyphosphate})_n + H_2O \rightleftharpoons (\text{polyphosphate})_{n-1}$$

Polyphosphatase occurs widely in polyphosphate accumulating organisms (Kulaev and Vagabov, 1983). In E. coli a significant amount of the polyphosphatase is membrane bound and appears on the outside of the plasma membrane (Nesmeyanova et al., 1974; Severin et al., 1976). The biosynthesis and secretion of polyphosphatase into the periplasma is repressed by external phosphate (Harold, 1966; Nesmeyanova et al., 1974; Maraeva et al., 1979) in certain organisms. On the other hand, the activity is stimulated by a lack of phosphate in the medium (Harold, 1964). This polyphosphatase enzyme in Aerobacter aerogenes catalyses the chief degradation reaction and indicates that the primary function of polyphosphate is to regulate intracellular phosphate levels rather than to act directly as a phosphagen (Harold and Harold, 1965). Reversal of the inhibition of the E. coli polyphosphatase by orthophosphate starvation has also been observed (Yagil, 1975). The A. calcoaceticus enzyme is initially stimulated by anaerobic conditions but after 4 hours the activity decreases to a level below that of the aerobic control (Ohtake et al., 1984).

2.5.2 Carbon Metabolism

The complete oxidation of carbon substrates to carbon dioxide and water is the most important source of ATP in obligate aerobic organisms. Oxidative phosphorylation to produce ATP is accomplished by the electron transfer path with simultaneous oxidation of NADH to NAD. Carbohydrate substrates are degraded by the Embden-Meyerhof or Entner-Doudoroff paths to pyruvate which enters the tricarboxylic acid cycle through acetyl CoA.

Where the substrate consists of two carbon containing compounds like acetic acid, both carbons are lost in the form of carbon dioxide through one pass of the cycle. To maintain the tricarboxylic acid cycle these decarboxylation reactions must be bypassed. The glyoxylic acid cycle performs this function (see Figure 2.2).

Micro-organisms also have the ability to accumulate carbon. For the purpose of this study only the accumulation products, polyhydroxybutyrate and polysaccharides, will be discussed.

2.5.2.1 Polyhydroxybutyrate metabolism

A wide variety of micro-organisms accumulate polyhydroxybutyrate (PHB) (Dawes and Senior, 1973). This polymer usually occurs in granules which are intensely stained by Sudan Black in fixed bacterial preparations. The accumulation of PHB in batch and continuous culture has been thoroughly reviewed by Dawes and Senior(1973), and will only be discussed here in relation to the activated sludge process.

The biosynthesis and degradation of PHB has been studied in depth in Azotobacter beijerinckii and will be used to illustrate the enzymatic reactions (see Figure 2.3)(Senior and Dawes, 1971).

α-Ketothiolase, (α-Keto-acyl-CoA-thiolase) catalyses the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. Although the equilibrium constant for this reaction does not favour the formation of acetoacetyl-CoA, it is claimed that under conditions where acetyl-CoA and NAD(P)H concentrations are high and CoA concentrations low, the equilibrium will be shifted in the direction of acetoacetyl-CoA formation (Ritchie et al., 1971).

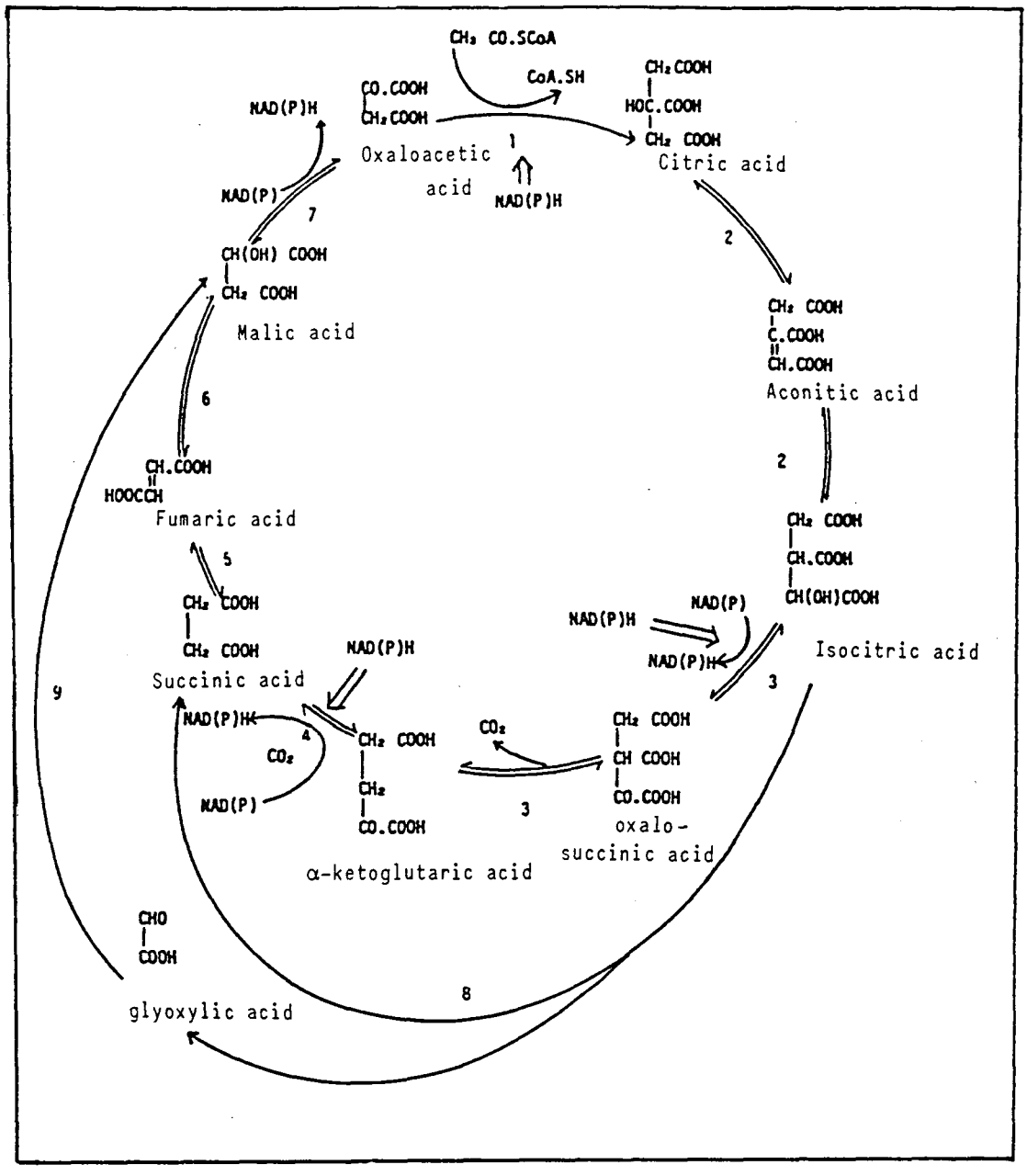


Figure 2.2 : Tricarboxylic acid and glyoxylic acid cycles

Key to enzymes :
1: Citrate synthase
2: Aconitase
3: Isocitrate dehydrogenase
4: β-ketoglutarate dehydrogenase
5: Succinate dehydrogenase
6: Fumarase
7; Malate dehydrogenase
8: Isocitratelyase
9: Malate synthase
⟹ Denotes inhibition

Tricarboxylic acid cycle : 1, 2, 3, 4, 5, 6, 7
Glyoxylic acid cycle : 1, 2, 7, 8, 9

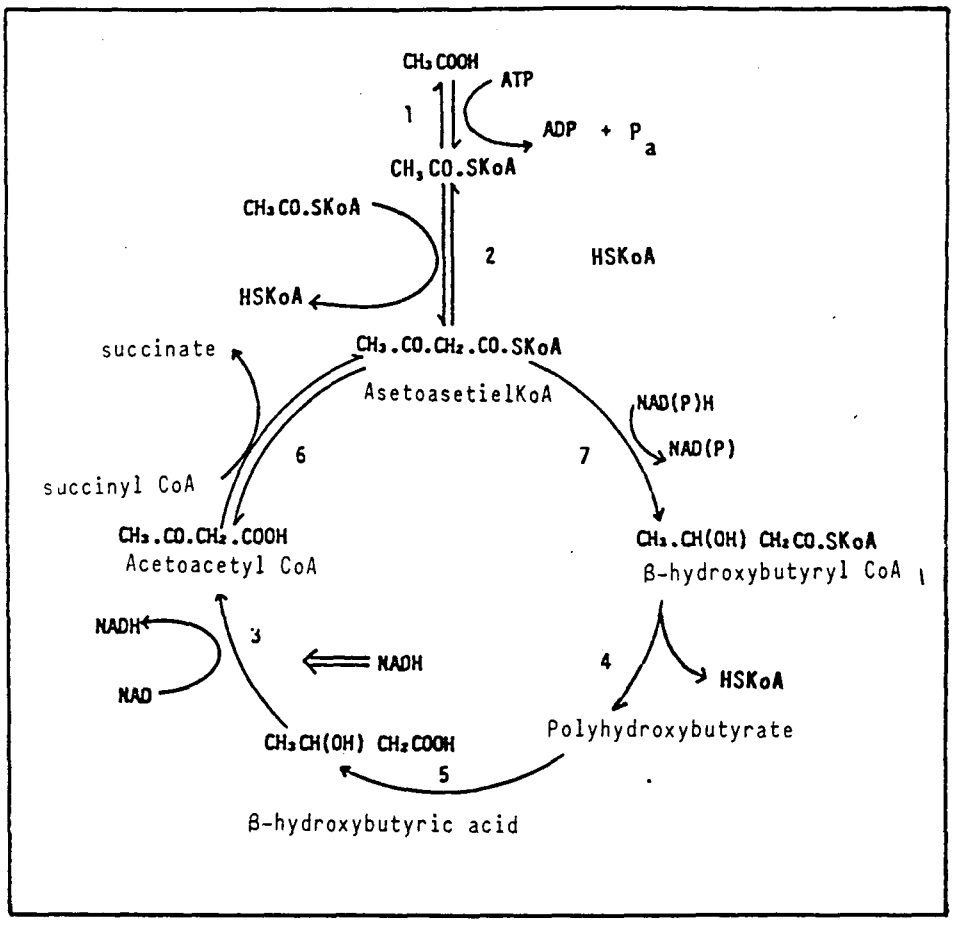


Figure 2.3 : Synthesis and degradation of polyhydroxybutyrate

Key to enzymes : 1: Thiokinase
2: β-ketothiolase
3: β-hydroxybutyrate dehydrogenase
4: β-hydroxybutyrate CoA polymerase
5: Poly-β-hydroxybutyrate depolymerase
Denotes inhibition

These researchers have observed an acetoacetyl-CoA reductase which catalyses the reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA. Subsequent studies with this enzyme have shown that the K_m for acetoacetyl-CoA is in the region of 1 to 2 μM and that inhibition occurs at high concentrations of acetoacetyl-CoA (10 μm) (Senior and Dawes, 1971).

The last reaction is catalysed by PHB granule-bound hydroxybutyryl-CoA polymerase. This enzyme uses

β-hydroxybutyryl-CoA as substrate and releases CoA-SH (Senior and Dawes, 1971).

The degradation of intracellular PHB apparently does not begin before all available exogenous carbon has been exhausted. There is no evidence to suggest that PHB undergoes conversion, particularly if its physical characteristics are taken into account (Alper et al., 1963). Some bacteria have shown a requirement for the presence of oxygen for degradation (Macrae and Wilkinson, 1958; Stockdale et al., 1968).

The initial hydrolysis of PHB involves the production of β-hydroxybutyric acid catalysed by a PHB depolymerase.

In contrast with the synthetic pathway, degradation occurs via the free acids (Macrae and Wilkinson, 1958; Sierra and Gibbons, 1962; Wong and Evans, 1971).

Every bacteria which has the ability to accumulate PHB contains a β-hydroxybutyrate dehydrogenase enzyme which catalyses the oxidation of the acid to acetoacetic acid (Dawes and Senior, 1973). The final step in the degradation path in A. beijerinckii is the conversion of acetoacetic acid to acetyl-CoA through the action of acetoacetate : succinyl CoA : CoA transferase (Dawes and Senior, 1973) (see Figure 2.3). The complete metabolism of acetyl-CoA is summarised in Figure 2.4.

2.5.2.2 Extracellular polysaccharides

Extracellular polysaccharides can, for convenience, be divided into cell wall material and exoglycans (material which is excreted by the cell into the growth medium). This discussion is restricted to the latter group.

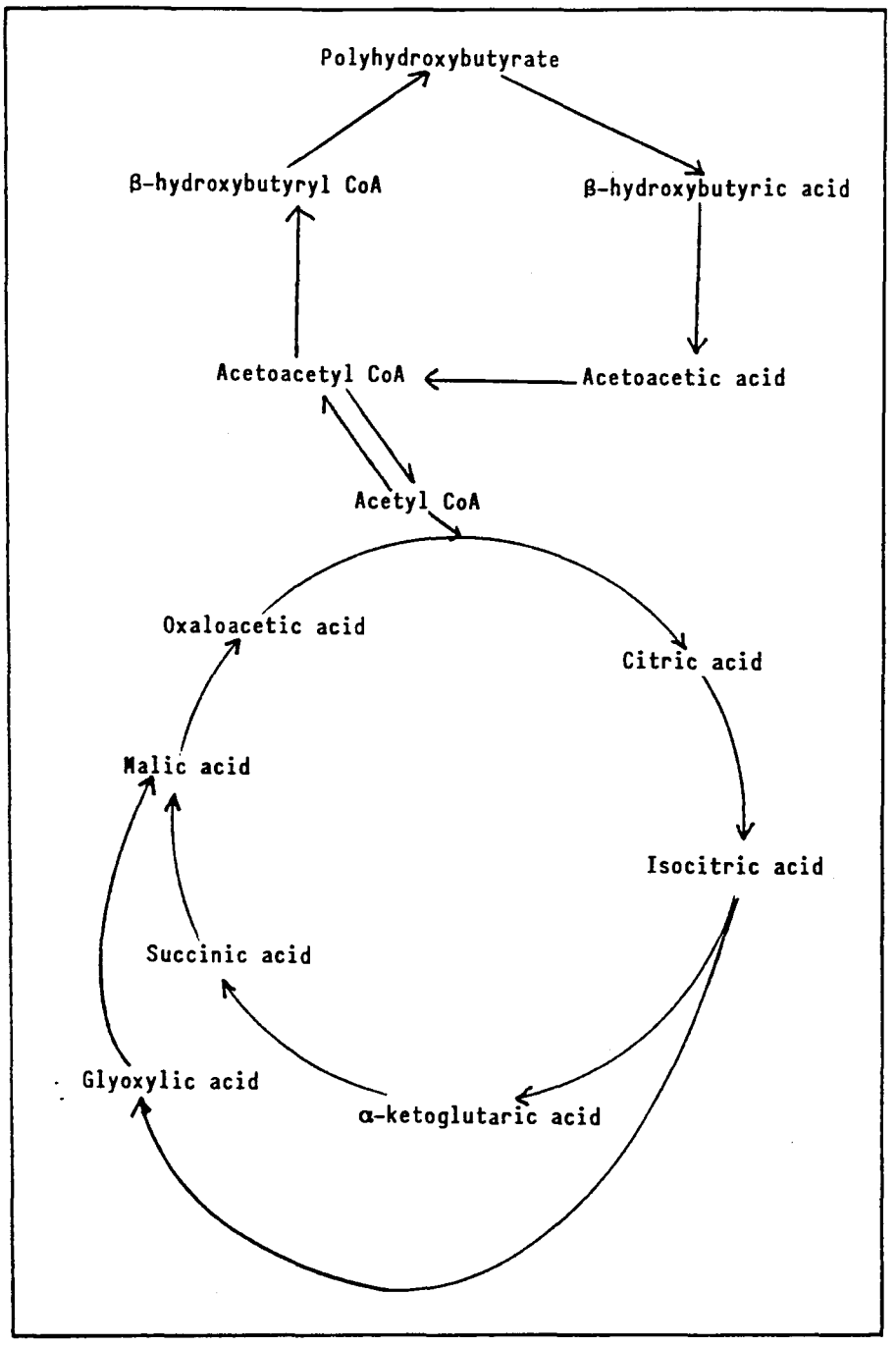


Figure 2.4 : Summary of acetyl CoA metabolism

In contrast with the extracellular polysaccharides which function as food reserves, the exoglycans in general, cannot be used by the micro-organisms which synthesise them as potential energy and carbon sources. Most microbial exocellular polysaccharides are hetero-polysaccharides which comprise a variety of mono-saccharide residues.

The polysaccharides which are produced by Pseudomonas aeruginosa comprise for example, D-glucose, D-galactose, D-mannose, L-rhamnose and D-glucuronic acid residues while Chlamydomonas ulvaensis excretes a polysaccharide which consists of glucose and xylose (Woodside and Kwapenski, 1974). Morphological and biochemical studies of floc-forming bacteria indicate the exocellular polymers are responsible for the flocculating growth habits of these bacteria (Friedman et al., 1969).

Bacterial aggregation is generally ascribed to exo-cellular polysaccharides, although polyamino acids could also play a functional role in this respect. These compounds are excreted from the surface of the cell under different physiological conditions. The availability of certain substrates and relative nutrient concentrations also influences the composition and concentration of exocellular polymers (Harris and Mitchell, 1973). Emulsan, the extracellular polymeric emulsifier which is produced by certain isolates of A. calcoaceticus occurs in the cell-free and cell-associated form (Goldman et al., 1982). The main portion of the cell capsule consists of the cell-associated emulsan. A reduction in this form occurs simultaneously with an increase in extracellular emuslifying activity (Pines et al., 1983). It appears as if this polymer has two roles to play, namely, encapsulation of the cell and emulsifying of hydrocarbon substrates.

Emulsan production is not unique to isolates which grow on hydrocarbon substrates (Sar and Rosenberg, 1983). The flocculating ability of activated sludge bacteria is without doubt an important factor in successful secondary settling, before discharge of the effluent to the river. The settling characteristics of flocculating bacteria are improved with an increase in the concentration of exocellular polymer (Sheintuch et al., 1986).

In this review the synthesis of polysaccharides from monosaccharides is discussed. Mutual conversion of aldoses and ketoses is accomplished by a series of isomerases which are present in micro-organisms. Enzymes which catalyse the amination and acetylation of sugars are also known (Hassid, 1971).

In general, the syntheses of polysaccharides follow similar paths. The monosaccharide residues are sequentially added to an existing chain. This reaction which usually involves the transfer of the monosaccharide residue from a carrier nucleotide diphosphate, is catalysed by a specific enzyme as follows (Mahler and Cordes, 1971) :

Polysaccharide chain + NDP sugar $\rightleftharpoons$ Polysaccharide chain sugar + NDP etc

2.5.3 Metabolite Transport

Researchers in the field of membrane transport agree that the proton-motive force is the most important energy source in bacterial active transport (Brodie et al., 1972; Boyer, 1977; Wilson, 1978; Jain and Wagner, 1980). The so-called primary active transport systems generally involve the creation of the proton-motive force. The two most important energy sources in this process, are ATP hydrolysis and

substrate oxidation, coupled to an electron flow (Brodie et al., 1972; Harold, 1974). Mitchell's chemical osmotic theory (1977) is largely responsible for the concept of proton-motive force. According to Mitchell (1968; 1977), electron transfer chains and ATP-ase complexes are incorporated in the membrane where they act as electrogenic pumps (Harold, 1974; Konings et al., 1981). In the bacterial membrane protons are translocated from the cytoplasm to the external medium by these proton pumps. The cytoplasmic membrane is, practically speaking, impermeable for ions, particularly protons and hydroxyl ions. The translocation of protons results in the creation of two gradients. A pH gradient or chemical proton gradient is formed as a result of the removal of protons from the cytoplasma, and an electrical potential is formed by the loss of positive charges from the cytoplasma to the medium (Harold, 1974; Konings et al., 1981).

Both gradients exert an inward force on the protons which is known as the proton-motive force (μH^+). The generation of this force is summarised in Figure 2.5.

2.5.3.1 Cation transport

Potassium transport has been well-studied in bacteria. Three separate potassium transport systems have been observed in E. coli. One of the systems is a high affinity trace potassium system which permits growth to take place at low external potassium levels and is inhibited by growth in high potassium (Silver, 1978). This system is unusual in that it does not appear to possess a periplasmic protein component. In addition, potassium uptake is not linked to sodium excretion but possibly to excretion of another cation (Laimins et al., 1978).

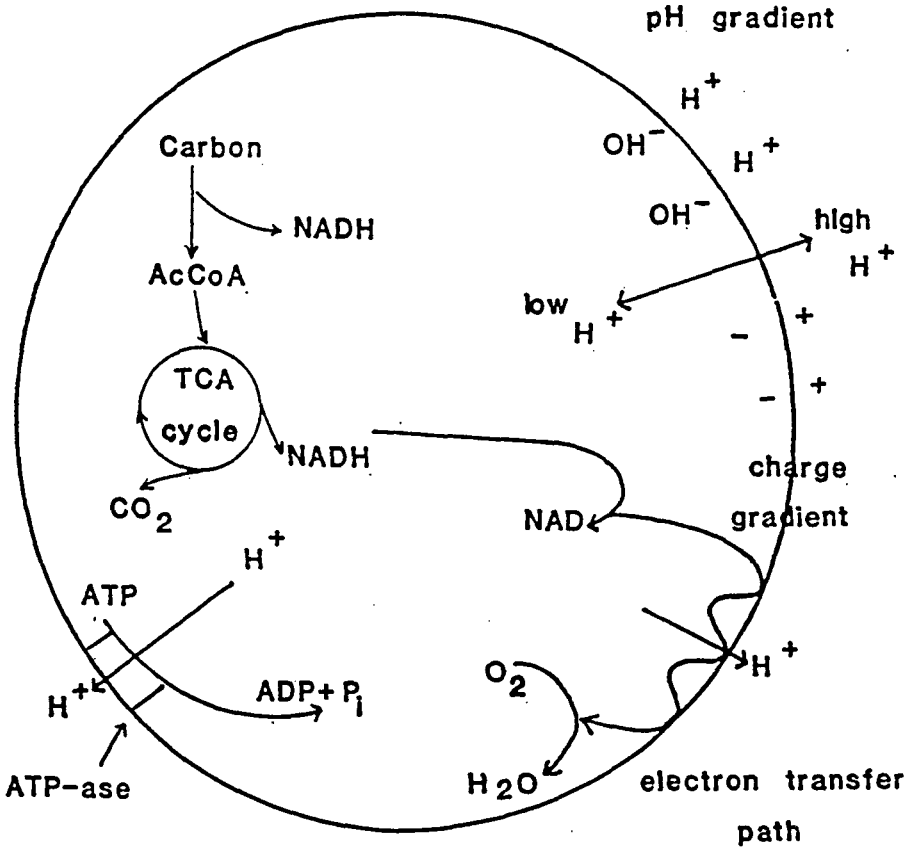


Figure 2.5 : Schematic representation of the generation of the proton motive force (Conseau et al. 1985)

Harold (1977) earlier suggested an energy transductive reversible ATP-ase for E. coli which excretes protons in reaction to potassium uptake. In contrast, S. faecalis, which possesses three systems similar to those that have been described for E. coli takes up potassium with simultaneous excretion of equivalent amounts of hydrogen and sodium ions (Harold et al., 1970)

The chief potassium system is synthesised constitutively and dominates the potassium economy at medium or high external potassium levels. The third system is also synthesised constitutively at low and high potassium concentrations. Potassium movement appears to be regulated by osmotic pressure (Silver, 1978).

In order to carry out its energy-generating function, the components of the proton-motive force must be present in sufficient quantities to buffer the variation rate in the hydrogen utilisation and generating processes (Skulachev, 1977). Skulachev (1978) further postulated that a sodium/potassium gradient existed as a membrane-linked buffer system. Electrophoretic potassium ingress results in the conversion of electrical potential to pH differences, thereby increasing the total capacity of the system which has the result that it offers more resistance to variations. The pH can be converted to pNa by an NA^+/H^+ antiport system. An increase in the intracellular concentration of potassium is compensated to a certain extent by sodium.

Magnesium is the chief intracellular divalent cation in all living cells. In bacterial cells the magnesium content is equivalent to 1 to 2 millimolar magnesium in contrast to 100 to 500 micromolar levels for potassium. Although most of the intracellular magnesium is bound so that it is not osmotically active, it remains relatively easily exchangeable. In-depth investigations into magnesium transport in E. coli and other microbial cells have exposed the presence of two systems. The cobalt resistant system is synthesised constitutively during growth in low or high magnesium concentrations. It transports magnesium as primary substrate but also accumulates cobalt, nickel and manganese. The other system is specific for magnesium and is inhibited by growth in high magnesium concentrations (Park et al., 1976).

Transport of iron, particularly under iron starvation conditions, involves a series of high affinity iron binding chelates which are secreted in iron deficient media. After binding with the iron the ferrichelate is

taken up by highly specific transport systems (Silver, 1978). One of these systems is responsible for the uptake of iron which is chelated with hydroxyamino-acids.

High affinity transport systems for the uptake of traces of manganese which supply most bacteria with their growth requirements exist. Other elements which appear to be essential for the growth of certain bacteria include copper, nickel, chrome and cobalt, for which evidence of specific transport systems has not been obtained (Silver, 1978).

All bacteria apparently possess outwardly orientated energy dependant calcium transport systems (Silver, 1978). The transport system is orientated in the cell membrane so that the intracellular calcium level is reduced to a level lower than that of the external medium.

Research with E. coli indicates that calcium is excreted in antiport for protons with the proton circulation serving as the only energy source (Rosen and McClees, 1974; Tsuchiya and Rosen, 1976). In contrast with this, Kobayasi et al., (1978) proposed an ATP-linked pump which excretes calcium by electro-neutral exchange for protons.

Bacterial cells also excrete sodium. Under physiologically active conditions a sodium to sodium gradient of between 1 : 3 and 1 : 50 is maintained.

In the case of E. coli, sodium egress occurs via a sodium hydrogen antiport system which translocates sodium outside the cell under the influence of an inwardly orientated hydrogen gradient (West and

and Papineau (1972) proposed a somewhat different system for S. faecalis, namely, that the cells excrete sodium in exchange for hydrogen via an ATP-linked sodium pump (Heefner and Harold, 1982), and that the hydrogen ions are then excreted by the proton pump.

2.5.3.2 Anion transport

Four transport systems exist to transport orthophosphate across the cell membrane of E. coli. There are two constitutive systems of which one operates at five times higher levels after phosphate starvation (Rosenberg et al., 1977) and two inducible organo-phosphate systems which can transport orthophosphate as alternative substrate (Bennett and Malamey, 1970). The Pst system has the basic characteristics of an ATP-driven system, while the Pit system appears to be linked to the proton motive gradient (Rosenberg et al., 1977). Orthophosphate uptake in E. coli requires hydrogen ions for symport with dihydrogen phosphate and extracellular potassium (Harold, 1977).

Phosphate uptake in S. faecalis is dependant on the capacity of the cells to maintain a neutral or alkali cytoplasma. The ATP-driven phosphate accumulation is apparently an electron neutral exchange for hydroxyl ions (Harold and Spitz, 1975). Phosphate transport in Parracoccus denitrificans occurs in the same manner (Harold, 1977).

The two constitutive phosphate accumulation systems appear to be sensitive to intracellular and extracellular phosphate levels in respect of the initial uptake rate. In phosphate deficient cells of Corynebacterium bovis the initial rate is dependant on the external phosphate concentration and is reciprocally related to the amount of intracellular phosphate (Chen,

1974). In Bacillus cereus the uptake rate is doubled in phosphate-starved cells, as a result of the requirement that the primary phosphate pool be filled before metabolism can be initiated. As soon as the primary pool has been filled, the uptake rate drops to about half of the initial value (Rosenberg et al., 1969). The same pattern is observed in starved E. coli cells. In this case however, two kinetically independant low and high affinity systems have been recognised (Medveczky and Rosenberg, 1971).

As in the case of phosphate, the sulphate ion requires a specific binding protein for transport (Oxender, 1972). It has already been shown that sulphate transport in P. denitrificans is sensitive to uncouplers and can be driven by respiration or a trans-membrane pH gradient. Based on experimental data, it is proposed that sulphate transport can be driven by a mechanism of electro-neutral symport in any direction. Increased uptake occurs through respiration-driven proton excretion (Burnell et al., 1975). In certain fungi a synergistic relationship between calcium and sulphate uptake has been observed (Cuppoletti and Segel, 1975).

Studies with a number of bacteria indicate that bacterial cells take up acetic acid in its undissociated form (Visser and Postma, 1973; Kell et al., 1981; Konings et al., 1981). The uptake may occur via facilitated diffusion. This possibility is supported by the fact that acetate and propionate share a common uptake system in E. coli with K_m values of 36 and 220 µM respectively (Kay, 1972). Whether uptake occurs via free diffusion as proposed for butyric acid and valeric acid (Salanitro and Weginer, 1971), or by facilitated diffusion, the acids are taken up in the undissociated form.

Fumerate, malate and succinate are taken up in E. coli by an active transport system. The system requires simultaneous uptake of two protons for every acid molecule (Gutowski and Rosenberg, 1975). One of the most important transport systems for carbohydrate uptake by bacteria is the phospho-enol pyruvate phosphotransferase system. A membrane-bound enzyme catalyses phosphorylation and the transfer of the sugar across the membrane (Kaback, 1968; Postma and Roseman, 1976).

2.5.4 Intracellular Metabolic Control

Metabolic control mechanisms are essential for the orderly continuation of life. In bacteria, as well as other cells, control mechanisms can be divided into two main groups, namely :

- alteration in enzyme activity
- alteration in the number of enzyme molecules.

Enzyme activity can simply be changed by the presence of different substrate concentrations or the accumulation of products. The enzyme can also be activated or inhibited by a small molecule, often an inorganic ion or substrate analogue, in that this molecule can bind with the enzyme directly near the active centre.

This direct modifying action differs from a second, more complex type of control, so-called allosteric control, in that the modifying molecule is not only bound near the active centre but also indirectly at distant secondary allosteric sites.

In this case, the term activator and inhibitor is not sufficient. The effector usually acts by modifying the

reaction components. The terms, positive and negative effector, indicate whether the enzyme affinity for the substrate is increased or decreased by the effector. The addition of a positive or negative effector does not change the maximum velocity, but shifts the whole substrate saturation curve to left or right. By switch-type control mechanisms where a single effector is required for activity, the maximum velocity of the enzyme reaction is usually increased.

Enzyme activity can also, as already mentioned, be controlled by regulating the number of enzyme molecules.

Enzyme synthesis involves the following steps :

$$\text{DNA} \xrightarrow{\text{transcription}} \text{RNA} \xrightarrow{\text{translation}} \text{protein}$$

Control over enzyme levels does not only occur on the level of transcription or translation, but also by altering the rate of degradation of mRNA or proteins. (See Figure 2.6).

Enzymes for which the structural gene is situated in the operon are induced or repressed by low molecular mass compounds, while enzymes for which the gene is not situated on the operon, can be induced or repressed to different degrees, so-called, repression or induction respectively.

The structural gene is a requirement for the synthesis of an enzyme. In many cases, the presence of a low molecular mass compound which is related to the substrate, is required for synthesis. This process is called induction.

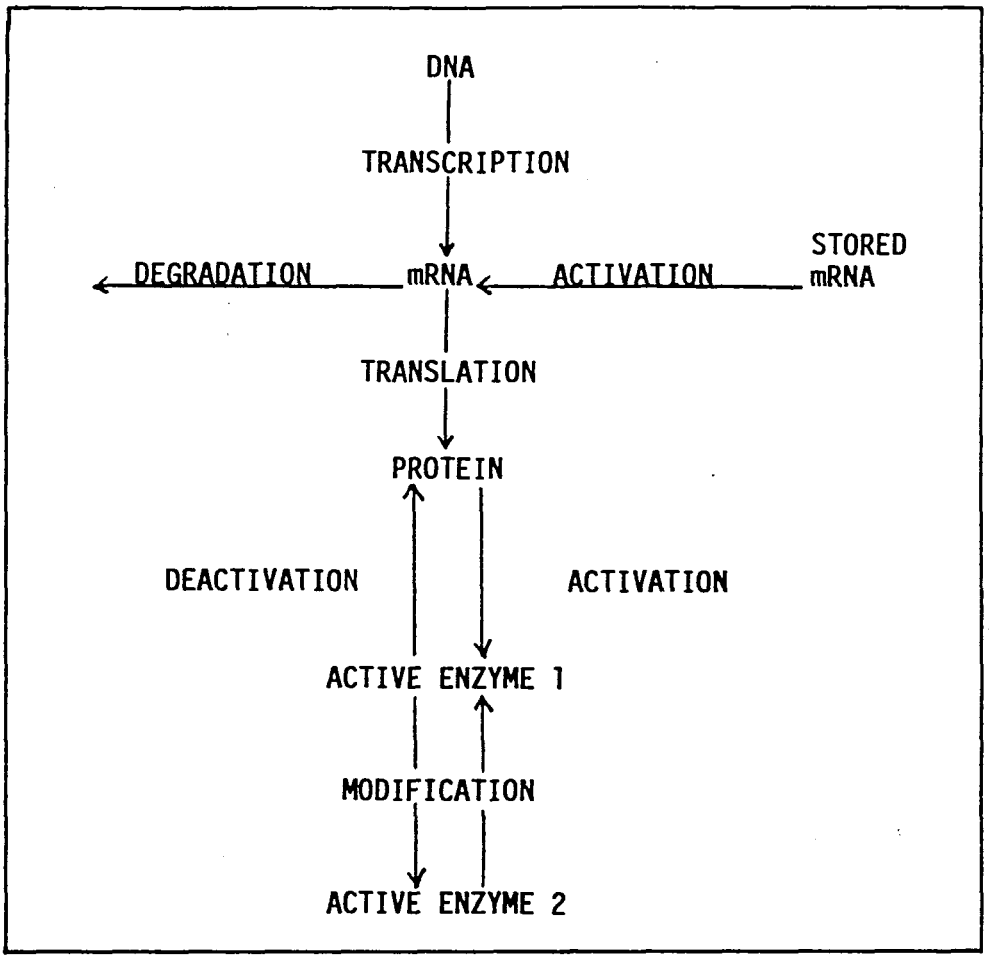


Figure 2.6 : Centres for the possible regulation of enzyme levels(Dubery, 1985)

Other low molecular mass compounds which are either products of the reaction or related to the products, act as co-repressors by preventing enzymes synthesis in the presence of the structural gene. This process is called repression. Structural genes do not exert any influence on the rate of synthesis.

The regulator genes control the rate of enzyme synthesis. Induction or repression exerts a direct effect on transcription which has a subsequent effect on translation. Enzyme synthesis can be switched on and off by these processes depending on the presence of metabolites.

Observations that cyclic AMP levels in bacteria vary with changes in the external carbon substrate level, led to the postulation that cyclic AMP plays a role in enzyme induction.

Further research in this direction has indicated that cyclic AMP regulates the synthesis of a number of enzymes (de Crombrugghe and Pastan, 1973).

Most known control mechanisms occur on the level of enzymatic conversion of the substrate. Metabolism can also be efficiently controlled by regulating the flow of metabolites to the conversion centre, which is considered the first step in metabolic conversion. Possible centres of control are indicated in Figure 2.7.

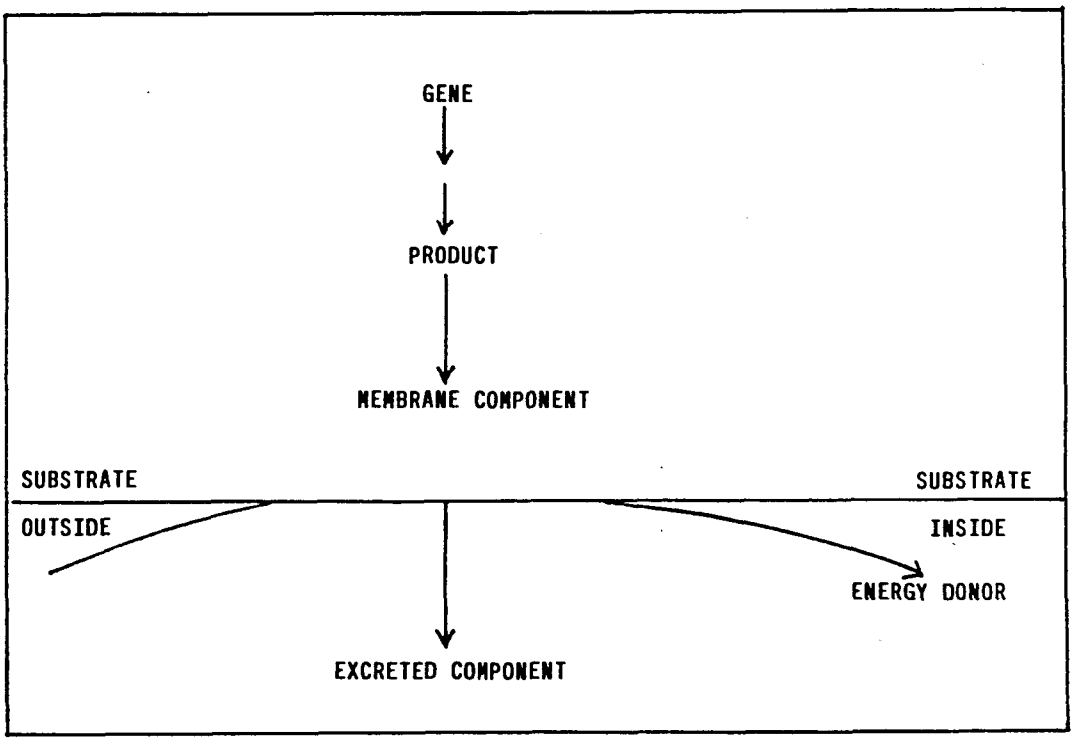


Figure 2.7 : Possible centres for the regulation of metabolite transport across the cell membrane

Transport systems can be induced in the same manner as enzymes. The control can be carried out by external or internal metabolites. So, for example, the internal

induction of galactose and malate transport in bacteria has been observed. Transport of citrate is inducible but not that of succinate. In contrast, the glucose-6-phosphate transport system in E. coli is induced by exogenous glucose-6-phosphate (Pardie and Palmer, 1973).

In general, metabolic control mechanisms react to modify the expected control which is exerted by substrate concentration. If an excess end product accumulates, one of the first steps in the metabolic pathway is inhibited and the metabolite levels return to normal. This control can be achieved by feedback inhibition or repression (see Figure 2.8). If this product is present the reaction rate falls by feedback inhibition and is then normalised by feedback inhibition and repression. Much better control is obtained with combined feedback and repression. An increase in the intracellular level of the substrate in the presence of an intermediate results in almost the same type of pattern, except that the final control by feedback, combined with repression, is not as normal as in the first mentioned case (see Figure 2.9), (Larner, 1971).

Feedback inhibition in bacterial systems can be described by four examples.

. Enzyme multiplicity

This form of feedback inhibition can be explained using an example. The phosphorylation of aspartic acid to aspartyl phosphate is the first reaction in the biosynthesis of lycine, methionine, threonine and isoleucine. In order to control this highly branched pathway, three different aspartokinase enzymes exist which are regulated by different effector compounds. One of these enzymes is inhibited specifically and completely by lycine.

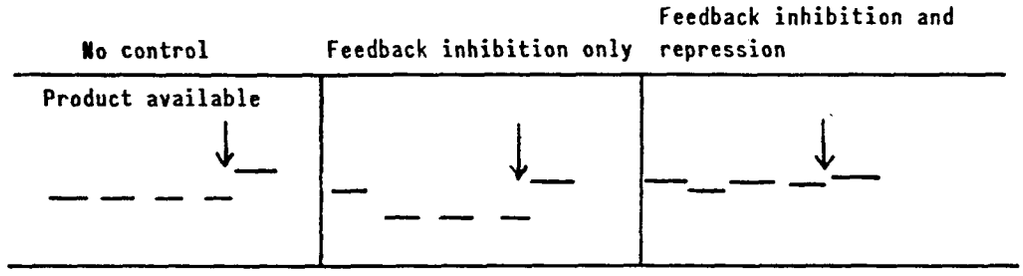


Figure 2.8 : Substrate levels in controlled and uncontrolled systems with product available. ↓ Indicates control point.

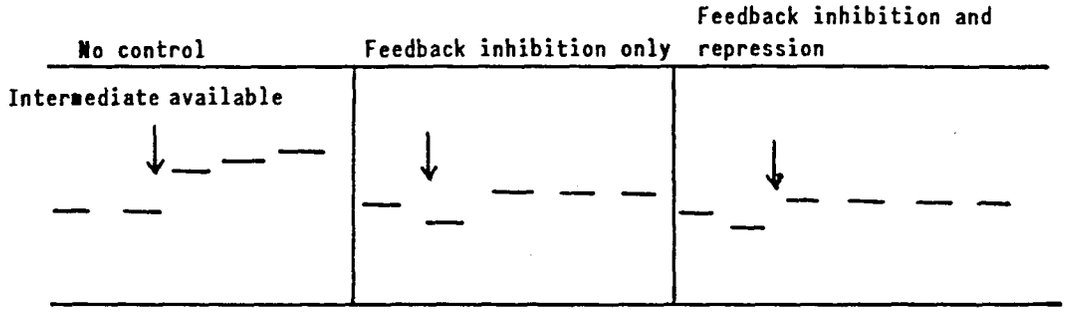


Figure 2.9 : Substrate levels in controlled and uncontrolled systems with intermediate product available. ↓ Indicates control point.

The synthesis of this enzyme is also repressed by lycine. The second enzyme is only present in small quantities and is specifically inhibited by homoserine. The third enzyme is inhibited specifically and completely by threonine. Thus, the synthesis of aspartyl phosphate is ensured even in the presence of an excess of one of the amino acid end products. The whole rate of aspartyl synthesis is dependant on the regulation of all three enzymes.

. Combined feedback inhibition

In this type of inhibition two or more end products must be present simultaneously and in such levels as to inhibit the key enzyme. This mechanism does not allow the fine regulation of enzyme multiplicity, but allows the metabolic flow to continue in the presence of only one of the end products in excess.

. Co-operative feedback inhibition

In this case, each end product causes partial inhibition of the key enzyme, but the simultaneous presence of two or more end products causes co-operative inhibition so that the total inhibition is greater than the single sum of the individual effects.

. Cumulative product inhibition

In this mechanism the inhibition occurs through each product independantly and separately, and appears to be used when a single reaction is responsible for a number of end products.

Control of enzyme activity by pH is another example of direct interaction of a small molecule with an active centre of the enzyme.

The number of pH optima which have been reported for intracellular enzymes exceed the micro-milieus which can be present in the cell. Enzymes normally function away from their pH optima and are therefore very sensitive to pH changes. Factors which have an effect on cytoplasmic pH can also influence enzyme activity (Padan et al., 1981). Most intracellular conversions are directly or indirectly dependant on the adenine nucleotides.

Nett synthesis of ATP from ADP occurs during catabolism. The ATP is used to drive endergonic biosynthetic pathways and is converted to ADP and AMP in the process. The adenine nucleotides therefore have a unique regulatory role in cell. In general, catabolic enzymes are activated by ADP or AMP and inhibited by ATP. In the intracellular environment all three adenonine nucleotides are present.

An alteration in the concentration of one influences the other two. This phenomenon has led to the formulation of the adenylate energy charge (AEC)

$$AEC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

of which the values vary between 0 (all AMP) and 1 (all ATP), (Atkinson, 1968).

Considering that the energy charge is dependant on the balance between ATP utilising and ATP generating reactions, it would be beneficial if the reaction rates were controlled by the energy charge.

The proposed pattern of this type of control is shown in Figure 2.10. Similar curves have already been observed for enzymes from ATP utilising and generating pathways. Metabolism is however, not so simple, and the metabolites also exert a modifying effect on the reaction rate. Figure 2.10 can therefore be modified as shown in Figures 2.11 and 2.12 (Atkinson, 1971). The effect of the energy charge is however, stronger than the effect of one of the nucleotides alone (Swedes et al., 1975).

Up to this stage the discussion of alterations in enzyme activity has been limited to non-covalent bonding of modifiers. Reversible covalent bonding has come to the fore as an important form of enzyme modification.

The question arises as to why covalent bonding as a control mechanism is necessary, as the non-covalent ligand bonding of allosteric control appears to be efficient. Krebs (1985) believes that the two types of mechanisms developed to handle two different situations; allosteric control in response to intracellular signals, and covalent modification in response to extracellular signals.

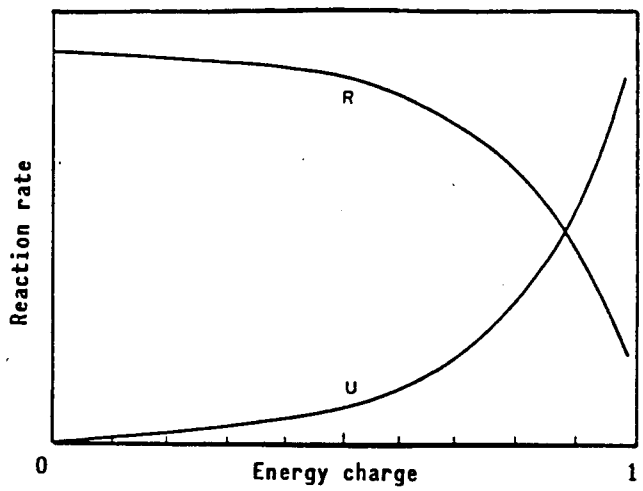


Figure 2.10 : General response of the adenylate energy charge for enzymes involved in ATP-generating (R) and ATP-utilising (U) reactions

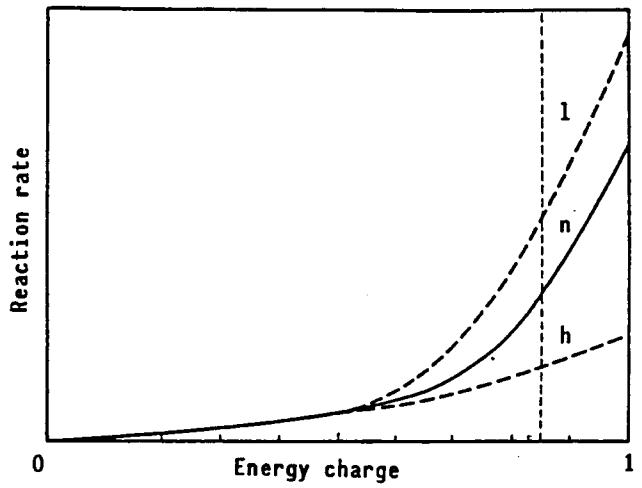


Figure 2.11 : General interaction between adenylate energy charge and the concentration of a metabolic modifier which controls a regulating enzyme in an amphibolic series
l : low; n : normal; h : high concentrations of the modifier

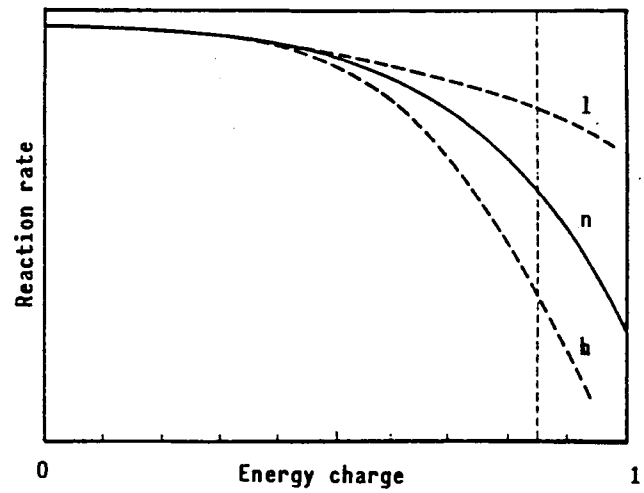
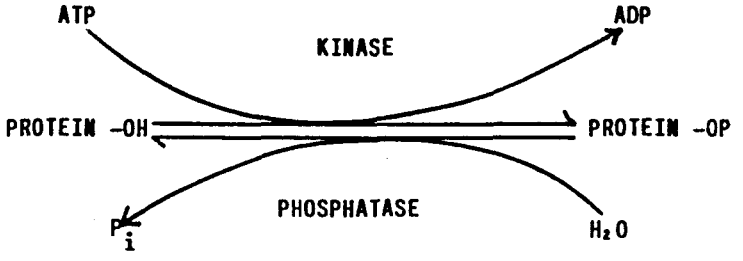


Figure 2.12 : General interaction between adenylate energy charge and concentration of an end product which controls a regulatory enzyme in a biosynthetic series of reactions
l : low; n : normal; h : high concentrations of the modifier

The six reversible covalent protein modifications which have already been described; phosphorylation/dephosphorylation; acetylation/deacetylation; adenylation/deadenylation; uridylation/deuridylation; methylation/demethylation and S-S/SH conversions (Krebs and Beavo, 1979), occur much more often in eucaryotic cells than in procaryotic cells (Krebs, 1985), which correlates with the thought that this type of control is essential in more complex organisms where extracellular signals are important.

Protein phosphorylation normally involves the protein kinase catalysed transfer of a gamma-phosphate residue from an ATP to a serine, threonine or tyrosine residue of the protein. This modification is reversed by protein phosphatase which hydrolyses the phosphate bond (see Scheme 2.1), (Hoffman, 1982).



Scheme 2.1 : Phosphorylation / dephosphorylation of a protein.

Although allosteric control is very well developed in bacterial systems, cases of phosphorylation and dephosphorylation particularly, are not unknown. In E. coli the branch point of the tricarboxylic and glyoxylic acid cycles is controlled by the reversible phosphorylation of isocitrate dehydrogenase (Garnak and Reeves, 1978; Walsh and Koshland, 1985). The isocitrate dehydrogenase kinase and gamma-phosphatase enzymes which are responsible for the conversion reactions have already been isolated from E. coli (Nimmo et al., 1984). Evidence for protein phosphorylation has also been

observed in Salmonella typhimurium (Wang and Koshland, 1978).

2.5.5 The Effect of Oxygen Levels on Metabolic Activity

Oxygen is essential for many micro-organisms, while it is toxic for others, even in small quantities. The chief requirement for oxygen is a terminal electron acceptor for the electron transfer path, the final step in substrate oxidation. Nitrate can replace oxygen as terminal electron acceptor in certain organisms (Hughes and Wimpenny, 1969; Stanier et al., 1980). The oxygen concentration in the medium affects the oxygen utilisation rate, biomass yield, cell composition, use of carbon sources and enzyme synthesis (Gaudy and Gaudy, 1981). The effect of oxygen concentration on respiration rate has been thoroughly studied. The respiration tempo of facultative anaerobic bacteria increases with increased oxygen concentration. This stimulation of respiration varies from species to species and with the nature of the carbon source. In most cases, very low levels of dissolved oxygen are involved in the increase in respiration rate. Above a certain critical dissolved oxygen level, increased dissolved oxygen levels contribute very little to respiration tempo (Rickard and Gaudy, 1968). The critical value is usually lower for dispersed cells than flocculated cells. The critical dissolved oxygen level for dispersed cells is less than 0,1 mg/ℓ in contrast with 0,5 mg/ℓ for flocculated heterogenous populations (Gaudy and Gaudy, 1981). Changes in the cytochrome levels of a number of organisms have been summarised by Harrison (1972). Cytochrome levels have been shown to be higher at low oxygen concentrations for more than half the bacteria studied.

Differences in enzyme activity between aerobic and anaerobic conditions are well-known in facultative anaerobes (Wimpenny and Necklen, 1981; Thomas et al., 1972). This change in enzymatic activity often promotes the conversion from respiration to fermentation in facultative organisms (Thomas et al., 1972).

The biosynthesis of tricarboxylic acid cycle enzymes is affected by the presence or absence of oxygen. The cycle still functions under anaerobic conditions but at a slower rate as a result of lower enzyme levels (Gray et al., 1966). Although the effect of low oxygen concentrations has been thoroughly investigated in facultative organisms, very little is known about the effect of low oxygen concentrations on obligate aerobes. Certain obligate aerobes survive anaerobic conditions by increased respiration of oxygen-containing substrates (Lloyd et al., 1983). The obligate aerobe Acinetobacter survives anaerobic conditions in laboratory studies (Murphy and Lötter, 1986), and in the natural habitat of the activated sludge plant (Lötter and Murphy, 1985).

The effect of oxygen deficiency on nicotine-amide co-enzymes has been thoroughly studied in plant tissue. When oxidation reactions cease as a result of oxygen deficiency, the tissue will continue to metabolise for a short time at a low efficiency, until the NADP and NAD levels begin to decrease (see Figure 2.13).

If oxygen becomes available again before total exhaustion of the NAD, oxidation will occur again. (Yamamoto and Tezuki, 1972). The NADP and NADPH levels in E. coli increase in the presence of oxygen and substrate, which indicates an oxygen requirement by an NAD kinase which catalyses the coversion of NAD to NADP (Yamamoto, 1966).

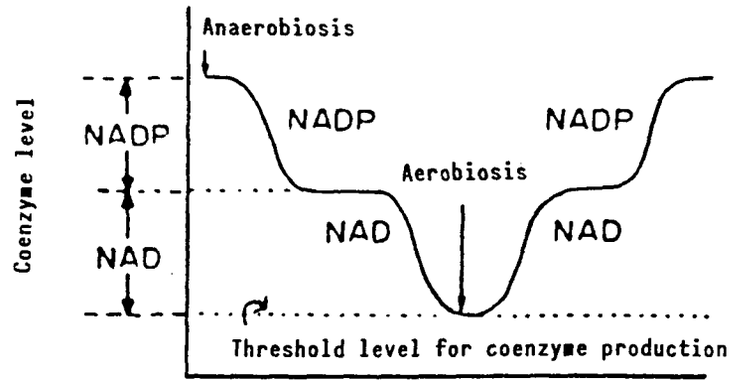


Figure 2.13 : Schematic representation of the effect of oxygen deficiency on nicotinamide co-enzyme synthesis (Yamamoto and Tezuka, 1972)

2.5.6 Biochemical Model

In 1983, Marais et al. declared that in their opinion "any significant advances in the use of this phenomenon (biological excess phosphate removal) in wastewater treatment will be contingent on greater understanding of the biochemical mechanisms controlling the phenomenon".

In the period before this statement, a number of attempts to explain plant observations in terms of the underlying biochemical processes had been made. Fuhs and Chen can be considered as fathers of the microbiological and biochemical concepts of excess phosphate removal. During studies into the phenomenon of phosphate uptake and release, they came to the conclusion that a single micro-organism or closely related species, was responsible for the phenomenon. These bacteria accumulated both polyhydroxybutyrate and polyphosphate; the former served as energy source for polyphosphate accumulation. During a study of the nutrient requirements of the polyphosphate accumulating bacteria, they found that volatile fatty acids, particularly acetic acid, were preferential substrates.

In contrast with this observation, Nicholls and Osborn (1979), postulated a conceptual model where glucose was taken up in the anaerobic zone and metabolised via the Embden-Meyeroff path to acetyl-CoA. The acetyl CoA serves as an electron sink by a reduction to polyhydroxybutyrate. Hydrolysis of ATP for cell maintenance results in phosphate release to the external medium. During subsequent aeration, the stored polyhydroxybutyrate is used for polyphosphate synthesis.

Rensink (1981) modified the model of Nicholls and Osborn (1979), in a number of ways. The former, with Fuhs and Chen (1975), proposed volatile fatty acids as substrate for polyphosphate accumulating organisms which store polyhydroxybutyrate in the anaerobic zone. The energy requirements for PHB synthesis are provided by the hydrolysis of polyphosphate, which results in phosphate release.

Marais et al., (1983), postulated that polyphosphate accumulation in the polyphosphate organisms served as energy source for cell maintenance and PHB synthesis under anaerobic conditions. In their investigations into possible pathways for PHB synthesis under anaerobic conditions, these researchers could only postulate the pathways with glucose as substrate, and acetate was not considered in this role.

Comeau et al., (1985), postulated a model where polyphosphate serves as an energy source for reinstatement of the proton motive force, and for substrate accumulation under anaerobic conditions. The anaerobic zone is essential for the optimisation of organic substrate accumulation by polyphosphate bacteria (see Figure 2.14).

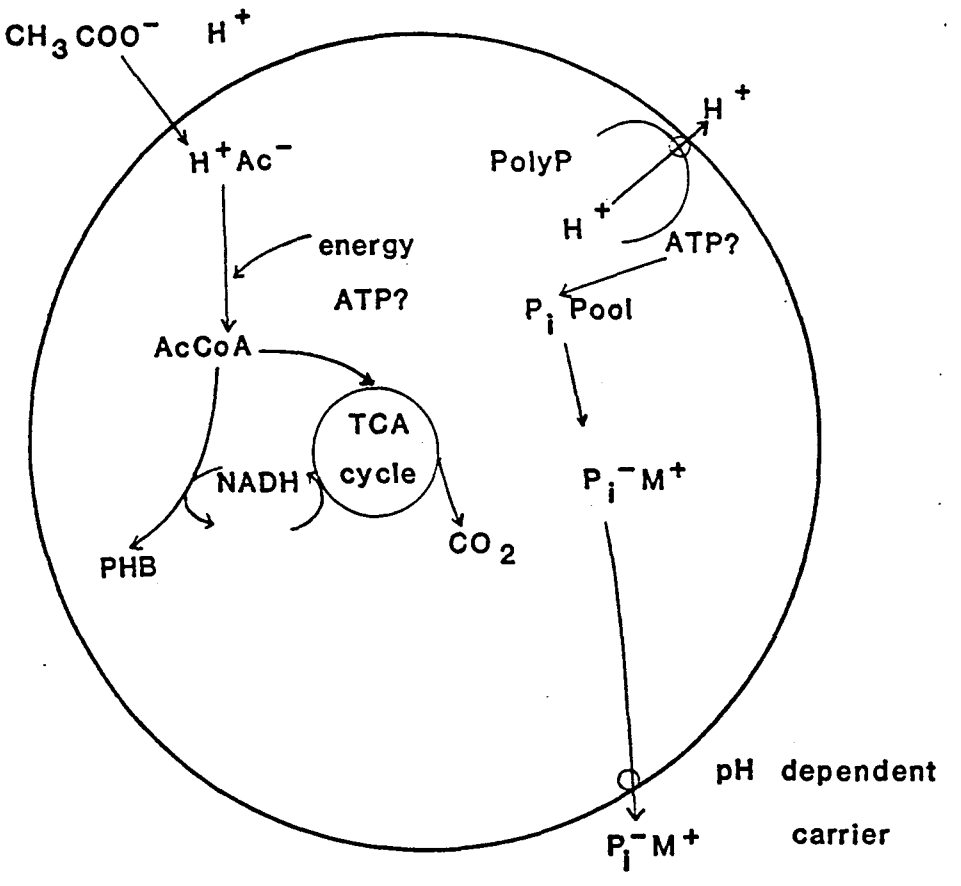


Figure 2.14 : Comeau model for anaerobic metabolism (Comeau et al., 1985)

In this model, undissociated acetic acid is transported across the membrane, with a decrease of one H^+ in the pH gradient for every acetate which is transported. Polyphosphate is either used directly for the excretion of H^+ across the membrane to extrude protons, or for ATP synthesis. Degradation of polyphosphate leads to accumulation of the intracellular orthophosphate which is released by the action of a pH sensitive carrier. Polyphosphate provides energy for polyhydroxybutyrate synthesis. Acetyl-CoA is partially metabolised by the tricarboxylic acid cycle to provide NADH for the reduction of acetoacetyl-CoA in PHB synthesis. The PHB carbon reserves are used under aerobic conditions to take up phosphate and metabolise it to polyphosphate (see Figure 2.15), (Comeau et al., 1985).

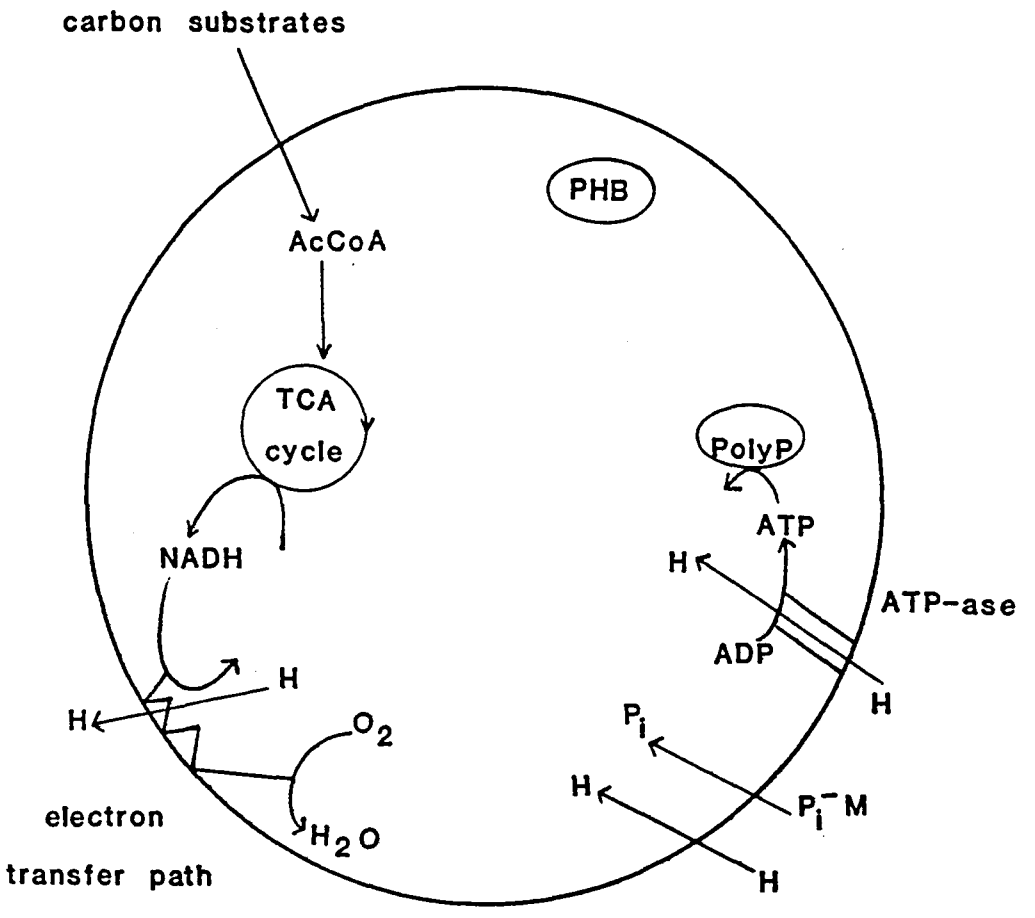


Figure 2.15 : Comeau model for aerobic metabolism

This model differs from that of Wentzel et al.,(1986), in that the mechanisms which control cellular processes under different environmental conditions, are not discussed. Wentzel et al., (1986), postulated that ATP/ADP and NADH/NAD ratios control polyphosphate and PHB synthesis and degradation.

These researchers postulate that the NADH/NAD ratio rises under anaerobic conditions as a result of the lack of a terminal electron acceptor. Repression of oxidative phosphorylation leads to a decrease in ATP/ADP. Citrate synthase is inhibited by high values of NADH/NAD and ATP/ADP (Weitzman and Jones, 1968; Weitzman and Dunmore, 1969). These alterations control the tricarboxylic acid cycle and PHB synthesis and ensure that acetyl CoA-is shared between the two pathways (see Figure 2.16).

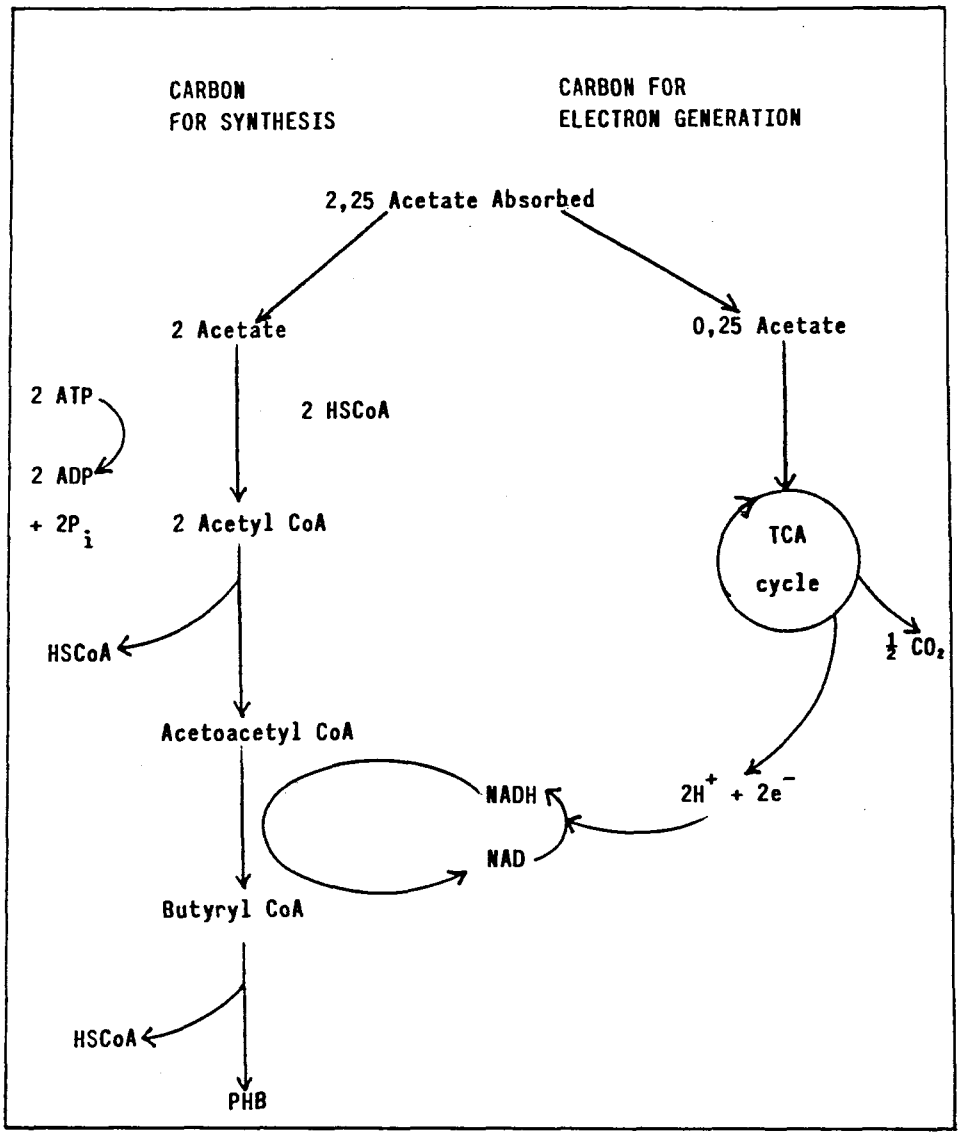


Figure 2.16 : Division of acetate between tricarboxylic acid cycle and PHB synthesis

The proton motive force which is dissipated by the uptake of acetate is reinstated by the uptake of hydroxyl ions in antiport for phosphate ions (see Fig 2.17).

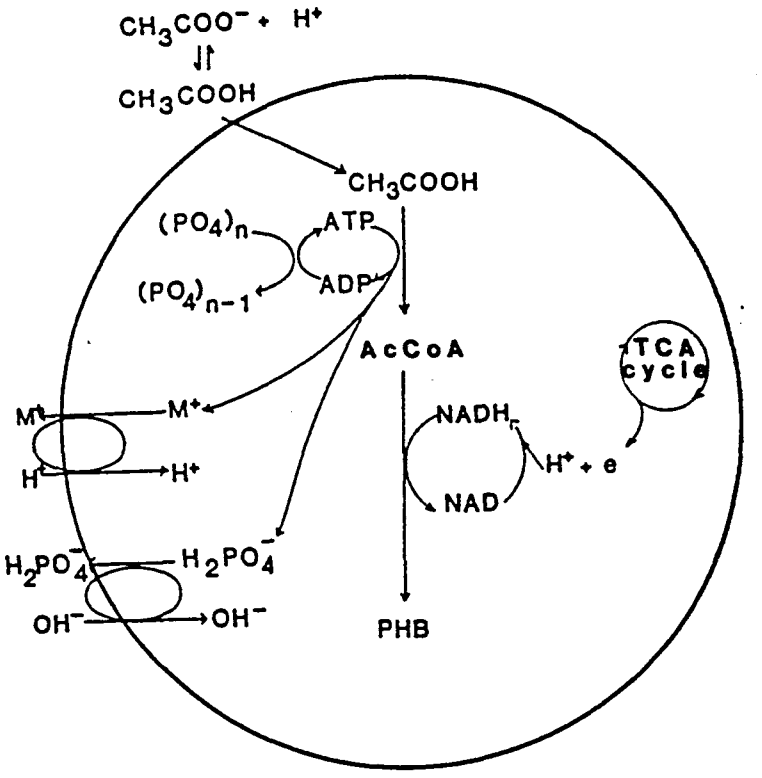


Figure 2.17 : Anaerobic metabolism according to Wentzel et al. (1986)

Under subsequent aerobic conditions where no exogenous carbon is available, PHB degradation is stimulated by the decrease in the NADH/NAD ratio, which also activates the tricarboxylic and glyoxylic acid cycles. Degradation of PHB provides the cell with energy. The resultant increase in the ATP/ADP ratio stimulates polyphosphate synthesis (see Figure 2.18).

The model of Wentzel et al., (1986) and indirectly, the model of Comeau et al., (1985), is based chiefly on metabolic control mechanisms which have already been observed in other organisms. In order to determine whether these mechanisms are also present in the dominant activated sludge organism, research on the intracellular regulation in Acinetobacter was necessary. The first step in the testing of the model must determine the nature of the intracellular metabolic control mechanisms.

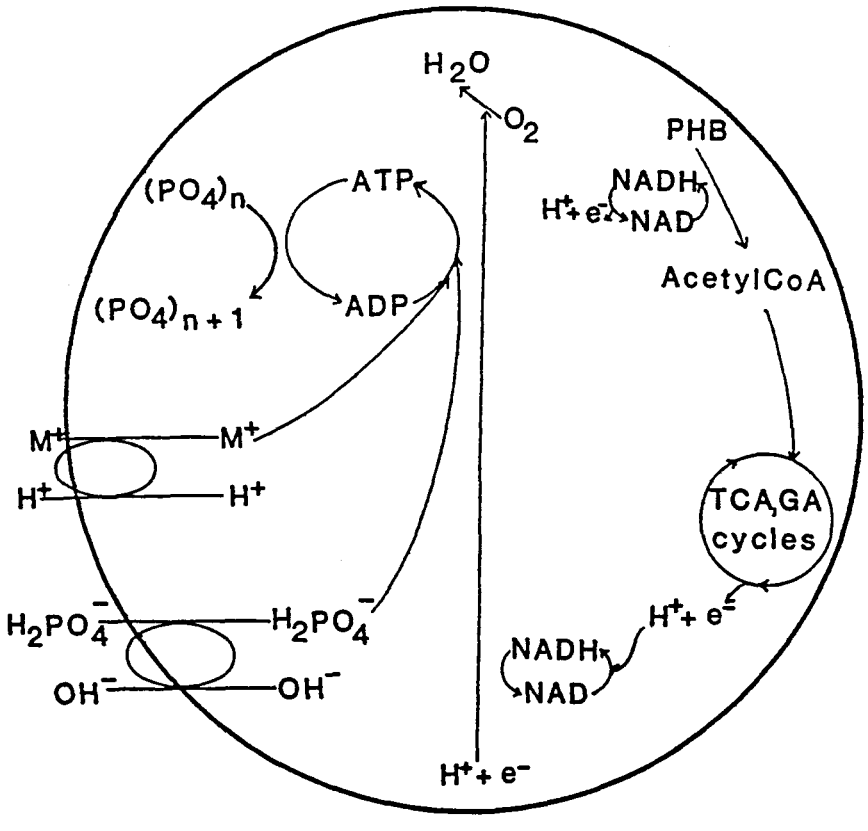


Figure 2.18 : Aerobic metabolism according to Wentzel et al. (1986)

The question arises whether metabolic control occurs at the level of protein synthesis or protein modification, or feedback inhibition. The model requires that control occurs by feedback mechanisms. If it is accepted that metabolic control occurs by means of feedback inhibition, then the arguments of the model can be summarised as follows :

. Anaerobic conditions

Uptake of acetate causes phosphate release, acetate is converted to polyhydroxybutyrate, acetate is oxidized by the tricarboxylic acid cycle to provide NADH for PHB synthesis. Initial high levels of NADH stimulate PHB synthesis. Low ATP levels stimulate

polyphosphate degradation to provide the cell with energy.

. Aerobic conditions

Phosphate is taken up and high ATP levels stimulate conversion to polyphosphate. The low NADH levels stimulate degradation of PHB. Lack of external carbon leads to low acetyl co-enzyme levels which stimulate PHB degradation.

The aim of this study was therefore, to investigate the following :

Nature of metabolic control which is maintained in Acinetobacter under activated sludge conditions.

Uptake and metabolism of acetate under anaerobic conditions.

Dependance of phosphate release on acetate uptake.

Polyphosphate metabolism under anaerobic and aerobic conditions.

Polyhydroxybutyrate metabolism under anaerobic and aerobic conditions.

Tricarboxylic acid and glyoxylic acid cycle activity under anaerobic conditions.

CHAPTER THREE

Experimental procedures

3.1 Bacterial Isolates

3.1.1 Sample Preparation

Mixed liquor from the aerobic zone of one of the modules of the Johannesburg Northern Works (see Figure 3.1), was used as a bacterial source.

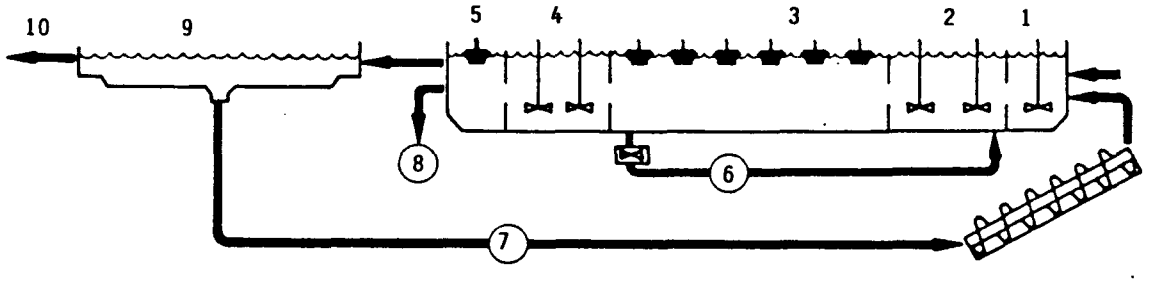


Figure 3.1 : Flow diagram of Northern Works, Module 3

1: Anaerobic zone	**2: First anoxic zone**
3: Main aeration zone	**4: Second anoxic zone**
5: Re-aeration zone	**6: Return sludge**
7: Return activated sludge	**8: Waste activated sludge**
9: Clarification	**10: Effluent**

A 40 mℓ aliquot of a well-mixed mixed liquor sample was centrifuged at 35 000 g. The residue was resuspended in 40 mℓ tripolyphosphate (5 mg/ℓ) in a plastic beaker. The suspension was kept in a Branson ultrasonic bath for 15 minutes at 20 °C. Serial dilutions (10^{-2} - 10^{-9}) were made of the suspensions.

3.1.2 Isolation of Bacteria

0,1 mℓ of each dilution was streaked on individual glycerol casitone yeast (GCY) agar plates (Pike et al., 1972). After 5 days incubation at 20 °C the plate which had the nearest to one hundred colonies was removed for further studies. Each of the 100 colonies was individually placed on a GCY agar plate. After a further 2 days incubation at 35 °C the isolates were identified.

3.1.3 Identification of Bacterial Isolates

The isolates were Gram stained (Society of American Bacteriologists, 1957) and the Gram negative bacteria retained. The Acinetobacter spp were identified by their positive reaction with a fluorescent Acinetobacter antibody (Cloete et al., 1985; Lötter and Murphy, 1985). The positive identification was confirmed by using the API 20E test (Analytlab, 1977).

Isolates were tested for their ability to accumulate polyphosphate (poly P) by incubating the isolates on a fermented sewage medium (Fuhs and Chen, 1975), for 5 days and then examining the cultures microscopically for accumulation of polyphosphate after staining by the Neisser technique (Society of American Bacteriologists, 1957). The ability to accumulate polyhydroxybutyrate was evaluated by growing the isolates in a β-hydroxybutyric acid medium for 5 days (Bovre and Henriksen, 1976) followed by staining with Sudan Black, and examination of the bacterial cells.

An isolate with the ability to accumulate polyphosphate and polyhydroxybutyrate was chosen for the metabolic studies. The isolate which was chosen for further studies was catalase positive, citrate oxidase negative, showed no hemolytic activity or liquifaction of gelatine and could reduce nitrate to nitrite.

3.1.4 Stock Culture

A stock culture of the selected isolate was made by inoculating twenty GCY plates with the isolate and incubating the plates for 5 days at 35 °C. The bacteria on the plates were then washed off in 500 mℓ of medium with the following composition :

Sodium acetate ($CH_3COOH.3H_2O$)	5 g
Ammonium sulphate $(NH_4)_2SO_4$)	2 g
Magnesium sulphate ($MgSO_4.7H_2O$	0,5 g
Monobasic potassium orthophosphate (KH_2PO_4)	0,25 g
Calcium chloride ($CaCl_2.2H_2O$)	0,2 g
Sterile distilled water	800 mℓ
Sterile fermented sludge	200 mℓ
pH	7,0

Every two days the volume was doubled by the addition of an amount of medium until a final volume of 10 ℓ was achieved.

The culture was maintained by removing 2 ℓ of the suspension and replacing with 2 ℓ of medium every week.

3.2 **Polyphosphate Dynamics**

3.2.1 Sample Treatment

In order to study the polyphosphate dynamics of activated sludge, mixed liquor samples from the aerobic zone of a five-phase Bardenpho plant were taken. The volatile suspended solids content of the mixed liquor was determined by filtering the suspension through a glass fibre filter. The filter was then dried at 105 °C and the dry residue ignited at 550 °C (American Public Health Association, 1981). Five hundred millilitres of mixed liquor was used for every experiment. Control samples were tested simultaneously and all experiments were carried out in

duplicate. The treatment of the samples included the addition of acetate to a final concentration of 100 mg/ℓ, resuspension of the mixed liquor solids in an acetate or acid sludge medium (see 3.1.4), without phosphate or fermented sludge. Forty millilitre aliquots were taken from every experimental batch at intervals of 40 minutes, over the experimental period of 160 minutes.

At the end of this period each sample was centrifuged at 5 000 g and the residue resuspended in 250 mℓ of acetate, or sludge medium (see 3.1.4), without fermented sludge. The resuspended samples were aerated at room temperature and 40 mℓ aliquots were again taken at intervals of 40 minutes, for 160 minutes. The phosphate fractions in the samples were determined as described in 3.2.2. An Acinetobacter isolate was used to inoculate the same media.

Suspensions for the experiments were taken during the stationary phase of the stock culture. Five hundred millilitre aliquots were used for each treatment. The sample taking and resuspension in acetate medium was carried out as described for the mixed liquor. In addition to the treatments which have already been described, succinate at a concentration of 100 mg/ℓ was also added to duplicate samples.

3.2.2 Phosphate Fractionation and Determinations

The samples were centrifuged for 10 minutes at 10 000 g. The residues were washed with sodium chloride solution (9 g/ℓ) and the resulting suspension centrifuged again. Fractionation of the phosphate compounds in the sludge residue was carried out according to the method of Harold (1960). The residue was shaken with 40 mℓ cold 0,5M perchloric acid, the suspensions was centrifuged and the extraction repeated.

The extracts were combined and the residue was extracted with 40 mℓ ethanol for 30 minutes. After centrifugation, 40 mℓ ethanol:diethyl ether (3 : 1) was added to the residue. This mixture was boiled for one minute, then centrifuged. The residue was then extracted twice with 40 mℓ aliquots of warm (70°C) 0,5M perchloric acid. An aliquot of the cold perchloric acid extract was taken for orthophosphate and total phosphorus determinations. Approximately 500 mg of phosphate-free carbon was added to the extract and the suspension shaken well. Total phosphorus and polyphosphate was determined in the filtrate.

The orthophosphate determination provides the intracellular phosphate level, and the difference between the total phosphorus before and after carbon treatment, indicates the nucleotide phosphorus level. The hot perchloric acid extract was treated in the same manner as the cold extract, and subjected to carbon treament total phosphorus determinations. The difference in total phosphorus, before and after carbon treatment, provide the nucleic acid phosphorus level. The polyphosphate in this fraction is defined as acid insoluble polyphosphate, while that in the cold extract is known as acid soluble polyphosphate.

Total phosphorus determinations are carried out by the digestion procedure of Jirka et al., (1976), followed by the orthophosphate determination of Canelli and Mitchell (1975). Polyphosphate was determined by hydrolysis in 1M hydrochloric acid at 70 °C for 15 minutes, followed by orthophosphate determination. The Acinetobacter samples were treated in exactly the same manner, except that 10 mℓ of each reagent instead of 40 mℓ were used.

3.3 Polyhydroxybutyrate Metabolism

3.3.1 Laboratory Studies

Five hundred millilitres of stock culture was centrifuged at 2 000 g for 5 minutes and the cells added to 2 ℓ medium containing $(NH_4)_2SO_4$, 2 g/ℓ; $MgSO_4$, 0,5 g/ℓ; KH_2PO_4, 0,25 g/ℓ; $CaCl_2.2H_2O$, 0,2 g/ℓ. The suspension was stirred for 1 hour to acclimatise the cells to the new medium. Acetate was added to a final concentration of 5 g/ℓ. A sample was taken at time 0 and the culture divided in three.

The first portion was purged with nitrogen gas and allowed to stand for 3 hours under anaerobic conditions, prior to aeration for 21 hours. The second aliquot was also purged with nitrogen gas and left anaerobic for 24 hours. Anaerobiosis was determined by the use of Anaerotest strips (Merck). The third aliquot was aerated for 24 hours. Samples were taken at three-hourly intervals over the 24 hour period. A 10 mℓ aliquot was taken and centrifuged at 10 000 g for 10 minutes.

The cell mass was determined by reading the optical density at 520 nm and preparing a calibration curve from which the mass was read. The acetate concentration in the cell-free medium (supernatant), was determined by gas chromatography. Acidified samples were steam distilled and the distillates titrated against 0,1M sodium hydroxide. The neutral distillates were dried at 60 °C under vacuum. Dichloroacetic acid and acetone were added to the residue (Horwitz, 1980) and the solutions subjected to gas chromatographic analysis. A 10 m 530 micron Carbowax 20M column with temperature programming from 80 °C to 150 °C was used for the chromatography (Packett and McCune, 1965). The remaining cell suspension was freeze-dried.

The freeze-dried samples were suspended in 10 mℓ acidified methanol, plus 10 mℓ chloroform, in a sealed tube, and kept at 100 °C for 3 hours. After cooling, 10 mℓ water was added and the samples were shaken for 10 minutes. The chloroform layer was filtered through Whatman PS1 and the filtrate evaporated to dryness. The residue was dissolved in 100 microlitres chloroform and subjected to gas chromatographic analysis.

3.3.2 Plant Studies

Mixed liquor samples were taken from the anaerobic, primary anoxic and secondary aerobic zones of the Northern Works and Goudkoppies plant. The samples were analysed for polyhydroxybutyrate content by the method of Braunegg et al. (1978).

3.4 **Polyphosphate Kinase Activity**

3.4.1 Culture Conditions

In order to determine the effect of growth conditions on enzyme activity, 100 mℓ of the stock culture was added to 1 litre of an acetate medium with the following composition :

Sodium acetate ($CH_3COOH.3H_2O$	5 g
Ammonium sulphate $(NH_4)(_2SO_4)$	2 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0,5 g
Monobasic potassium orthophosphate (KH_2PO_4)	0,25 g
Calcium chloride ($CaCl_2.2H_2O$)	0,2 g
Sterile distilled water	1 000 mℓ

The bacterial suspension was aerated for 100 hours. Samples were taken immediately after inoculation and thereafter, at intervals during the incubation period. The optical density at 520 nm was measured and the polyphosphate kinase activity determined.

In order to monitor the effect of substrate on enzyme activity, 2 ℓ of stock culture were centrifuged at 15 000 g for 15 minutes, and resuspended in 2 x 1 ℓ of the acetate medium. A duplicate sample was resuspended in medium containing succinate instead of acetate. One acetate and one succinate suspension was aerated for 5 hours. The duplicate suspensions were allowed to stand anaerobic for one hour, whereafter they were aerated for 4 hours. Samples were taken after the first hour, and thereafter every 2 hours. The polyphosphate kinase activity in the bacteria was determined.

3.4.2 Sample Preparation

The bacterial suspensions were centrifuged at 15 000 g and resuspended in 5 mℓ of the extraction buffer. Glass beads were added to the suspensions in plastic containers and the suspensions were placed in an ultrasonic bath for 15 minutes. The bath was kept cool during the ultrasonification by the addition of ice. The suspensions were centrifuged at 20 000 g and the supernatant was retained for enzymatic assay.

Two different extraction buffers, A and B, at different concentrations were compared.

Extraction buffer A

100 or 250 mM sodium acetate containing
1 mM EDTA
0,7 mM 2 mercapto-ethanol
10^{-4}M phenylmethylsulphonylfluoride
pH 5,5

Extraction buffer B

5 or 10 mM Tris HCL containing
2 mM EDTA
PH 7,5

In the case of Buffer A, the supernatants were dialysed against a 10 mM potassium phosphate buffer containing 1 mM EDTA and 0,7 mM 2-mercapto-ethanol at pH 7,0 before enzyme determination. In the case of Buffer B, the extract was dialysed against a 5 mM potassium phosphate buffer which contained 1 mM EDTA and 10 mM dithioeritritol at pH 7,0.

3.4.3 Protein Determination

Protein concentrations were determined according to the dye-binding method of Bradford (1976). Protein binds with Coomassie Blue in a phosphoric acid medium to form a blue complex which absorbs at 595 nm. The method was standardised with bovine serum albumin.

3.4.4 Polyphosphate Kinase Determination

Polyphosphate kinase activity was determined by measuring the incorporation of radioactive phosphate in polyphosphate. The inhibitory effect of ADP on the activity was overcome by incorporating an ATP generating system as part of the reaction system, namely :

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow[\text{Creatine kinase}]{} \text{Creatine} + \text{ATP}$$

The reaction mixture thus had the following composition :
Forty mM potassium phosphate buffer, pH 6,0; 20 mM $MgCl_2$; 0,4 mM ^{32}P-ATP; 25 mM creatine phosphate; creatine phosphokinase (Boehringer Cat, Nr 126969) 20 units/mℓ.

The reaction was carried out at 30 °C for 30 minutes and then terminated by the addition of 1 mℓ perchloric acid (600 g/ℓ) and 1 mℓ bovine serum albumin (5 mg/mℓ). The polyphosphate which is precipitated is obtained by centrifugation and dissolved in 1 mℓ 1N NaOH. The polyphosphate solution was added to 10 mℓ of scintillation fluid (Packard Instafluor) and the gross activity in counts per minute was determined. This procedure is a combination of methods which were described by Nesmeyanova et al. (1973) and T'Seyen et al. (1985).

3.5 Electrophoretic Studies

3.5.1 Culture Conditions

Two ℓ of stock culture (3.1.4) was centrifuged at 8 300 g and the cells resuspended in 2 x 1 ℓ of acetate medium (3.2.1). One flask was left anaerobic and the second was aerated with sterile air. Both flasks were incubated at 37 °C for 24 hours.

The experiment was repeated separately under aerobic conditions, with acetate and succinate as substrate.

The abovementioned experimental conditions were repeated in the presence of 0,5 mCi [^{32}P] KH_2PO_4 (Amersham, England) in each flask.

3.5.2 Extraction Procedure

The cell suspension was centrifuged at 8 300 g for 20 minutes and the cells were resuspended in 25 mℓ of physiological saline. The cell suspension was then centrifuged at 18 000 g for 20 minutes and the cells resuspended in 5 mℓ 0,05 M phosphate buffer at pH 7,0. This suspension was subjected to ultrasonic disintegration. The cell suspension was kept below 5 °C during disintegration.

The supernatant which was obtained after centrifugation at 15 000 g for 30 minutes was stored frozen. The protein content of the extract was determined by the method described in 3.2.3.

3.5.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.5.3.1 Sample preparation

Electrophoresis was carried out by a modification of the method described by Laemmli (1970).

The cell extracts were diluted with 0,05 M phosphate buffer, pH 7,0 to give a protein concentration of 24 mg/mℓ. The diluted solution was filtered through a 0,45 µm Millex H4 filter unit. The filtrate was further diluted with sample buffer (3.3.4) and boiled for 3 - 4 minutes. The density was increased by the addition of a few crystals of sucrose.

Eighty µg protein per channel was applied and 20 µℓ of bromophenol blue solution (2 mg/mℓ ethanol) was added.

A standard solution with the following composition was applied with the samples :

Lysosyme	14,4 kDa
Soya bean trypsin inhibitor	21,5 kDa
Carbonic acid anhydrase	31,0 kDa
Ovalbumin	45,0 kDa
Bovine serum albumin	66,2 kDa
Phosphorilase b	92,5 kDa
ß-galactosidase	116,2 kDa
Myosin	200,0 kDa

3.5.3.2 Preparation of gel

The glass plates were thoroughly cleaned by allowing them to stand overnight in chromic acid, whereafter, they were rinsed with water and dried with acetone.

The following reagents were used for the preparation of the gel :

Stacking gel

Acrylamide	4,0 %
TEMED	0,1 %
Ammonium persulphate	1,0 %

Separation gel

Acrylamide	7,5 %
TEMED	0,1 %
Ammonium persulphate	1,0 %

Stacking gel buffer

Tris HCl	0,125 M
SDS	0,1 %
pH	6,8

Separation gel buffer

Tris HCl	0,375 M
SDS	0,1 %
pH	8,8

3.5.3.3 Electrophoretic conditions

A potential of 60 V was applied until the sample had moved through the stacking gel. Thereafter 220 V was applied until the marker front had migrated to 10 mm from the bottom end of the gel. The temperature was kept constant at 10 °C.

3.5.3.4 Electrophoretic buffer solutions

The following buffer solutions were used :

Electrophoresis buffer

Tris HCl	0,050 M
Glycine	0,384 M
SDS	0,1 %
pH	8,3

Sample buffer

Tris HCl	0,05 M
with mercapto-ethanol	5 %
SDS	2,3 %
pH	6,8

3.5.3.5 Protein fixing, staining and destaining

(a) Coomassie blue staining

Gels were fixed overnight in 40 % (v/v) methanol which contained 10 % acetic acid. Thereafter, they were left in the staining solution overnight. The staining solution consisted of 0,1 % (m/v) Coomassie blue (G-250) in 25 % (v/v) methanol containing 7,5 % (v/v) acetic acid.

Diffusion destaining of the gels was carried out in 25 % (v/v) methanol containing 7,5 % (v/v) glacial acetic acid. The gels were dried between cellophane on a Biorad gel drier under vacuum at 60 °C.

(b) Silver staining

The gels were fixed as described in 3.3.7.1 and then left in 10 % ethanol containing 5 % acetic acid for 15 minutes. After a second immersion in the ethanol acetic acid solution, the gels were washed with water.

The gels were then left in a dithriotretol solution (5 µg/mℓ) for 30 minutes and immediately thereafter, immersed in a silver nitrate solution (0,1 %) for 30 minutes.

After rinsing with water the gels were rinsed in a developer consisting of 3 % sodium carbonate containing 200 µℓ formaldehyde/400 mℓ. Thereafter, the gels were immersed in the developer until bands appeared. The gels were then left for 10 minutes in the developer to which 5 mℓ 2,3 M citric acid had been added. After rinsing with water the gels were allowed to stand in water for 30 minutes.

3.5.4 Polyacrylamide Electrophoresis

Sample preparation and electrophoresis was carried out as described in 3.5.3.

3.5.4.1 Enzymatic staining

The gels were fixed as described in 3.5.3.5 and thereafter, stained with a modification of the enzymatic staining technique of Gabriel (1971).

The staining solutions had the following composition :

DL isocitric acid or		
ß-hydroxybutyric acid	1 M	1,0 mℓ
NAD	10 mg/mℓ	1,0 mℓ
NaCl	0,1 M	1,0 mℓ
$MgCl_2$	5 mM	1,0 mℓ
Tris-HCl buffer, pH 7,4	0,5 M	2,5 mℓ
Nitroblue tetrazolium	1 mg/mℓ	2,5 mℓ
Phenasine metosulphate	1 mg/mℓ	0,25 mℓ

The gels were incubated in the staining solution at 30°C for 30 minutes in the dark. After colour development, the gels were rinsed sequentially in water and 7,5 % acetic acid. The efficiency of the staining technique was tested by analysing isocitric dehydrogenase from porcine heart and β-hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroides under the same electrophoretic conditions, whereafter, the gels were stained.

3.5.5 Autoradiography

3.5.5.1 Sample preparation and electrophoresis

Samples were prepared and electrophoresis carried out as described in 3.5.3.

[^{14}C] methylated protein standard (Amersham, England), were run with the samples. The standard mixture comprised the following :

[^{14}C] methylated lysozyme	14,3 kDa
[^{14}C] methylated carbonic acid anhydrase	30,0 kDa
[^{14}C] methylated ovalbumin	46,0 kDa
[^{14}C] methylated bovine serum albumin	69.0 kDa
[^{14}C] methylated phosphorylase b	92,5 kDa
[^{14}C] methylated myocine	100,0 kDa

3.5.5.2 Exposure and development of film

All manipulations were carried out in a dark room with a filter. Cronex-4 X-ray film was used. The dried gel was exposed to the film between two calcium tungstate (Corex-Hi-plus) intensifying screens for 48 hours in an X-ray film cassette at -20 °C.

After exposure the film was placed in Cronex HSD developer for 3 minutes. After rinsing with water the film was fixed in Cronex CPF.

3.6 β-hydroxybutyrate Dehydrogenase Activity

3.6.1 Choice of Isolate

Eleven A. calcoaceticus var lwoffi isolates from the activated sludge plant were grown on a 10 % serum-nutrient agar containing 0,5 % β-hydroxybutyric acid. The bacteria were stained every day with Sudan Black (Gurr, 1973) and examined microscopically for polyhydroxybutyrate accumulation.

The isolates which accumulated polyhydroxybutyrate under these conditions were incubated in the acetate containing medium which was described in 3.2.1 at 37 °C for three days.

The cells were collected by centrifugation and extracted by ultrasonification in 0,1 M Tris-HCl buffer containing 20 mM $MgCl_2$ and 1 mM EDTA at pH 8,0. The isolate with the highest β-hydroxybuyrate dehydrogenase activity was used for further studies.

3.6.2 Culture Conditions

The chosen isolate was cultured in the acetate medium as described in 3.4.1 for three days at 35 °C under aerobic conditions.

3.6.3 The Effect of Growth Substrate on Enzyme Activity

In order to determine the effect of different growth substrates on enzyme activity, the bacteria were also grown in a medium containing sodium succinate and glucose respectively, instead of sodium acetate.

3.6.4 Enzyme Isolation

3.6.4.1 Extraction

The cells were subjected to ultrasonic disintegration in 20 mM Tris-HCl buffer containing 20 mM $MgCl_2$ and 1 mM EDTA at pH 7,2. The cell suspension was kept below 5 °C by cooling during the disintegration. After ultrasonification for 9 minutes, the cell suspension was centrifuged at 20 000 g. The cell-free extract was retained for ammonium sulphate fractionation.

3.6.4.2 Ammonium sulphate treatment

The cell-free extract was saturated to 35, 45 and 55 % in respect of ammonium sulphate. The supernatant after centrifugation at 20 000 g, was again saturated to 55 and 65 % in respect of ammonium sulphate and the precipitate was kept after centrifugation. The precipitate was dissolved in 100 mM Tris-HCl buffer at pH 8,5 and dialysed against the same buffer.

3.6.5 Enzyme Determination

The enzyme activity was determined by monitoring the production of NADH at 340 nm during the oxidation of β-hydroxybutyrate to acetoacetate. One activity unit was defined as the amount of enzyme which was necessary to cause the absorbance at 340 nm to increase by one unit. The reaction mixture consisted of β-hydroxybutyrate, NAD and Tris-HCl buffer at pH 8,5. The reaction was followed at 25 °C for five minutes. The method is a modification of that described by Williamson and Mellanby (1974).

3.6.6 Production of Acetoacetate

Acetoacetate was prepared from ethylacetoacetate according to the procedure of Procos (1961).

Freshly distilled ethylacetoacetate (2,6 mℓ) was added to 20 millilitres of 1 N NaOH and incubated at 40 °C for 1½ hours. After cooling, the solution was extracted four times with diethyl ether to remove unreacted ethylacetoacetate. The aqueous solution was acidified and extracted four times with diethyl ether. The combined ether extracts were dried over anhydrous sodium sulphate and evaporated under vacuum. The redisue was dissolved in water and used for enzyme determinations at concentrations of 0 to 133 mM.

3.6.7 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out as described in 3.5.4. The enzymatic staining technique of Gabriel (1971), with β-hydroxybutyrate as substrate, was used to visualize the enzyme on the gels.

3.6.8 The Effect of Certain Metabolites on Enzyme Activity

In order to investigate the possible effect of certain metabolites on the activity of the enzyme, a number of modifications were made to the reaction mixture.

The β-hydroxybutyrate concentration was varied between 0 to 150 µM and NAD from 5 to 20 mM.

The specificity for NAD was determined by replacing NAD with NADP in the reaction mixture.

The reverse reaction, namely, the reduction of acetoacetate to β-hydroxybutyrate was measured by monitoring the disappearance of NADH at 340 nm.

NADH at concentrations between 5 and 15 mM was added to the reaction mixture to determine the effect of this product on the reaction. The reaction was carried out at different β-hydroxybutyrate concentrations.

The effect of acetyl-CoA and oxaloacetic acid on the enzyme activity was determined by adding these metabolites at concentrations of 0 to 20 mM to the reaction mixture. The reaction was also followed in the presence of the metabolites at different concentrations of -hydroxy-butyrate.

3.7 Isocitrate Dehydrogenase

3.7.1 Bacterial Isolate

The same isolate which was used for the ß-hydroxybutyrate dehydrogenase investigation was used. The Acinetobacter cells were grown in the acetate medium described in 3.4.1 for three days at 35 °C under aerobic conditions. A duplicate sample was allowed to go anaerobic for the last 24 hours of the three-day period.

3.7.2 The Effect of Growth Substrate on Enzyme Activity

In order to determine the effect of different growth substrates on the enzyme activity, the bacteria were also grown in a medium containing sodium succinate and glucose, instead of sodium acetate.

3.7.3 Enzyme Isolation

The enzyme was isolated as desribed in 3.6.4, except that the precipitate after ammonium sulphate treatment was dissolved in 75 mM Tris-HCl buffer at pH 7,5. Dialysis took place against the same buffer.

3.7.4 Enzyme Determination Method

The enzyme activity was determined by monitoring the release of NADPH at 340 nm during the oxidation of isocitrate to α-ketoglutarate (Borthwick et al., 1984). One activity unit

was defined as the amount of enzyme necessary to cause the absorbance at 340 nm to rise with one unit. The reaction mixture consisted of D,L isocitrate, NADP and 75 mM Tris buffer at pH 7,2.

3.7.5 Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out as described in 3.5.4. The enzymatic staining technique of Gabriel (1971), with D,L isocitrate as substrate, was used to visualize the enzyme on the gels.

3.7.6 The Effect of Certain Metabolites on Enzyme Activity

A number of modifications were made to the reaction mixture in order to investigate the possible effect of certain metabolites on enzyme activity. ATP and AMP were added to the reaction mixture at 10 mM,and oxaloacetic acid and NADPH were added at 10 mM.

CHAPTER FOUR

Results and discussion

4.1 Polyphosphate Dynamics

4.1.1 Activated Sludge

The results are expressed as mg P/g VSS (volatile suspended solids). Prelminary experiments showed that no significant differences in the nucleotide, nucleic acid and lipid phosphorus fractions were observed during the experimental period. The determination of these fractions were thus not carried out in the subsequent experiments which are described here. Phosphate starvation and acetate treatment caused a release of phosphate to the external medium. The effect of acetate is much more dramatic than that of phosphate starvation. Subsequent aeration of these samples caused uptake of phosphate in greater amounts than that which was released (see Table 4.1). A number of researchers have reported that pre-treatment of activated sludge is essential to stimulate phosphate uptake. Nicholls and Osborn (1979), considered the removal of oxygen in order to place the biomass under stress conditions as sufficient pre-treatment, while Marais and Ekama (1982), postulated simultaneous substrate uptake and phosphate release under anaerobic conditions, as prerequisite for phosphate uptake.

TABLE 4.1

PHOSPHATE RELEASED AND ABSORBED BY ACTIVATED SLUDGE DURING TREATMENT

	Phosphate released mg P/g VSS	Phosphate absorbed mg P/g VSS
Control	3	5
Acetate treatment	14	20
Phosphate starvation	5	12

Increased phosphate uptake after a period of phosphate starvation has been observed in a number of organisms (Rosenberg et al., 1969 ; Medveczky and Rosenberg, 1971). The higher phosphate uptake which is stimulated by acetate treatment, cannot only be explained by an apparent starvation effect as a result of the inability of the obligate aerobe to take up phosphate under anaerobic conditions. There appears to be a link between acetate and phosphate metabolism.

The variation in intracellular phosphates during the experiment is shown in Figures 4.1 and 4.2.

A number of changes occurred in the intracellular phosphate components during treatment. Phosphate starvation and acetate treatment caused a decrease in intracellular components which coincided with phosphate being released to the medium. It is clear from Figures 4.1 and 4.2 that acid insoluble and acid soluble polyphosphate contributed to the phosphate which was released.

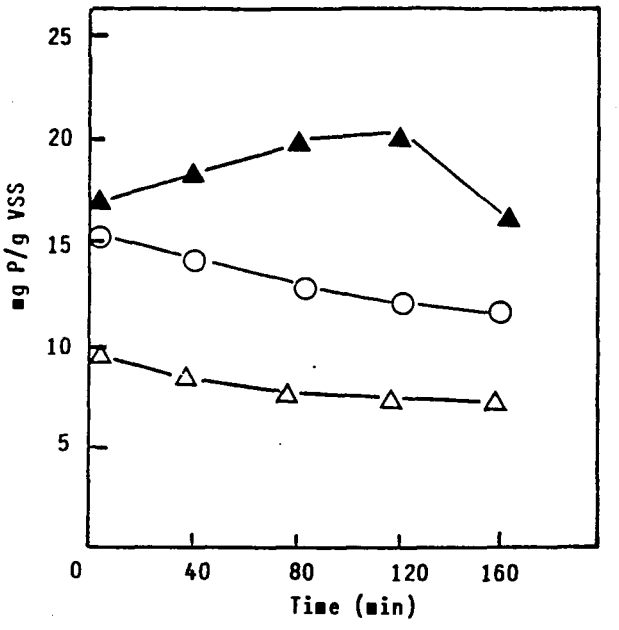


Figure 4.1 : Changes in intracellular phosphate in activated sludge resuspended in phosphate-free medium
○—○: Phosphate
▲—▲: Acid soluble polyphosphate
△—△: Acid insoluble polyphosphate

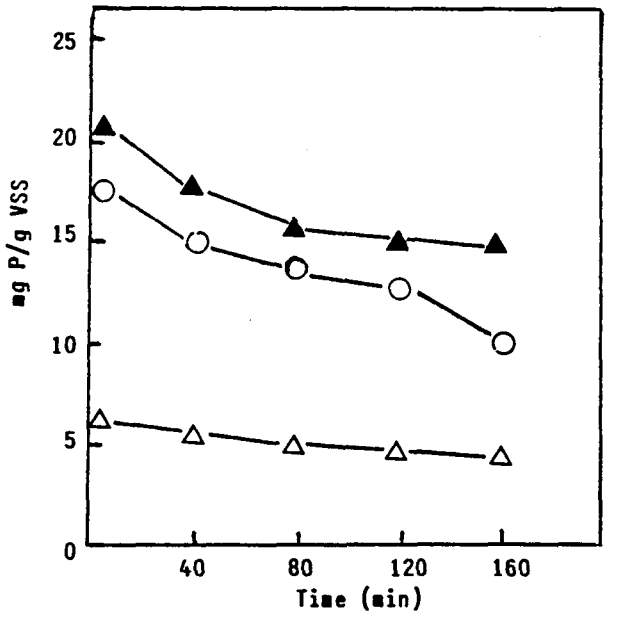


Figure 4.2 : Changes in intracellular phosphate in activated sludge during acetate treatment
○—○: Phosphate
▲—▲: Acid soluble polyphosphate
△—△: Acid insoluble polyphosphate

Although the role of polyphosphate in bacteria has not been satisfactorily clarified (Harold, 1962; van Stevenink and Booij, 1964; Kulaev and Vagabov, 1983), the hydrolysis of compounds under conditions of intracellular phosphate exhaustion support the theory of a regulatory role in intracellular phosphate levels (Harold, 1962); Nesmayanova et al., 1974; Kulaev, 1975).

Changes in the intracellular components in activated sludge during the aeration phase of the experiment are shown in Figure 4.3; 4.4 and 4.5. Figure 4.1 gives the results for the control samples.

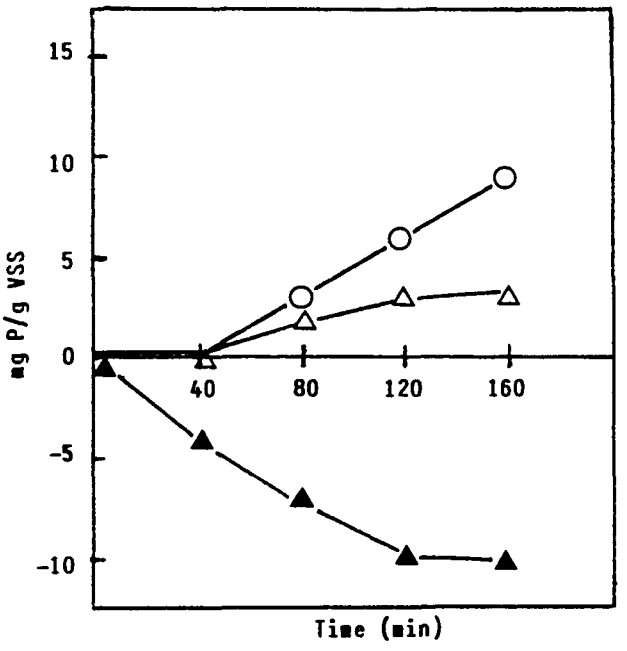


Figure 4.3 : Nett change in intracellular phosphate in control-activated sludge during aeration
○—○: Phosphate
▲—▲: Acid soluble polyphosphate
△—△: Acid insoluble polyphosphate

In phosphate starved and control samples, conversion between different fractions takes place.

An immediate increase in the polyphosphate fractions was observed in the acetate treated samples.

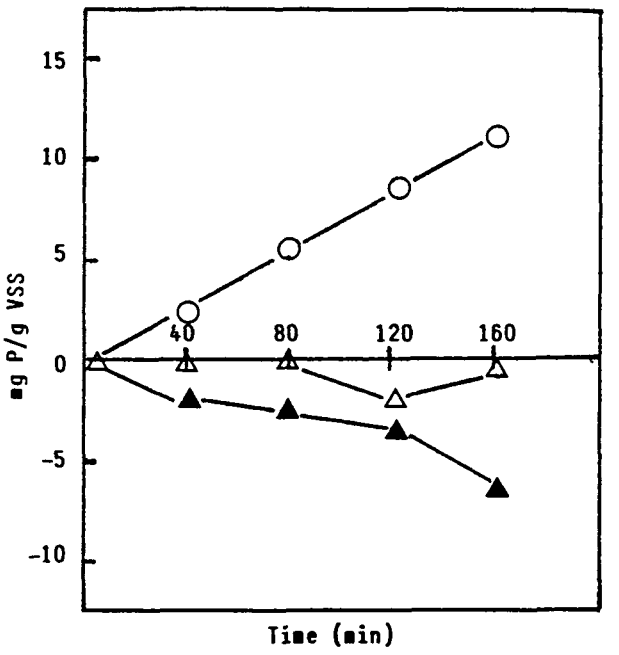


Figure 4.4 : Nett change in intracellular phosphate in activated sludge during aeration, afte suspension in phosphate-free medium
○—○: Phosphate
▲—▲: Acid soluble polyphosphate
△—△: Acid insoluble polyphosphate

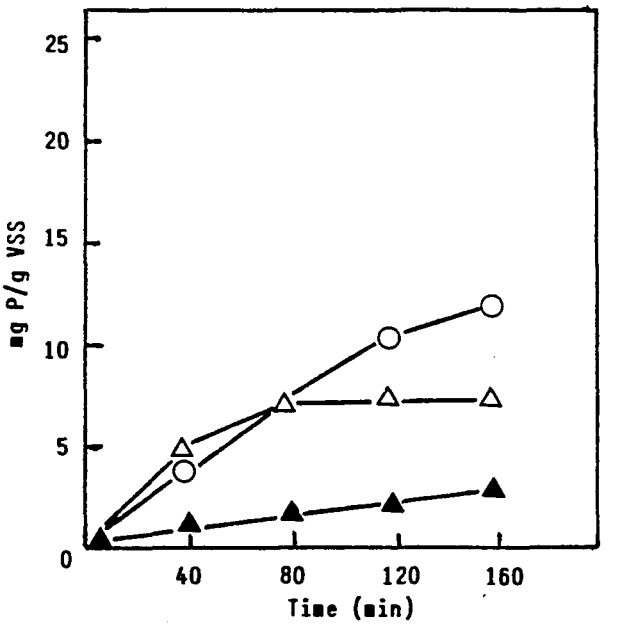


Figure 4.5 : Nett change in intracellular phosphate in acetate-treated activated sludge during aeration
○—○: Phosphate
▲—▲: Acid soluble polyphosphate
△—△: Acid insoluble polyphosphate

The continued decrease in acid soluble polyphosphate during aeration is apparently as a result of the low intracellular phosphate level at the start of aeration. In the acetate treated cells this could not occur, as most of the intracellular orthophosphate had been released to the external medium.

4.1.2 Acinetobacter

The results are expressed as mg P/g of cells. As in the case of the activated sludge sample, phosphate starvation and acetate treatment causes release of phosphate to the external medium. The simulatory effect of the acetate on phosphate release in the activated sludge has already been observed by a number of researchers (Wentzel et al., 1985; Lötter, 1985).

The similar behaviour which is observed with Acinetobacter suggests that these bacteria could be the chief source of phosphate release in the activated sludge (see Table 4.2).

TABLE 4.2

PHOSPHATE RELEASED AND ABSORBED BY ACINETOBACTER DURING TREATMENT

	Phosphate released mg P/g cells	Phosphate absorbed mg P/g cells
Control	2	3
Acetate treatment	32	43
Phosphate starvation	10	5

No difference between the control and succinate treated sample was observed. The difference in acetate and succinate behaviour is probably as a result of the

difference in their membrane-transport characteristics (Lötter, 1985). Again, enhanced phosphate uptake after aeration of the treated samples was observed (see Figures 4.6 and 4.7). The dramatic increase which was obtained with acetate (Figure 4.7), is again clear. The effect is however, more noticeable in this pure culture study, which indicates that the possible relationship between acetate and phosphate metabolism is restricted to Acinetobacter (compare Figures 4.5 and 4.7).

The higher stimulatory effect of phosphate starvation on the activated sludge samples (compare Figures 4.4 and 4.6), is apparently as a result of the presence of other bacteria which are also sensitive to this effect. The lack of change in intracellular components which was observed in the activated sludge control sample during treatment (Figure 4.1), was repeated in the pure culture control and succinate treated sample.

During phosphate starvation in activated sludge, acetate stimulated an increase in all three phosphate fractions (Figure 4.5). The phosphate starved cells again showed rapid conversion between fractions (Figure 4.6). It is clear from the results of this study, that the stimulation of phosphate uptake by acetate, which has been observed by certain researchers (Marais and Ekama, 1982), is not only as a result of phosphate starvation. The significant difference between acetate treatment and phosphate starvation indicates a direct relationship between acetate and polyphosphate metabolism. These results were qualitatively confirmed by light and electron microscopy (Murphy and Lötter, 1986).

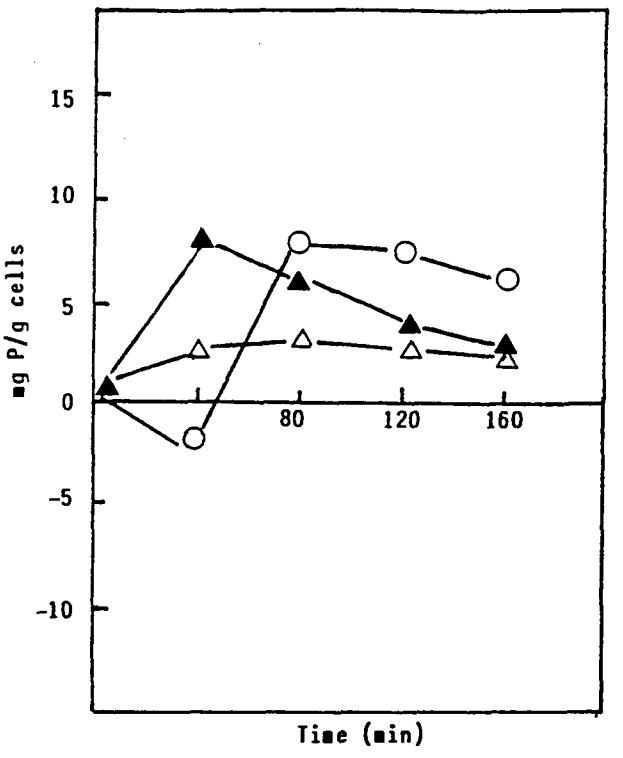


Figure 4.6 : Nett change in intracellular phosphate fractions of Acinetobacter during aeration, after suspension in phosphate-free medium
○—○: Phosphate
▲—▲: Acid soluble polyphosphate
△—△: Acid insoluble polyphosphate

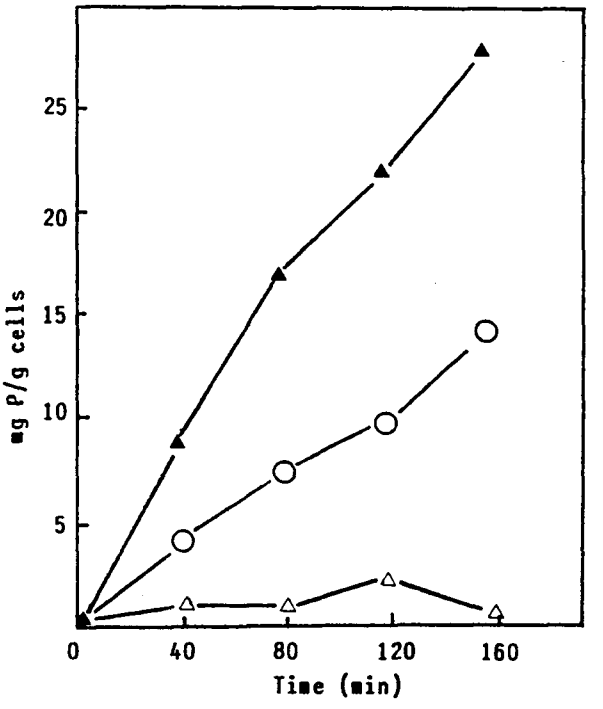


Figure 4.7 : Nett change in intracellular phosphate fractions in acetate treated Acinetobacter during aeration
○—○ : Phosphate
▲—▲ : Acid soluble polyphosphate
△—△ : Acid insoluble polyphosphate

4.2 Polyhydroxybutyrate Metabolism

4.2.1 Acinetobacter

The uptake of acetate under anaerobic conditions is shown in Figure 4.8.

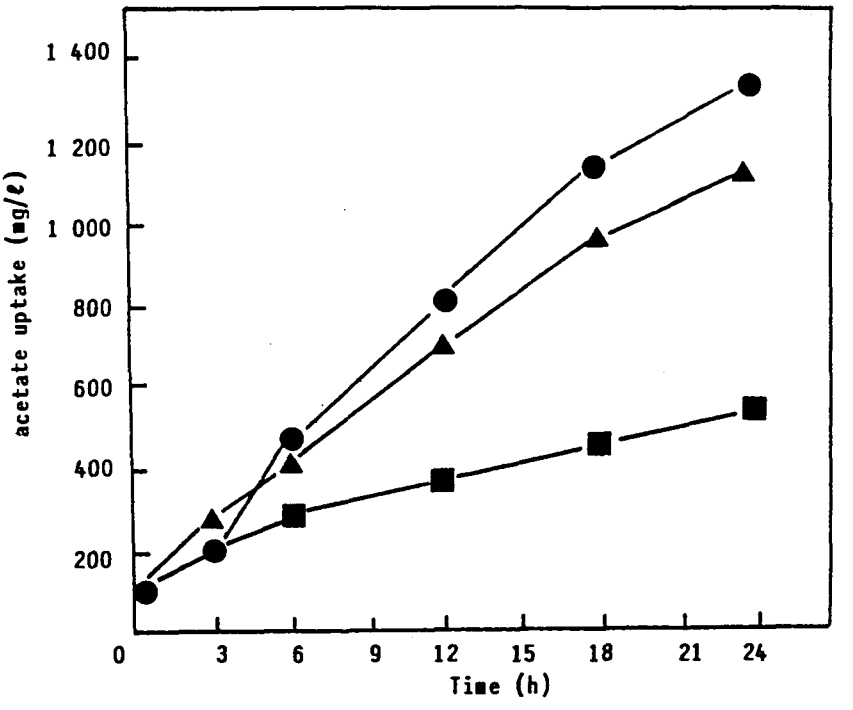


Figure 4.8 : Uptake of acetate by Acinetobacter under anaerobic ■—■ ; ●—● anaerobic/aerobic ; ▲—▲ aerobic conditions

Acetate uptake under aerobic conditions increases linearly over the 24 hour experimental period. Under anaerobic conditions the slower uptake also increases to a lower maximum than that which was observed for aerobic conditions. The initial period of anaerobiosis in the third sample apparently stimulated uptake under subsequent aeration. As Acinetobacter are obligate aerobes (Juni, 1978), growth cannot be expected under anaerobic conditions.

The increased uptake under aerobic conditions is however, at least partially, a result of the growth requirement for exogenous carbon, as shown in Figure 4.9.

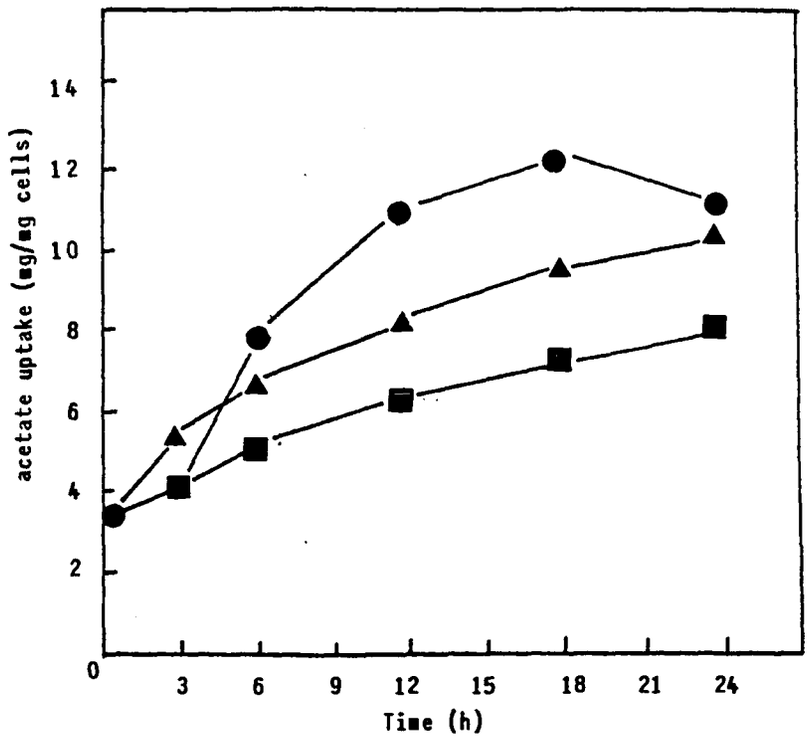


Figure 4.9 : Acetate uptake by Acinetobacter under anaerobic ■—■; anaerobic/aerobic ●—●; aerobic ▲—▲ conditions expressed as a fraction of the cell mass

The downward trend in the acetate uptake curve in the case of the anaerobically pretreated aerobic sample, indicates a growth rate faster than the uptake rate after 18 hours. The uptake of acetate under anaerobic conditions is not unexpected, as previous researchers have already reported the lack of an energy requirement for bacterial uptake of acetate (Konings et al., 1981).

The apparent stimulation of aerobic metabolism by anaerobic pretreatment in Acinetobacter requires further study. These results can be considered in the same light as the enhanced phosphate metabolism, which has been observed after anaerobic pretreatment (Deinema et al., 1980 and 4.1).

The polyhydroxybutyrate concentration changes under the experimental conditions are shown in Figure 4.10.

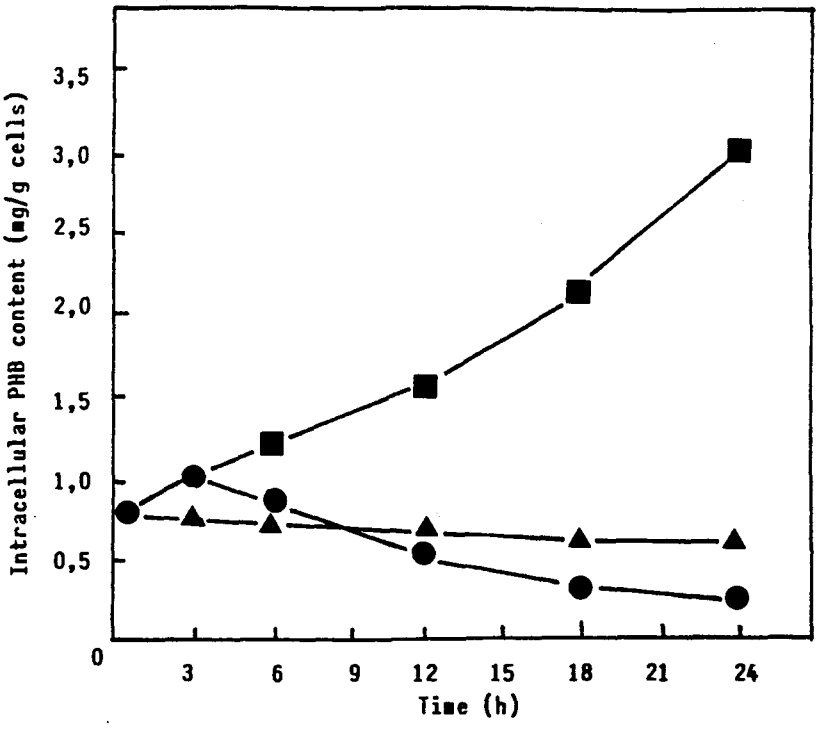


Figure 4.10 : PHB synthesis and utilisation in Acinetobacter under anaerobic ■—■: anaerobic/aerobic ●—● ; and aerobic ▲—▲conditions

In the continually aerated sample no significant difference in the polyhydroxybutyrate level was observed, while in the anaerobically pretreated sample, a slight decrease in polyhydroxybutyrate was observed during aeration. The initial utilisation of polyhydroxybutyrate can apparently be ascribed to the demand which is made on all available carbon sources after a stress period.

The synthesis of polyhydroxybutyrate which was observed under anaerobic conditions, confirm the postulate of Wentzel et al., (1986), who considered a regulatory role for polyhydroxybutyrate in the maintenance of the relationship between the reduced and oxidised forms of nicotine-amide adenine nucleotide (NAD) under anaerobic conditions. This study has shown that the obligate aerobe Acinetobacter has the ability to take up and accumulate acetate under anaerobic conditions.

4.2.2 Plant Studies

The polyhydroxybutyrate levels which are expressed as a fraction of the volatile suspended solids, are given in Table 4.3 for the different zones of the plant.

A decrease in the polyhydroxybutyrate concentration from the anaerobic zone to the aerobic zone is clear in all three plants.

TABLE 4.3

DISTRIBUTION OF POLYHYDROXYBUTYRATE LEVELS IN THREE PLANTS

Sample description	Polyhydroxybutyrate µg/g VSS	
	Minimum value	Maximum value
Goudkoppies Module 2		
Anaerobic zone	37	190
Anoxic zone	13	58
Primary aerobic zone	13	50
Secondary aerobic zone	12	40
Northern Works Module 2		
Anaerobic zone	15	79
Anoxic zone	20	49
Primary aerobic zone	12	51
Northern Works Module 3		
Anaerobic zone	21	55
Anoxic zone	20	44
Primary aerobic zone	4	44
Secondary anoxic zone	4	42

In order to evaluate the role of polyhydroxybutyrate in the process, the amount of this carbon storage product which is produced, had to be compared to the amount of volatile fatty acids which passed through the plant.

During the period under discussion, the Goudkoppies plant received more than 2 000 kg/d of volatile fatty acids, and the nitrate in the return sludge remained below 1 mg/ℓ as nitrogen. Under these conditions polyhydroxybutyrate was continually synthesised in the anaerobic zone and utilised in the anoxic and aerobic zones (see Figure 4.11).

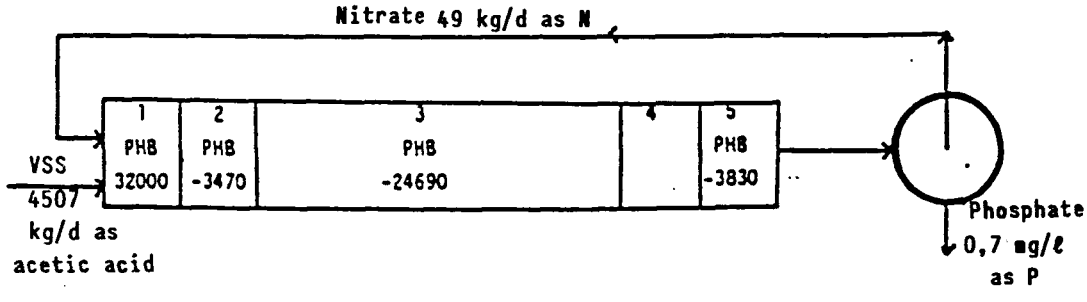


Figure 4.11 : Typical results of PHB synthesis and degradation in Goudkoppies activated sludge plant
- indicates degradation. Results in kg/d
1 : Anaerobic; 2 : Anoxic ; 3 : Aerobic ;
4 : Anoxic ; 5 : Aerobic

The utilisation of polyhydroxybutyrate in the anoxic zone of this plant indicates a high number of Acinetobacter spp which can reduce nitrate (Wentzel et al., 1986). The presence of these bacteria in this zone apparently contributes significantly to the efficient denitrification which is achieved by this plant. In the Northern Works Module 2, which is a three-phase Bardenpho process, the volatile fatty acid feed to the plant varied between 540 and 3 420 kg/d. The nitrate in the return sludge to the anaerobic zone varied between 0 and 690 kg/d (see Figure 4.12).

It is clear from these results that nitrate in the anaerobic zone and insufficient volatile fatty acids in the feed,

inhibits the synthesis of polyhydroxybutyrate. The same typical results were observed for Northern Works Module 3 (see Figure 4.13). The volatile fatty acid level was however, sufficient to initiate synthesis of polyhydroxybutyrate as long as the nitrate concentration was not excessively high.

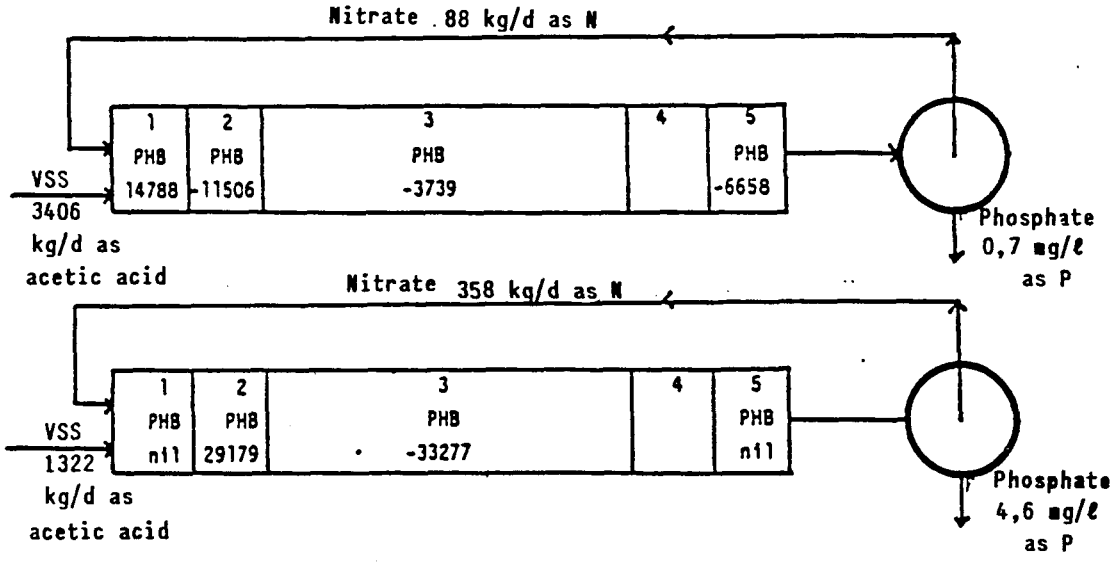


Figure 4.12 : Typical results of PHB synthesis and degradation in Northern Works, Module 2, activated sludge plant
- indicates degradation. Results in kg/d
1 : Anaerobic; 2 : Anoxic ; 3, 4, 5 : Aerobic

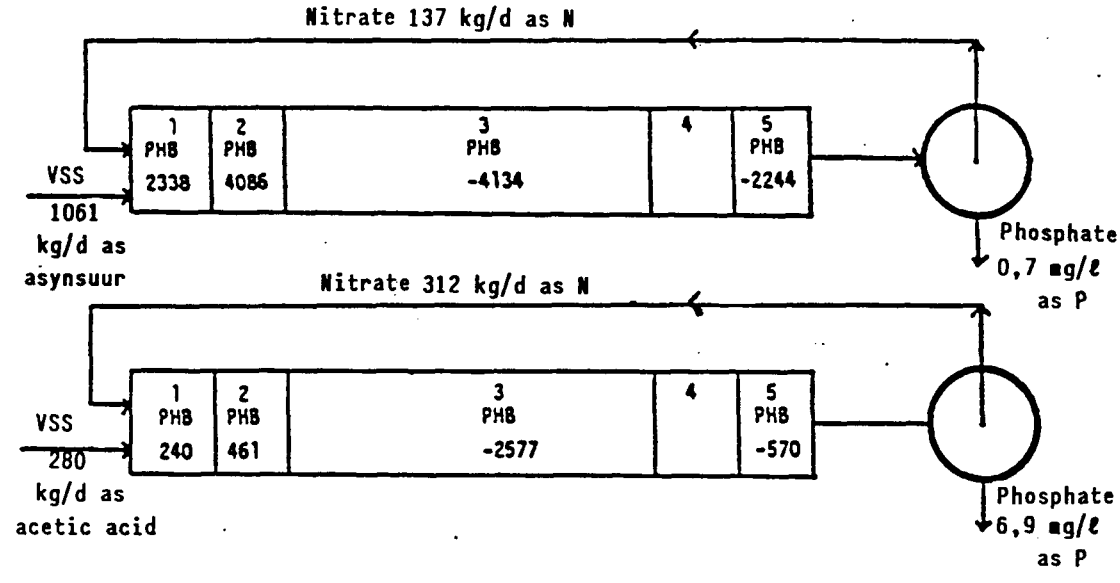


Figure 4.13 : Typical results of PHB synthesis and degradation in Northern Works, Module 3, activated sludge plant
- indicates degradation. Results in kg/d
1 : Anaerobic ; 2 : Anoxic ; 3 : Aerobic ;
4 : Anoxic ; 5 : Aerobic

No direct correlation between inflow of volatile fatty acids, effluent phosphate and nitrate levels and poly-hydroxybutyrate synthesis and utilisation were observed. The continued conversion between polyhydroxybutyrate synthesis and degradation was also observed over long periods with microscopic studies (Lötter and Murphy, 1987).

4.3 Polyphosphate Kinase Activity

4.3.1 Enzyme Extraction

The bacteria were grown for 50 and 100 hours respectively, and then extracted with different buffers. The results are given in Table 4.4.

TABLE 4.4

EFFECT OF EXTRACTION BUFFER ON ENZYME ACTIVITY

Buffer	Specific activity cpm*/mg protein	
Extraction buffer	aeration period	
	50 h	100 h
Extraction buffer A 5 mM	6165	2850
Extraction buffer A 10 mM	5683	1680
Extraction buffer B 5 mM	5299	1611
Extraction buffer B 10 mM	4957	1220
Without dialysis		
Extraction buffer A	-	333
Extraction buffer B	-	650

*cpm - counts per minute

The crude extracts apparently contained an inhibitory substance which was removed by dialysis. The highest

activity was obtained with 5 mM Tris-HCl buffer and this buffer was used throughout for further studies.

4.3.2 The Effect of Growth Conditions on Enzyme Activity

The changes in polyphosphate kinase activity with growth phase are shown in Figure 4.14.

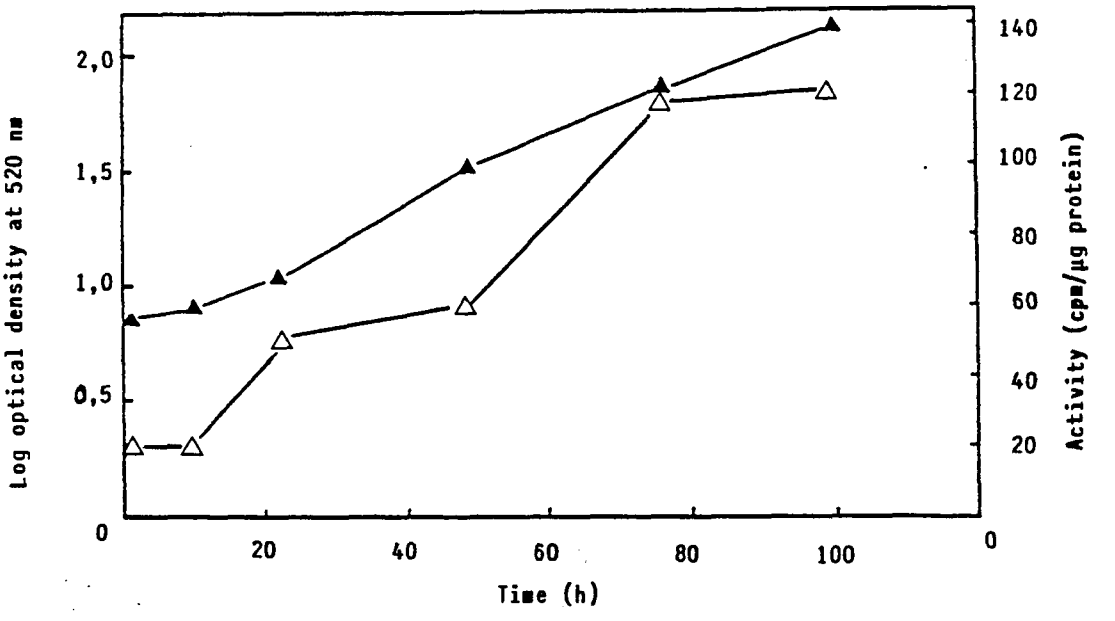


Figure 4.14 : Polyphosphate kinase activity at different growth phases
Growth : △—△ ; Enzyme activity : ▲—▲

As expected, the activity is at its highest at the end of the exponential growth phase, as the demand for ATP for cell synthesis is high during growth. At the end of the growth phase the demand for ATP is considerably lower and the nucleotide can be used for polyphosphate synthesis.

The accumulation of polyphosphate by the same Acinetobacter isolate has been observed at the end of the growth phase in a phosphate containing medium (Murphy and Lötter, 1986), (see Figure 4.7).

The implications for phosphate removal in the activated sludge process are clear. In this process, biomass is continually removed from the process in order to maintain

the process in a steady state condition. The bacterial population is thus held constantly in the stationary growth phase. The period during which ATP is required for normal cell synthesis is thus considerably shortened. The phosphate which is taken up can therefore almost immediately be converted to polyphosphate, and in this way more phosphate can be removed from the medium than that which is required for normal metabolic use.

4.3.3 The Effect of Substrate on Enzyme Activity

Growth in acetate after a period of anaerobic conditions stimulated polyphosphate kinase activity, while the same conditions with succinate as substrate, led to inhibition of the enzyme (Figures 4.15 and 4.16). The difference in behaviour of the two substrates can be explained by their different uptake characteristics. Succinate can only enter the cell by an active transport mechanism (Ramos and Kaback, 1977) and can thus not be taken up under anaerobic conditions by the obligate aerobe, Acinetobacter. In contrast, acetate is transported by a passive mechanism (Konings et al., 1981), and can thus enter the cell under anaerobic conditions which are energy limiting for an obligate aerobe. Acetate uptake by this bacteria has been observed under anaerobic conditions (see 4.2.1).

Cells which are incubated in acetate will therefore enter the aerobic phase with sufficient carbon for metabolic purposes, and ATP can be used for polyphosphate synthesis more rapidly than in the succinate-grown cells. This slow increase in polyphosphate kinase activity after anaerobiosis is evidence of this.

In addition, as a result of phosphate release by acetate addition (Wentzel et al., 1985), the high phosphate levels in the medium probably have a stimulatory effect on the phosphate-transport mechanism which results in rapid phosphate uptake and consequent availability for cell growth.

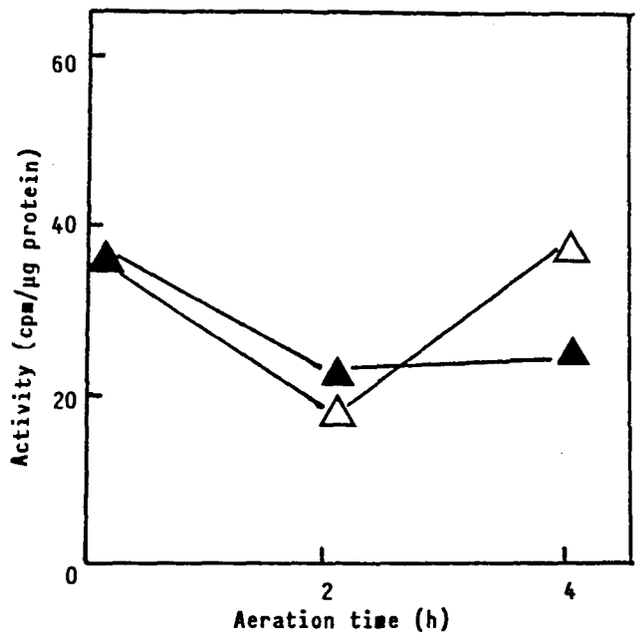


Figure 4.15 : Effect of anaerobic conditions on polyphosphate kinase activity in A. calcoaceticus var lwoffi grown in acetate.
After anaerobic treatment : △—△ ;
Aerobic control : ▲—▲

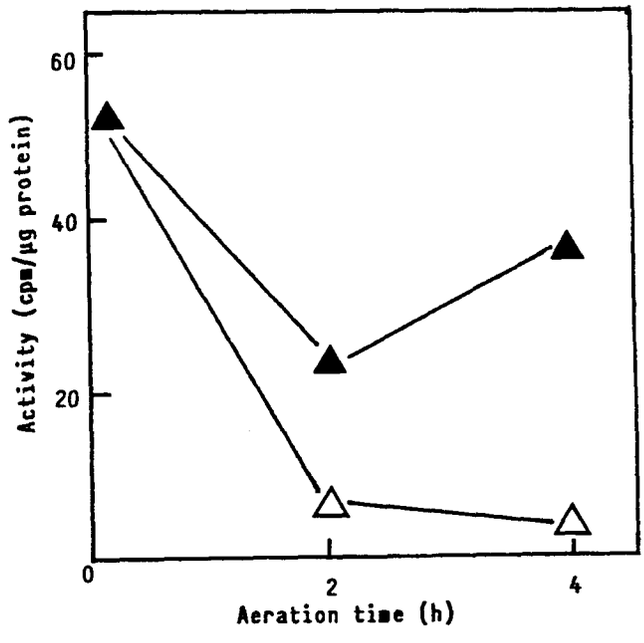


Figure 4.16 : Efect of anaerobic conditions on polyphosphate kinase activity in A. calcoaceticus var lwoffi grown in succinate
After anaerobic treatment : △—△ ;
Aerobic control : ▲—▲

Succinate which does not have the same effect on phosphate levels (see 4.1.2), will not stimulate phosphate uptake in the same manner. The stimulatory effect of acetate on polyphosphate kinase under sequential anaerobic and aerobic conditions has two forms. First, the ability to take up acetate under anaerobic conditions, provides the bacterium with an advantage for growth under aerobic conditions, which then stimulates metabolism. Secondly, the external phosphate levels stimulate the enzyme.

Bacteria which possess the polyphosphate kinase enzyme and which can metabolise acetate under anaerobic conditions will thus dominate the phosphate removal population.

4.4 Electrophoretic Studies

4.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

PAGE in multiphase buffer systems is today, one of the most powerful techniques available for the analysis of a complex mixture of biological macromolecules.

The technique has already been applied in studies on the occurrence and causes of molecular differences (Andrews, 1981). PAGE is further useful in the monitoring of changes in bacteriological activity which can be recognised by differences in protein composition (Law et al., 1977).

By carrying out electrophoresis in the presence of sodium dodecyl sulphate it is possible to determine the molecular mass of the different protein sub-units. This technique was therefore used to obtain as much information as possible from the experiment.

Slab gels were used to facilitate comparison of samples. Electrophoresis was carried out at two gel concentrations.

Preliminary experiments showed that 12,6 % and 7,0 % gels gave the best resolution of protein profiles. These concentrations were thus used throughout.

4.4.2 The Effect of Anaerobic Conditions on Protein Profiles

The Coommassie blue sensitive proteins are shown in Figures 4.17 and 4.18. The anaerobic conditions did not result in a change in the protein profile.

These findings were confirmed by double staining with silver and Coomassie blue, which is shown in Figure 4.19.

Induction or inhibition of protein synthesis is thus not influenced by a lack of oxygen. The changes in metabolic activity which are essential for survival under the anaerobic conditions, are thus apparently regulated on a level other than protein synthesis. The hypothesis of Wentzel et al., (1986), namely, that metabolic control occurs by variation in the ATP/ADP and NAD/NADH relationship, is not contradicted by these findings.

Levels of the nicotinamide adenine dinucleotide are influenced directly by environmental oxygen levels (Yamamoto and Tezuka, 1972). As enzyme synthesis does not play a role in metabolic control under these conditions, it is probable that the activities of the constitutive enzymes will be controlled by metabolites.

4.4.3 The Effect of Substrate on Protein Profiles

The replacement of acetate with succinate in the growth medium had an observable effect on the Coomassie blue stained protein profiles (see Figures 4.20 and 4.21).

Figure 4.17 : SDS-PAGE protein profiles of A. calcoaceticus var lwoffi under anaerobic and aerobic conditions. 12,6% gel stained with Coomassie blue

Figure 4.18 : SDS-PAGE protein profiles of A. calcoaceticus var lwoffi under anaerobic and aerobic conditions. 7% gel stained with Coomassie blue

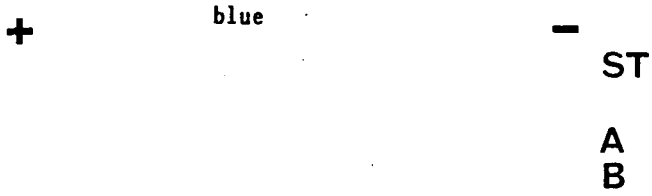


Figure 4.19 : SDS-PAGE protein profiles of A. calcoaceticus var lwoffi under anaerobic and aerobic conditions. 7 % gel stained with Coomassie blue followed by silver staining

Figure 4.20 :SDS-PAGE protein profiles of A. calcoaceticus var lwoffi grown in acetate and succinate. 12,6% gel stained with Coomassie blue

Figure 4.21 : SDS-PAGE protein profiles of A. calcoaceticus var lwoffi grown in acetate and succinate. 7% gel stained with Coomassie blue

Figure 4.22 : SDS-PAGE protein profiles of A. calcoacetius var lwoffi grown in acetate and succinate. 12,6% gel stained with Coomassie blue followed by silver staining

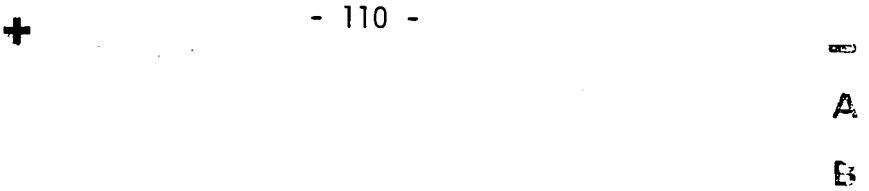


Figure 4.23 : SDS-PAGE protein profiles of A. calcoaceticus var lwoffi grown in acetate and succinate. 7% gel stained with Coomassie blue followed by silver staining

Figure 4.24 : PAGE of protein extract stained enzymatically with β-hydroxybutyrate as substrate

Figure 4.25 : PAGE of protein extract stained enzymatically with D,L isocitric acid as substrate

Figure 4.26 : Auto-radiogram of protein extracts from Acinetobacter grown in acetate and succinate. 12,6% gel

Figure 4.27 : Auto-radiogram of protein extracts of Acinetobacter grown in acetate and succinate. 7,0 % gel

Figure 4.28 : 12% PAGE of β-hydroxy-butyrate dehydrogenase

Figure 4.29 : 7% PAGE of β-hydroxy-butyrate dehydrogenase

Molecular masses were determined by the method of Weber and Osborn (1969). Bands with molecular masses of 70; 80; 84 and 105 kDa were only observed in the succinate-grown cells, while bands of 82 and 115 kDa were only present in the acetate-grown cells.

Silver staining produced positive (yellow-brown) and negative (no colour) bands. Subsequent restaining with Coomassie blue stains the negative bands blue. This differential staining has been described by Dzandu et al., (1984). These researchers were able to distinguish between sialoglycoproteins and lipoproteins and conventional Coomassie blue sensitive polypeptides.

No unique silver stained bands were observed in the succinate grown cells. In contrast, the acetate grown cells produced well-defined bands at 38; 44; 78; 80; 86 and 92 kDa. Two diffuse yellow-brown areas between 70 and 74 kDa and 62 and 66 kDa were also observed (see Figures 4.22 and 4.23).

4.4.4 Polyacrylamide Gel Electrophoresis With Enzymatic Staining

Enzymatic staining was carried out on 7 % gels of protein extracts from succinate and acetate grown cells. A strong band of activity with a molecular mass of approximately 96 kDa was observed with β-hydroxybutyrate as substrate. A much weaker band with a molecular mass of approximately 130 kDa was also observed (see Figure 4.24). The β-hydroxybutyrate dehydrogenase enzyme from Rhodopseudomonas sphaeroides has a molecular mass of 85 kDa (Bergmeyer et al., 1967).

By using isocitrate as substrate, a strong activity band of approximately 80 kDa and two weaker bands at 76 and 78 kDa was observed (see Figure 4.25). Molecular masses of 100 and 300 kDa has been observed for isoenzymes of isocitrate

dehydrogenase from Acinetobacter var lwoffi (sic) (Self and Weitzman, 1972).

4.4.5 Autoradiography

By placing the film between calcium tungstate intensifying screens and the sample, the sensitivity of autoradiography can be increased (Laskey and Mills, 1977). Comparative studies showed that calcium tungstate screens were the most effective in the determination of ^{32}P radiation (Swanstrom and Shank, 1978).

Phosphorylated protein bands which corresponded with different molecular masses were obtained in protein extracts from acetate and succinate grown cells. No difference between the two was observed (see Table 4.5 and Figures 4.26 and 4.27).

TABLE 4.5

SUMMARY OF PHOSPHORYLATED PROTEIN BANDS OBTAINED IN CELLS GROWN IN ACETATE AND SUCCINATE

Protein band	Molecular mass (kDa)
1	150
2	130
3	110
4	90

Reversible phosphorylation could not be shown at this stage. Anaerobiosis had no effect on protein phosphorylation.

4.5 β-hydroxybutyrate Dehydrogenase Activity

4.5.1 Comparison of Different Bacterial Isolates

Eight out of the 11 isolates which were tested accumulated polyhydroxybutyurate under the experimental conditions. The variation in enzyme activity among these 8 isolates are shown in Table 4.6.

TABLE 4.6

β-HYDROXYBUTYRATE DEHYDROGENASE ACTIVITY IN A. CALCOACETICUS VAR LWOFFI ISOLATES

Isolate	Specific activity AU/mg protein
NW01	1,71
NW04	0,02
NW06	0,05
NW07	0,23
NW08	0,72
NW09	0,06
NW10	0,04
NW11	0,46

Isolate NW01 was used for further studies

4.5.2 Enzyme Isolation

The ultrasonic disintegration in Tris buffer (3.6.4.1) successfully extracted β-hydroxybutyrate dehydrogenase as was shown in the case of activated sludge samples (Lötter and van der Merwe, 1987). The ammonium sulphate fractionation is shown in Table 4.7.

The supernatants which were obtained after centrifugation of the 0 to 45 % ammonium sulphate fractionation, were further fractionated, as shown in Table 4.8.

TABLE 4.7

AMMONIUM SULPHATE FRACTIONATION OF THE CELL-FREE EXTRACT

$(NH_4)_2SO_4$ Saturation %	Specific activity in supernatant AU/mg protein
0 - 35	0,890
0 - 45	0,968
0 - 55	0,578

TABLE 4.8

SECOND AMMONIUM SULPHATE FRACTIONATION

$(NH_4)_2SO_4$ Saturation %	Specific activity in supernatant AU/mg protein
45 - 55	5,21
45 - 65	7,18

The highest yield of β-hydroxybutyrate dehydrogenase was obtained in the protein fraction which precipitates between 45 and 65 % ammonium sulphate saturation.

The enrichment which was obtained with this procedure is summarised in Table 4.9.

TABLE 4.9

ENRICHMENT TABLE FOR THE ISOLATION OF β-HYDROXYBUTYRATE DEHYDROGENASE

	Step	Volume (m)	Total protein (mg)	Total activity ncat	Specific activity ncat/mg	Purification (-times)
1	Crude extract	40	24	31,2	1,3	1
2	0 - 45% $(NH_4)_2SO_4$ supernatant	39	10,9	18,8	1,7	1,3
3	0 - 65% $(NH_4)_2SO_4$ precipitate	5	6,72	52,3	7,8	6,0

4.5.2.1 Polyacrylamide electrophoresis

One band was observed on the 12 % gel after enzymatic staining (Figure 4.28). Electrophoresis on a 7 % gel resulted in two bands (Figure 4.29).

More than 90 % of the activity was present in the stronger band. Calibration of the gel with a series of proteins of known molecular mass, indicates a molecular mass for the enzyme of approximately 85 kDa (Figure 4.30). This extract was used for metabolic studies.

4.5.3 The Effect of Certain Growth Substrates on Enzyme Activity

The method of Williamson and Mellanby (1974), for mammalian β-hydroxybutyrate dehydrogenase was used for the activity determination. The nature of the carbon source had an effect on the specific activity of the enzyme. The results expressed as a percentage of the highest specific activity, which was obtained with acetate, are shown in Figure 4.31.

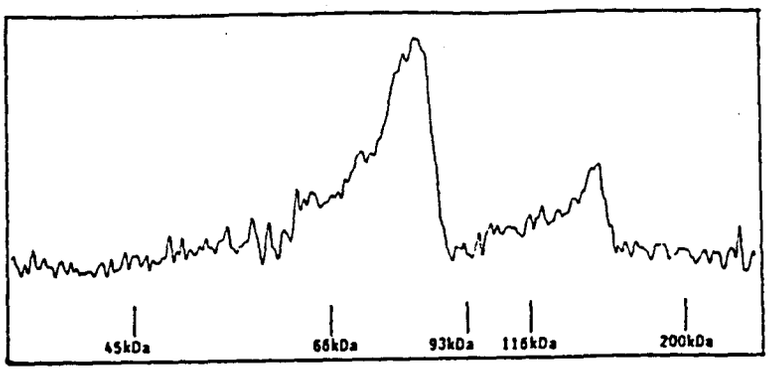


Figure 4.30 :Densitogram of 7 % PAGE of β-hydroxy-butyrate dehydrogenase

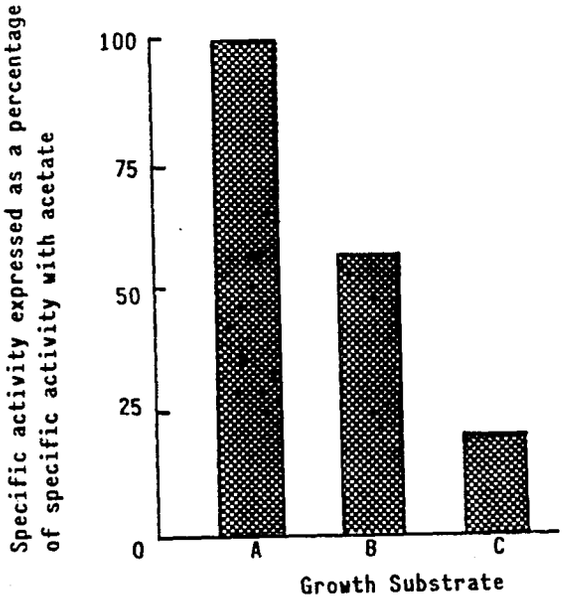


Figure 4.31 : Effect of growth substrate on β-hydroxy-butyrate dehydrogenase
A: Acetate; B: Succinate; C: Glucose

Acetate is a very favourable substrate for Acinetobacter spp and is completely utilised under aerobic conditions (Abbott, 1973; Schutte, 1987).

Under these conditions polyhydroxybutyrate is accumulated and high activities of the enzymes involved in this metabolism are expected. Acetate can, after activation to acetyl-CoA, either enter the tricarboxylic acid cycle directly, or be converted to polyhydroxybutyrate (Mahler and

Cordes, 1971; Dawes and Senior, 1973), while succinate and glucose first have to be further metabolised before polyhydroxybutyrate can be synthesised.

4.5.4 The Effect of Certain Metabolites on Enzyme Activity

Initial reaction rates for the enzyme were initially determined at a β-hydroxybutyrate concentration range of 50 to 150 µM at different cofactor concentrations. The dependance of the enzyme activity on substrate concentration at different cofactor concentrations are shown in Figure 4.32. Substrate inhibition was observed at β-hydroxybutyrate concentrations above 100 µM, as is clearly shown in the Lineweaver-Burk manipulation of the data obtained at 1 cofactor concentration (see Figure 4.33).

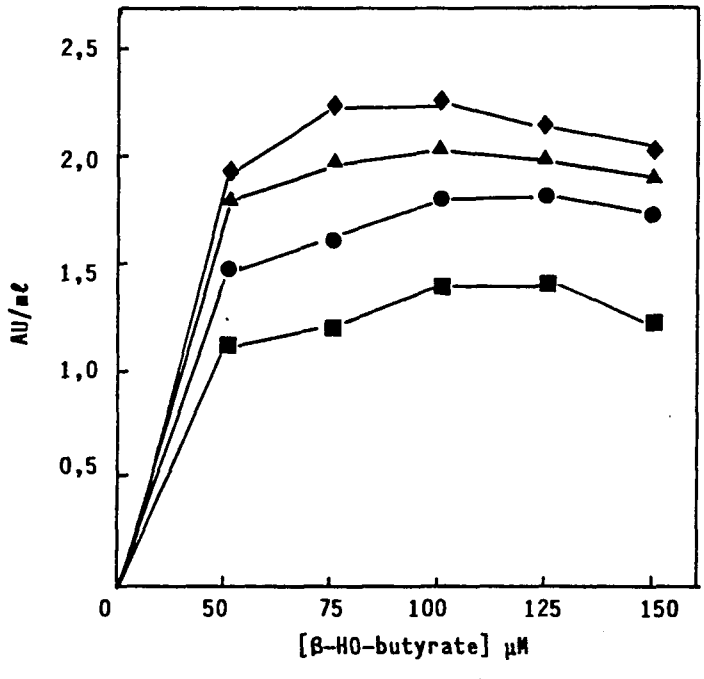


Figure 4.32 : Saturation curves of β-hydroxybutyrate dehydrogenase with β-hydroxybutyrate as substrate and 5 mM NAD ■—■ ; 10 mM NAD ●—● ; 15 mM NAD ▲—▲ ; 20 mM NAD ◆—◆

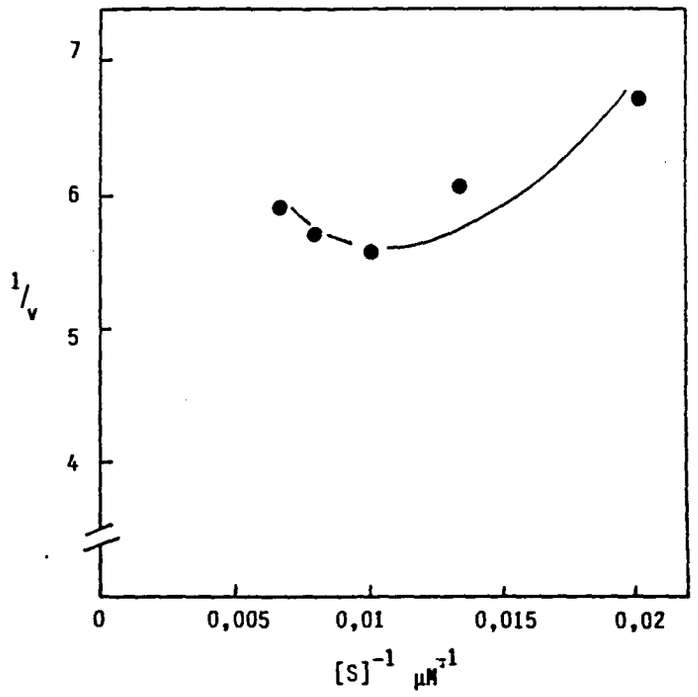


Figure 4.33 : Lineweaver-Burk manipulation of saturation curves obtained with β-hydroxybutyrate and 15 mM NAD as co-factor

The saturation curve obtained with varying NAD concentrations at 100 µM β-hydroxybutyrate shows the typical Michaelis-Menten form (see Figure 4.34), but the Lineweaver-Burk manipulation of the data, shows that the data deviates from simple Michaelis-Menten kinetics, in that the graph shows a downwards concave tendency (see Figure 4.35).

The initial rates were consequently determined over a β-hydroxybutyrate concentration range of 0 to 80 µM. The results are shown in Figure 4.36.

The Lineweaver-Burk manipulation of the data is shown in Figure 4.37.

In order to determine whether NADP can replace NAD as cofactor, the enzyme was incubated with β-hydroxybutyrate at 100 mM and NADP at 15 mM, under the standard test conditions.

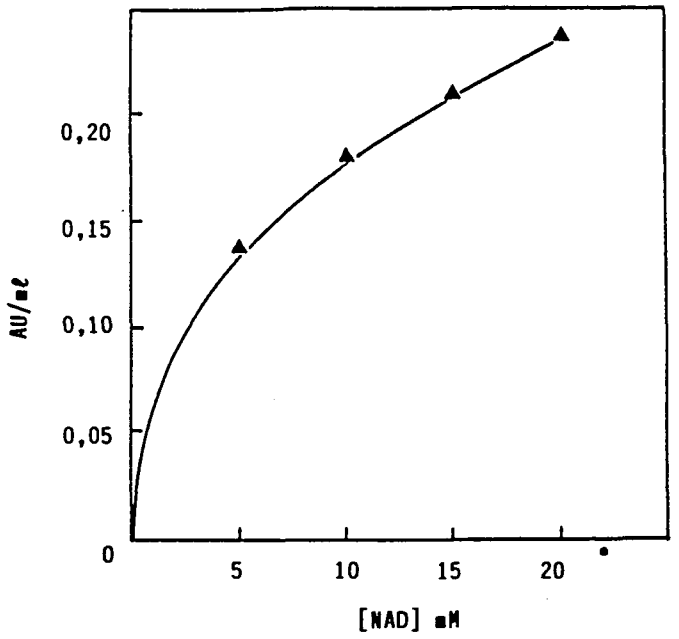


Figure 4.34 : Effect of NAD concentration on enzyme activity with β-hydroxybutyrate as substrate at a concentration of 100 µM

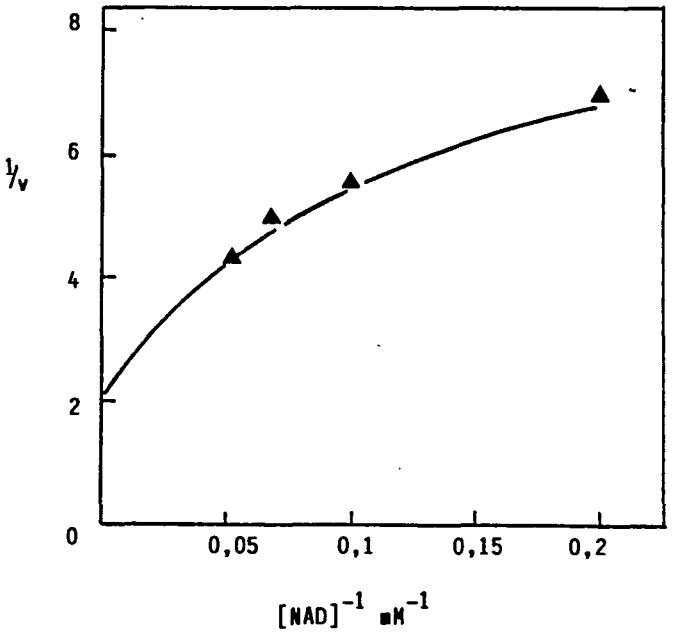


Figure 4.35 : Lineweaver-Burk manipulation of the effect of NAD concentration on enzyme activity with β-hydroxybutyrate as substrate at a concentration of 100 µM

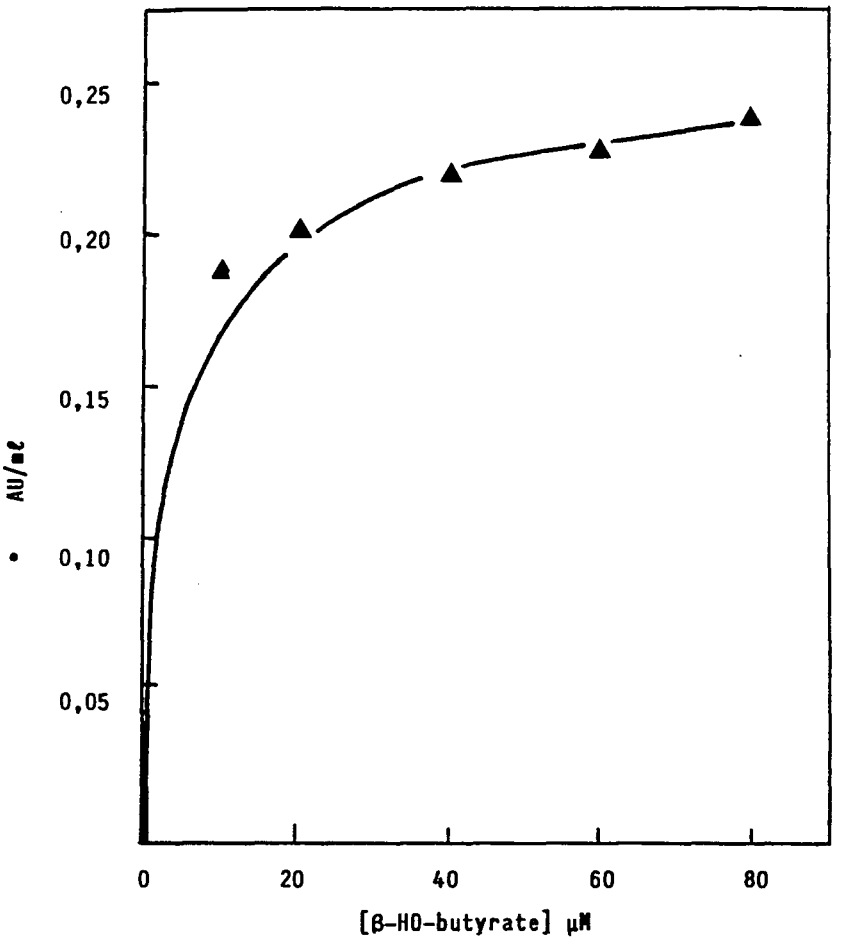


Figure 4.36 : Saturation curve of β-hydroxybutyrate dehydrogenase with β-hydroxybutyrate as substrate and 15 mM NAD as co-factor

No activity was observed. The enzyme from A. calcoaceticus var lwoffi thus has a specific requirement for NAD, on common with enzymes from Hydrogenomonas spp (Ahrens and Schlegel, 1966), Rhodopseudomonas sphaeroides (Bergmeyer et al., 1967) and Azotobacter beijerinckii (Dawes and Senior, 1973).

The reverse reaction, namely, the reduction of the acetoacetate to -hydroxybutyrate, was determined by incubating the enzyme with acetoacetate at a concentration range of 34 to 133 mM, and 15 mM NADH. The saturation curves obtained with different concentrations of acetoacetate and 15 mM NAD, also appear to exhibit simple

Michaelis-Menten kinetics (see Figure 4.38), which is only contradicted by Lineweaver-Burk manipulation of the data (see Figure 4.39).

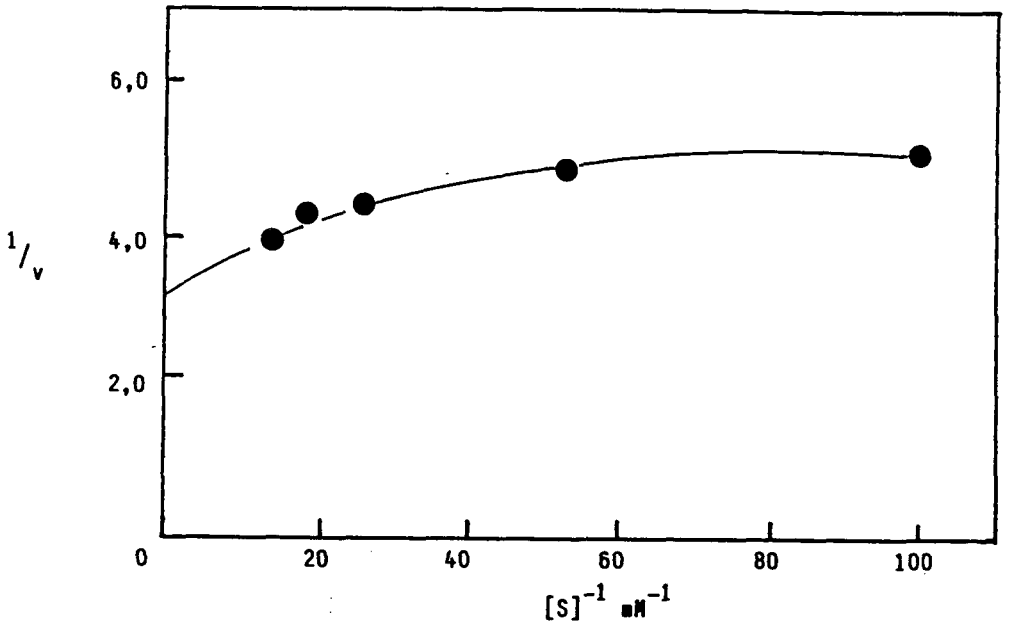


Figure 4.37 : Lineweaver-Burk manipulation of the data in Figure 4.36

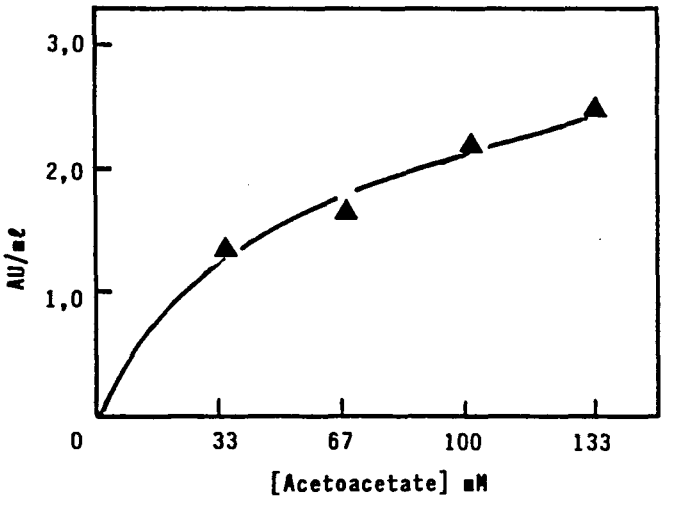


Figure 4.38 : Saturation curve of enzyme activity with acetoacetate as substrate and 15 mM NADH as co-factor

The same pattern is obtained with NADH as varying substrate (see Figures 4.40 and 4.41).

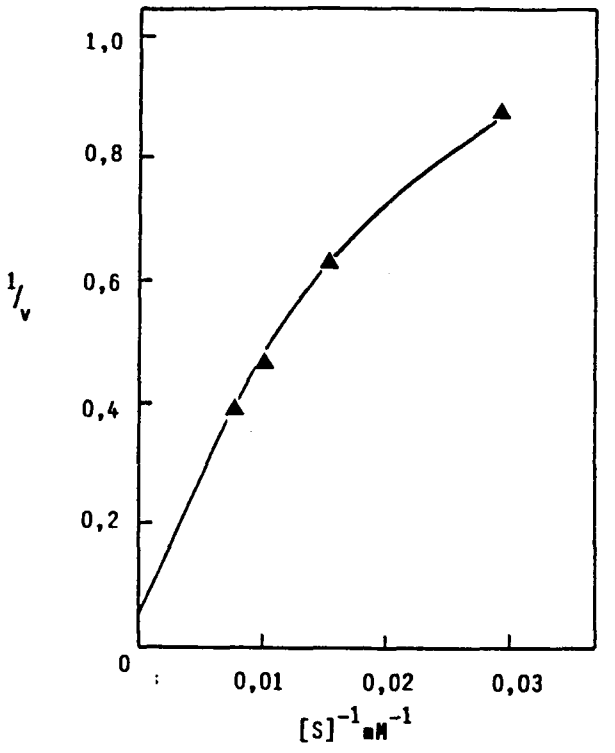


Figure 4.39 : Lineweaver-Burk manipulation of the data in Figure 4.38

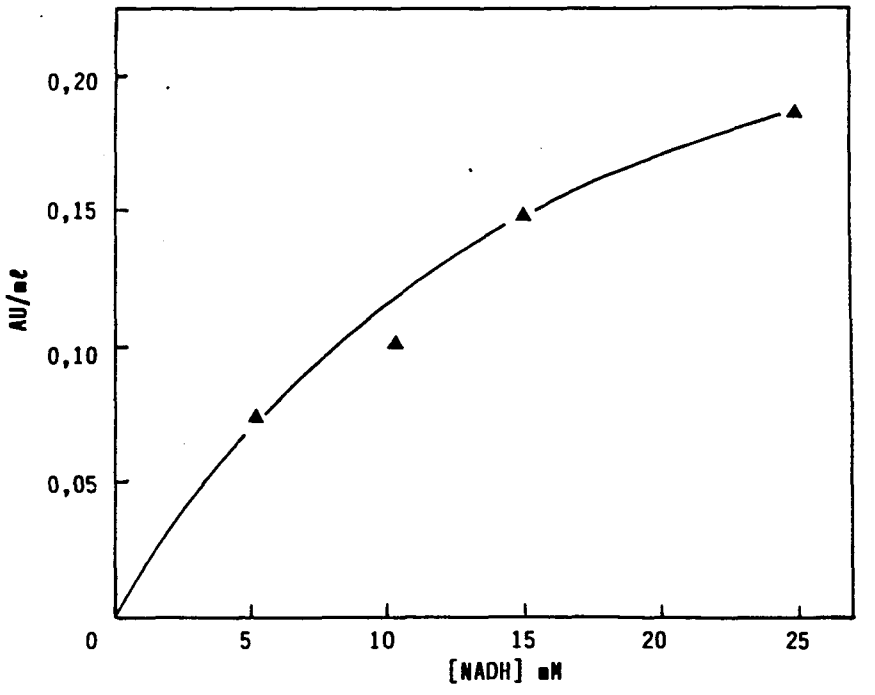


Figure 4.40 : The effect of NADH concentration on enzyme activity with aceto-acetate as substrate at a concentration of 100 mM

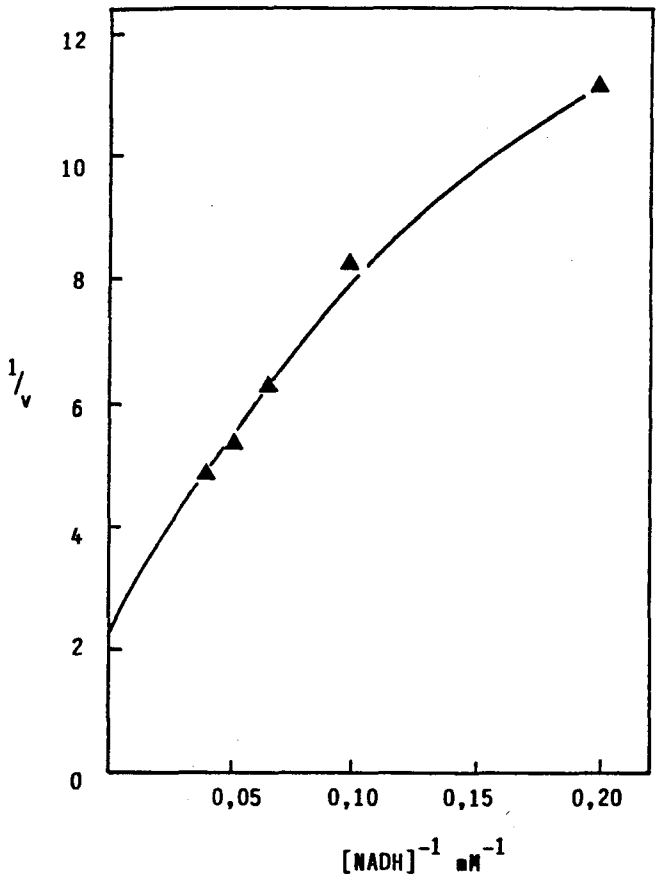


Figure 4.41 : Lineweaver-Burk manipulation of the data in Figure 4.40

The catalysis of the reverse reaction has already been observed in other bacteria (Bergmeyer et al., 1967). Although β-hydroxybutyrate dehydrogenase can catalyse the reverse reaction of acetoacetate to β-hydroxybutyrate, it is unlikely that synthesis occurs according to this route. In other organisms which accumulate PHB, this step in the synthesis pathway is catalysed by an acetoacetyl-CoA reductase (Ritchie et al., 1971), an enzyme, which has still to be detected in Acinetobacter.

The effect of NADH on β-hydroxybutyrate dehydrogenase activity with β-hydroxybutyrate as substrate, is shown in Figures 4.42 and 4.43. The reduced form of the nicotinamide has a strong inhibitory effect on the enzyme.

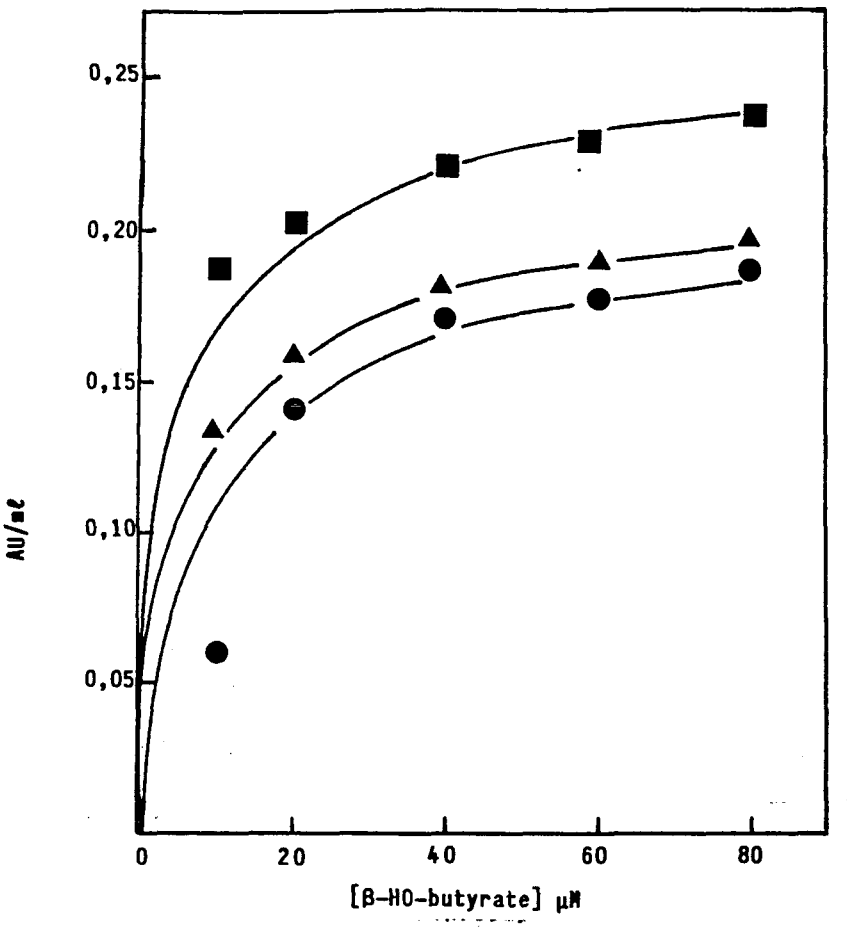


Figure 4.42 : The effect of NADH on β-hydroxybutyrate dehydrogenase activity with β-hydroxybutyrate as substrate. ■—■ : 0 mM NADH; ▲—▲ : 5 mM NADH ; ●—● : 15 mM NADH

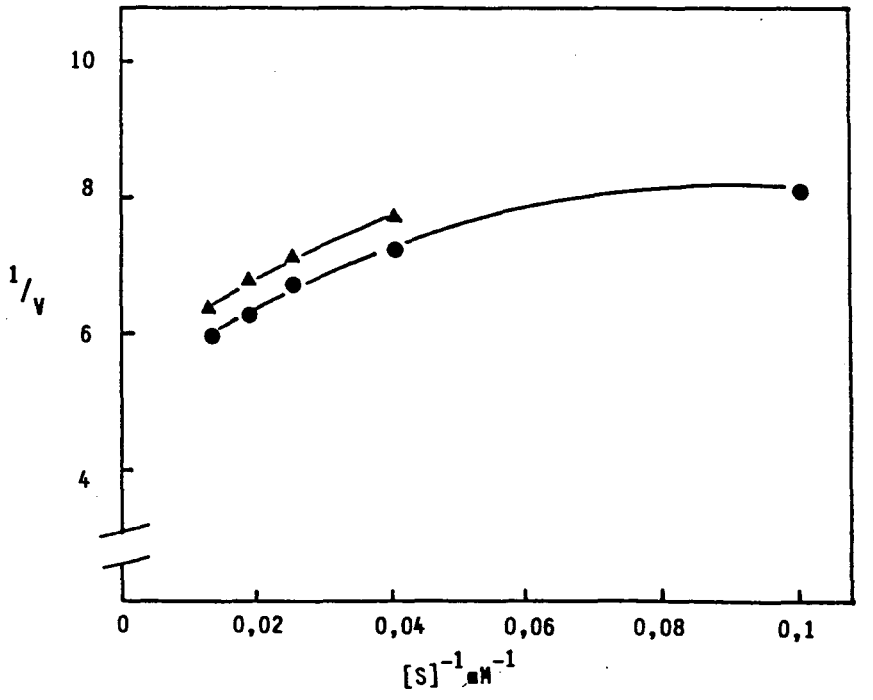


Figure 4.43 : Lineweaver-Burk manipulation of the data in Figure 4.42

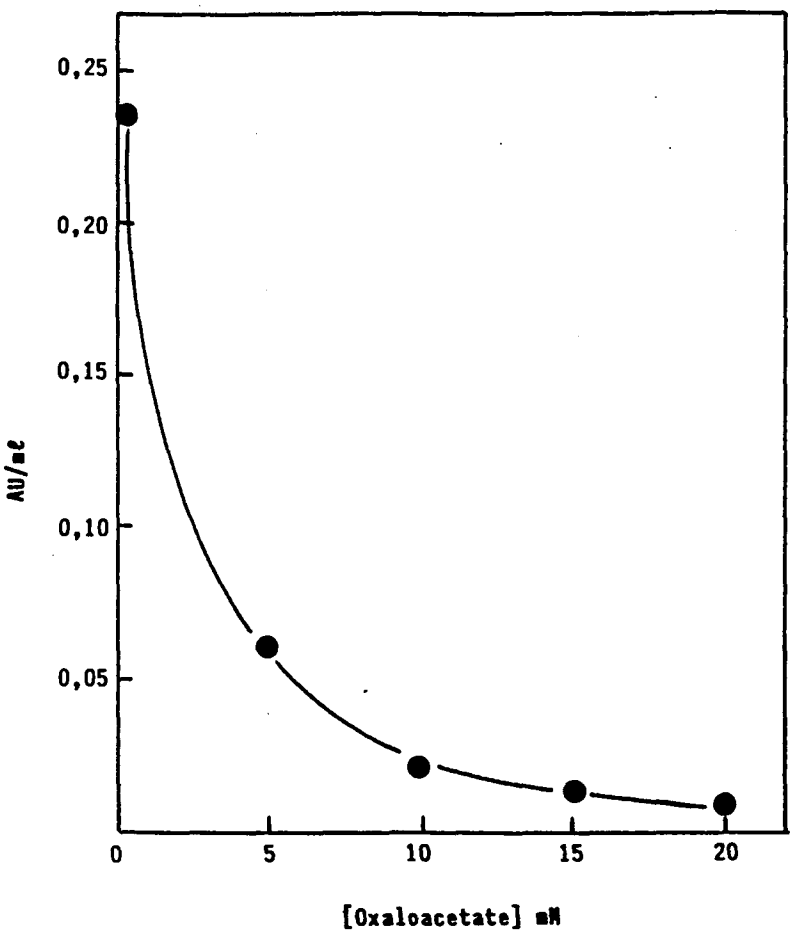


Figure 4.45 : The effect of different concentrations of oxaloacetic acid on β-hydroxybutyrate dehydrogenase activity with β-hydroxybutyrate as substrate

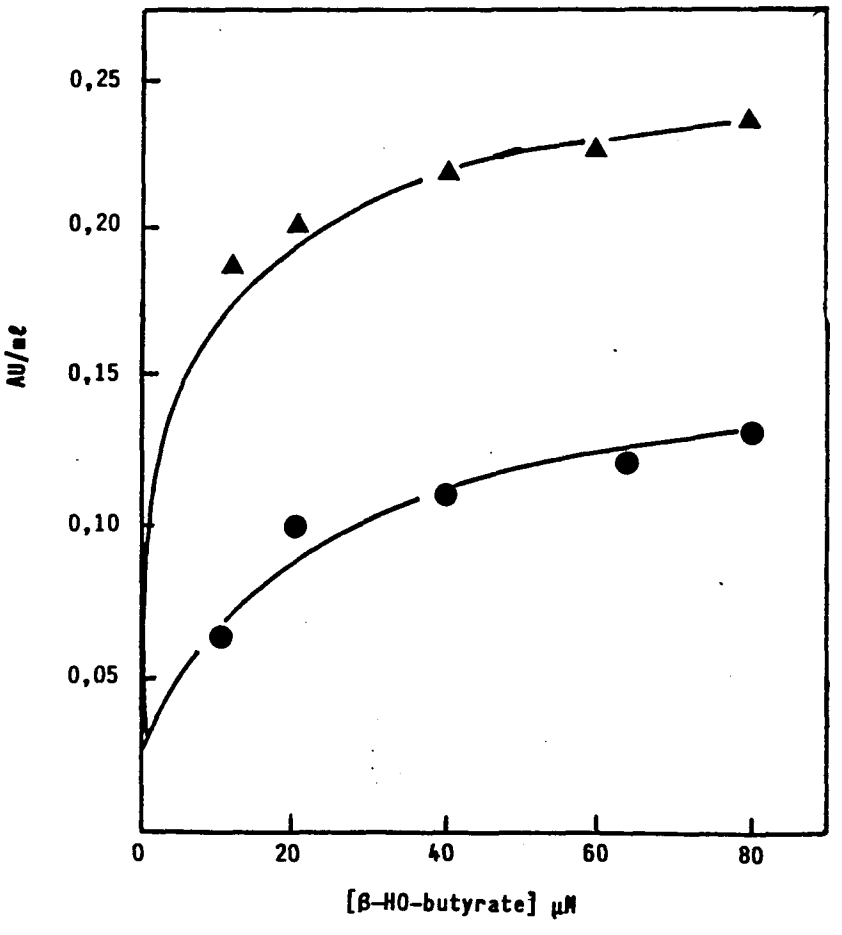


Figure 4.44 : The effect of oxaloacetic acid on β-hydroxybutyrate dehydrogenase activity

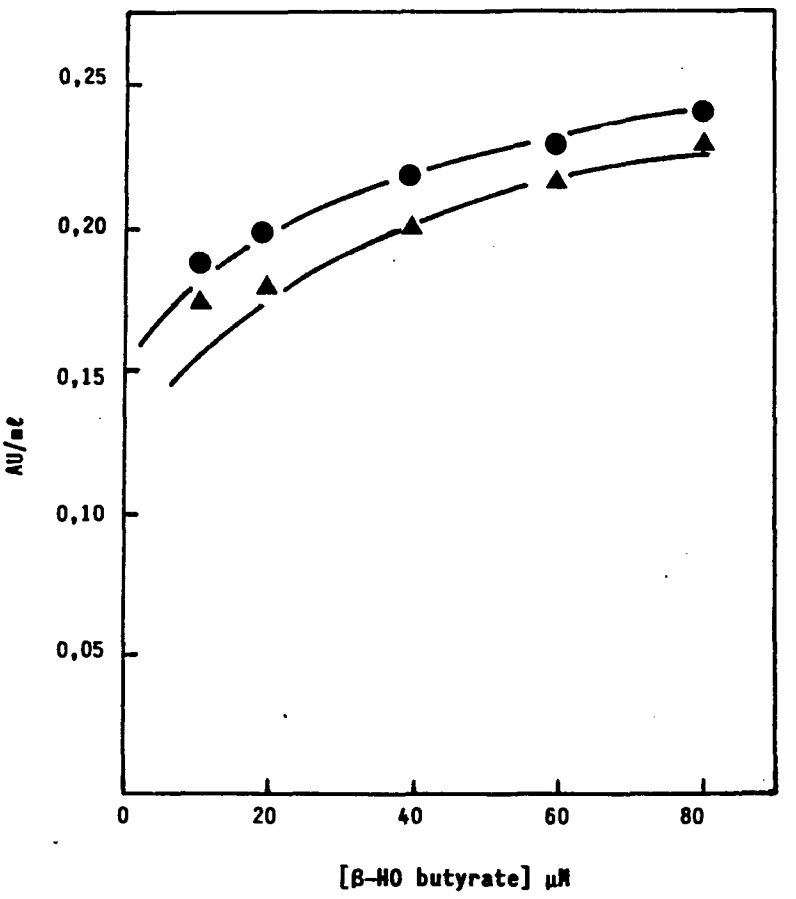


Figure 4.46 : The effect of acetyl CoA on β-hydroxybutyrate dehydrogenase activity at different β-hydroxybutyrate concentrations.
▲—▲ : 25 mM Acetyl-CoA ;
●—● : 0 mM Acetyl-CoA

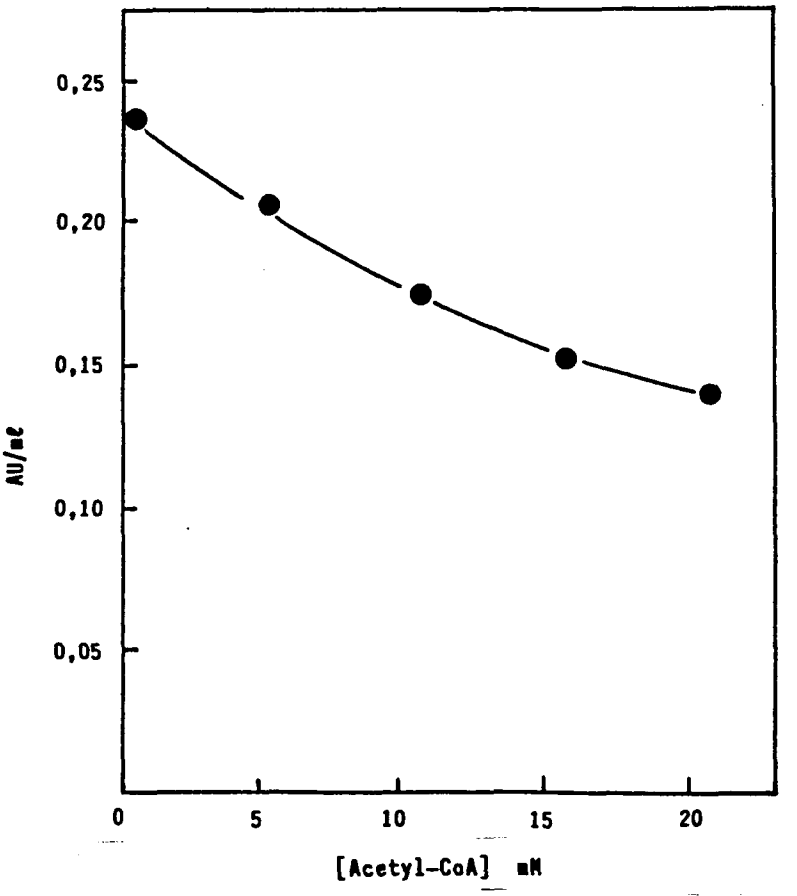


Figure 4.47 : The effect of different concentrations of acetyl CoA on β-hydroxybutyrate dehydrogenase with β-hydroxybutyrate as substrate

The metabolites oxaloacetate and acetyl-CoA, both have an inhibitory effect on the oxidation of β-hydroxybutyrate by the enzyme (see Figures 4.44 to 4.47). β-hydroxybutyrate dehydrogenase enzymes from different sources are inhibited by different metabolites.

The enzyme from Hydrogenomonas eutropha is inhibited by NADH, pyruvate and oxaloacetic acid (Oeding and Schlegel, 1973), while the Azotobacter beijerinckii enzyme is inhibited by NADH and pyruvate, but not by oxaloacetate (Dawes and Senior, 1973). The enzyme from Rhodospeudomonas sphaeroides is strongly inhibited by malonic acid and lactic acid (Bergmeyer et al., 1967).

The different metabolic paths which prevail in these bacteria are probably responsible for the different inhibition patterns. The same is valid for Acinetobacter spp.

4.6 Isocitrate Dehydrogenase

4.6.1 Effect of Anaerobic Conditions on Enzyme Activity

Incubation of Acinetobacter cells under anaerobic conditions results in a lower specific isocitrate dehydrogenase activity (see Table 4.10).

The lower activity which prevails under anaerobic conditions is not unexpected, as the obligate aerobe's metabolic activity under these unfavourable conditions will be reduced. The results show however, that this enzyme is still active under anaerobic conditions, which indicates that the tricarboxylic acid cycle activity remains, even in the absence of a terminal electron acceptor. This latter observation corresponds with the findings of Florentz and Harteman (1982), who observed citrate synthase, α-keto-glutarate dehydrogenase and isocitrate dehydrogenase activity in activated sludge under anaerobic conditions.

TABLE 4.10

THE EFFECT OF ANAEROBIC CONDITIONS ON THE SPECIFIC ACTIVITY OF ISOCITRATE DEHYDROGENASE

Incubation conditions	Specific activity AU/mg protein
Aerobic	0,117
Anaerobic	0,045

4.6.2 Enzyme Isolation

Once again, ultrasonic disintegration in Tris buffer (3.6.4), was found to be a successful extraction procedure. The results of the first ammonium sulphate fractionation are given in Table 4.11.

The supernatant obtained after centrifugation of 0 to 45 % ammonium sulphate fractionation was further fractionated as shown in Table 4.12.

TABLE 4.11

AMMONIUM SULPHATE FRACTIONATION OF THE CELL-FREE EXTRACT

$(NH_4)_2SO_4$ saturation	Specific activity in supernatant AU/mg protein
0 - 35	0,177
0 - 45	0,224
0 - 55	0,152

TABLE 4.12

SECOND AMMONIUM SULPHATE FRACTIONATION

$(NH_4)_2SO_4$	Specific activity in supernatant AU/mg protein
45 - 55	0,163
45 - 65	0,970

The highest yield of the enzyme was between obtained between 45 and 65 % saturation. The enrichment which was obtained with this procedure is summarised in Table 4.13.

TABLE 4.13

ENRICHMENT TABLE FOR THE ISOLATION OF ISOCITRATE DEHYDROGENASE

	Step	Volume (m)	Total protein (mg)	Total activity AU	Specific activity AU/mg	Purification (-times)
1	Crude extract	40	13	28,5	2,19	1
2	0 - 45 % $(NH_4)_2SO_4$ supernatant	20	3,5	7,95	2,26	1,03
3	45 - 65 % $(NH_4)_2SO_4$ precipitate	5	0,325	3,15	9,69	9,4

Electrophoresis of the extract on a 7 % polyacrylamide gel produced two bands, which are apparently the two isoenzymes of isocitrate dehydrogenase which have already been detected in Acinetobacter lwoffi (Parker and Weitzman, 1970) (see Figure 4.48).

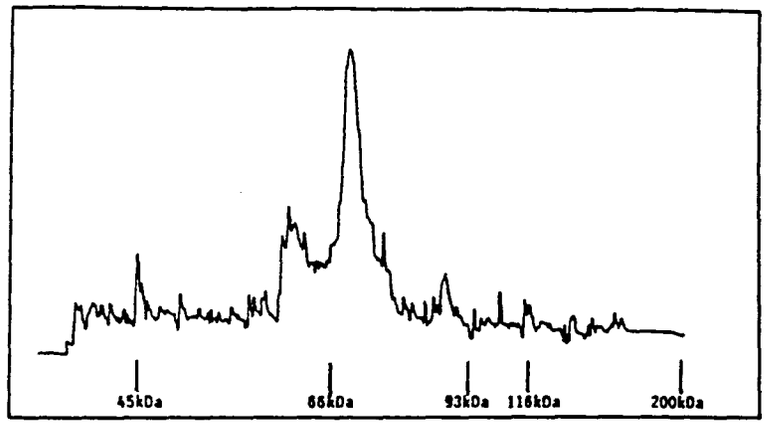


Figure 4.48 : Densitogram of electrophoresis pattern of protein extract on a 7 % gel showing isocitrate dehydrogenase peaks

This extract was used for further preliminary metabolic studies.

4.6.3 The Effect of Growth Substrate on Enzyme Activity

As in the case of β-hydroxybutyrate dehydrogenase the activity of the isocitrate dehydrogenase is affected by growth substrate. The specific activity was once again expressed as a percentage of the specific activity which was obtained with acetate (see Figure 4.49).

The reversible phosphorylation of isocitrate dehydrogenase in acetate medium which led to a decrease in activity in other organisms (Garnak and Reeves, 1987; Wang and Koshland, 1978), was not observed here. The absence of enzyme phosphorylation with the changeover from succinate to acetate as carbon source, has already been discussed (4.45). Previous researchers (Reeves et al., 1983; Reeves et al., 1986), reported the unique behaviour of an organism which

they identified as Acinetobacter lwoffi. As described in their studies, growth in an acetate medium stimulated isocitrate dehydrogenase activity in Acinetobacter lwoffi.

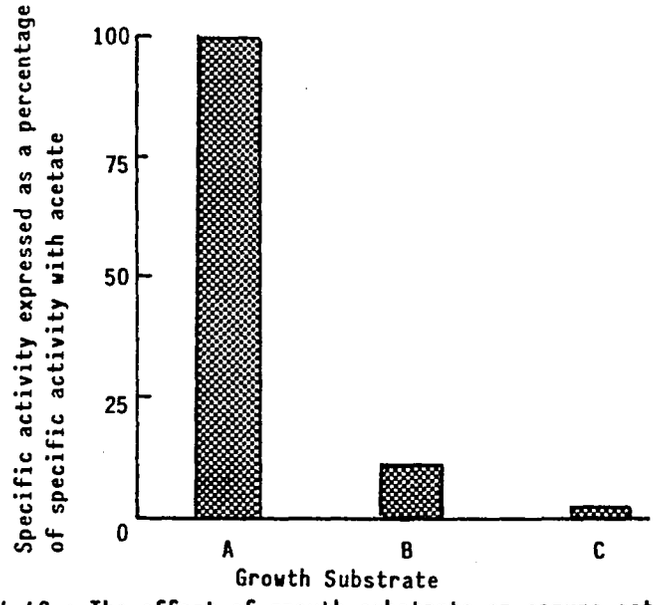


Figure 4.49 : The effect of growth substrate on enzyme activity
A: Acetate; B : Succinate; C : Glucose

4.6.4 The Effect of Certain Metabolites on Enzyme Activity

The effect of NADP and isocitrate concentrations on the enzyme activity are shown in Figures 4.50 and 4.51.

Only preliminary studies were carried out with certain metabolites, to gain an idea of which compounds required further study. The results are shown in Figure 4.52.

The enzyme is inhibited by the reduced form of NADP. Inhibition of this enzyme by NADPH and oxaloacetate has already been observed in other organisms (Marr and Weber, 1969). Inhibition by oxaloacetate is not unexpected, as high concentrations of this compound indicates sufficient carbon to maintain the tricarboxylic acid cycle. The enzyme is stimulated by AMP, a typical pattern for a catabolic enzyme (Atkinson, 1968).

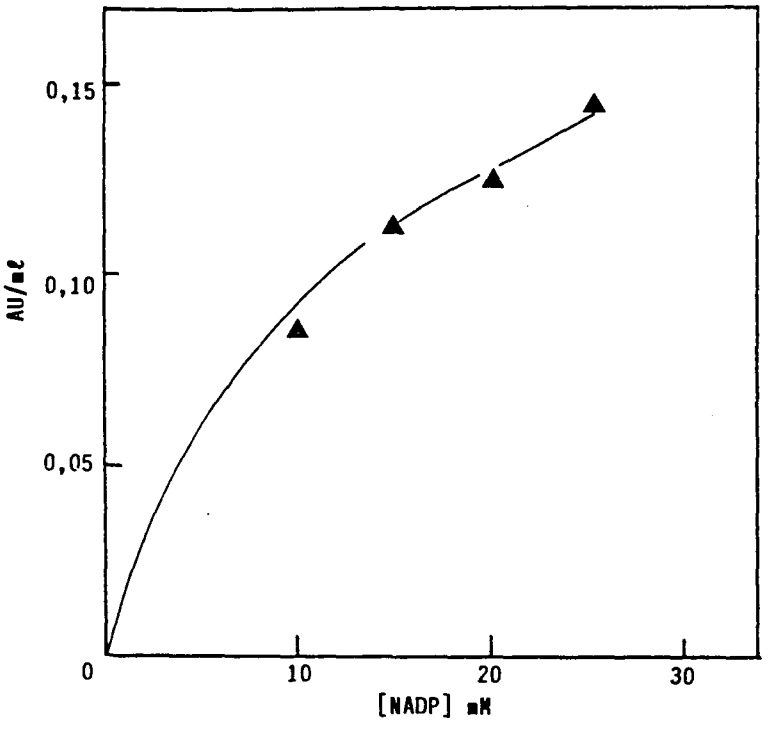


Figure 4.50 : The effect of different NADP concentrations on isocitrate dehydrogenase activity with 30 mM isocitrate as substrate

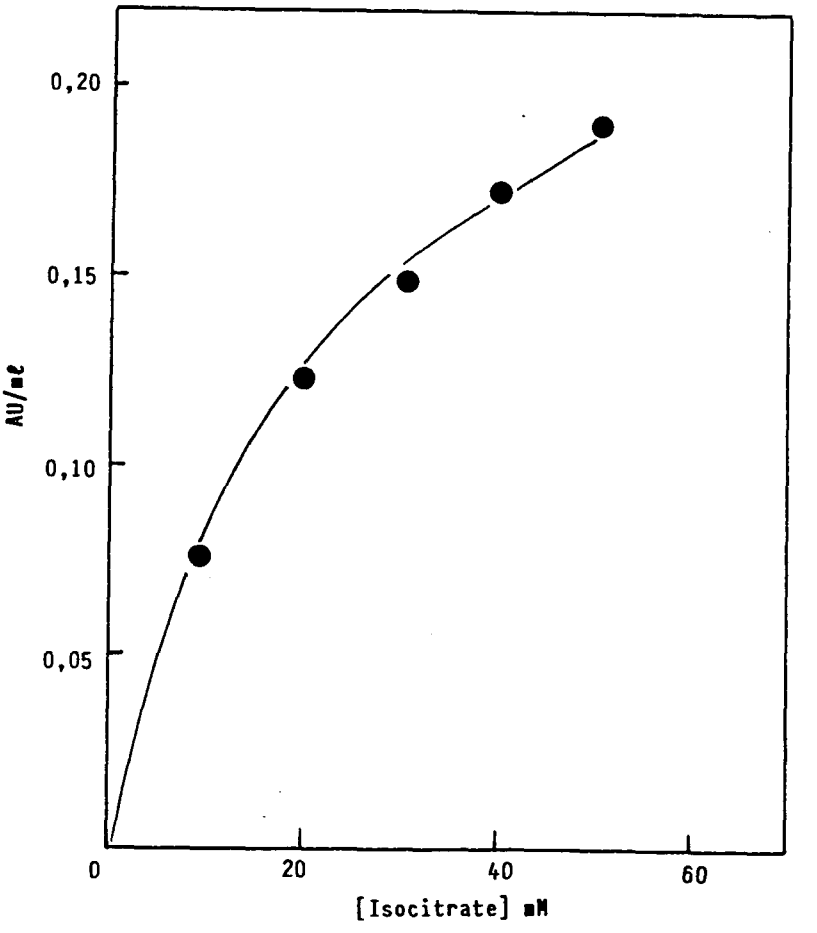


Figure 4.51 : The effect of different isocitrate concentrations on isocitrate dehydrogenase activity with 25 mM NADP as co-factor

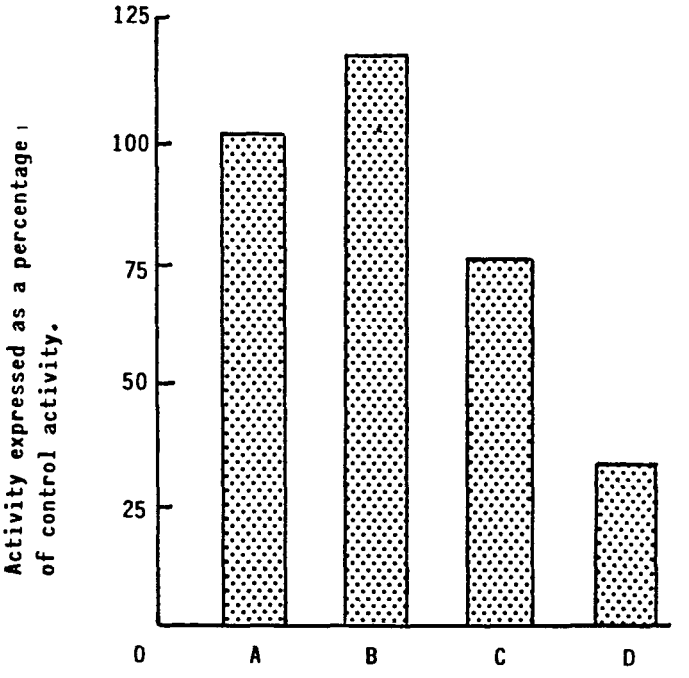


Figure 4.52 : The effect of certain metabolites on enzyme activity
A : Control; B : AMP ;
C : ATP : D : Oxaloacetate

The oxidation of isocitrate is the first step in the tricarboxylic acid cycle from where reduced nicotinamide cofactors are transferred to the electron transfer pathway for oxidation. During this oxidation process, ATP is manufactured.

The modifying effect of ATP and AMP possibly form an integral part of a feedback control mechanism which maintains the correct balance between the tricarboxylic acid cycle and the electron transfer pathway. Similar modifying effects by nucleoside phosphates have been observed in other bacteria (Marr and Weber, 1969; Parker and Weitzman, 1970).

Further research on this enzyme from Acinetobacter calcoaceticus var lwoffi is clearly essential in order to investigate the implications for biological phosphate removal.

CHAPTER FIVE

Conclusions and Recommendations

5.1 Polyphosphate Metabolism

The relationship between polyphosphate metabolism and other intracellular processes which have been observed for other micro-organisms (Mudd et al., 1958; Kaltwasser, 1962; Harold, 1963; Eigener and Bock, 1972), are also valid for Acinetobacter calcoaceticus var lwoffi. The oxygen level and the nature of the carbon source, both had an influence on polyphosphate synthesis and degradation. Polyphosphate was degraded in the presence of substrate in the absence of oxygen and accumulated under aerobic conditions. A graphical representation of this is shown in Figure 5.1.

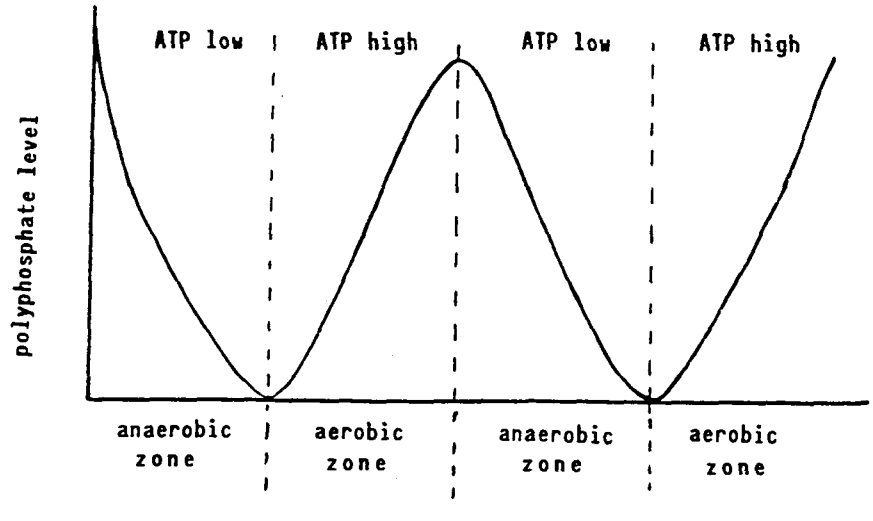


Figure 5.1 : Graphical representation of polyphosphate metabolism under aerobic and anaerobic conditions

The most important synthesis enzyme, polyphosphate kinase (Kornberg et al., 1956; Harold and Harold, 1965; Suresh et al, 1985), has been observed in the Acinetobacter calcoaceticus var lwoffi which accumulates polyphosphate, which coincides with the findings of T'Seyen et al. (1985), who observed the enzyme in unidentified Acinetobacter spp from activated sludge.

The modification of enzyme activity by the carbon growth source emphasizes the relationship between polyphosphate metabolism and other intracelluar processes.

5.2 Carbon Metabolism

Acetate uptake under anaerobic conditions was observed in the Acinetobacter calcoaceticus var lwoffi isolate. Under anaerobic conditions, polyhydroxybutyrate was accumulated, but during the subsequent aeration, the polyhydroxybutyrate was degraded. The same synthesis and degradation pattern was observed under sequential anaerobic and aerobic conditions in activated sludge plants (see Figure 5.2).

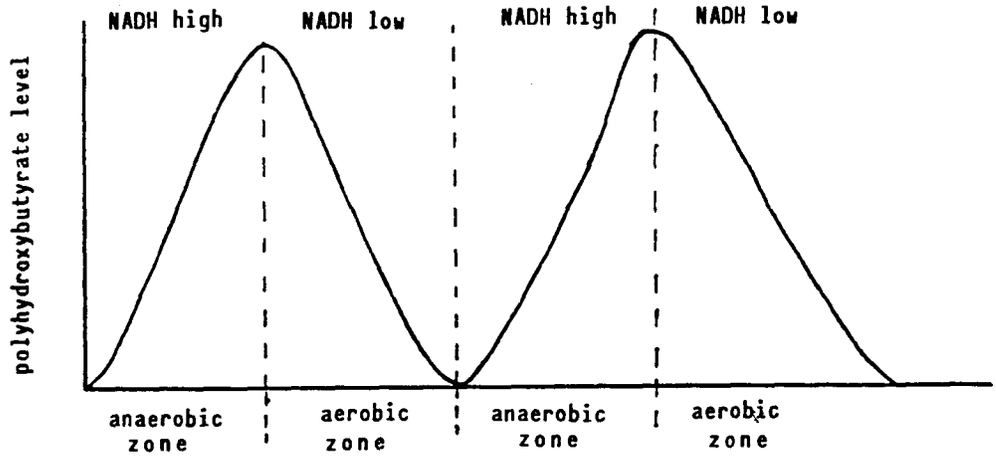


Figure 5.2 : Graphical representation of polyhydroxybutyrate metabolism under aerobic and anaerobic conditions

The presence of one of the key enzymes in polyhydroxybutyrate degradation, β-hydroxybutyrate dehydrogenase, was demonstrated in Acinetobacter calcoaceticus var lwoffi isolates from activated

sludge. This coincides with previous findings that micro-organisms which accumulate polyhydroxybutyrate possess this enzyme (Dawes and Senior, 1973).

Several metabolites had a modifying effect on enzyme activity, and it is clear that the enzyme is subject to feedback inhibition.

The same goes for the isocitrate dehydrogenase enzyme, where the activity was also modified by a number of metabolites.

5.3 Metabolic Control

The sequential exposure of the Acinetobacter cells to anaerobic and aerobic conditions did not exert a direct modifying effect on enzyme activity, in other words, enzyme synthesis was not altered and enzymes were not phosphorylated.

The statement of Krebs (1985), namely, that allosteric control is dependant on intracellular signals and covalent modification occurs as a result of extracellular signals, is borne out in this case. The intracellular environment in respect of nicotinamide adenine dinucleotides and the adenosine phosphates is directly dependant on the external oxygen level. The fluctuation in the levels of these metabolites is responsible for the fine metabolic control which is practised by this organism to survive sequential anaerobic/aerobic conditions.

5.4 Biochemical Model

In order to place the results of this study in perspective, it was decided to test the findings against the postulates of Wentzel et al. (1986). The anaerobic and the aerobic phases are discussed separately.

5.4.1 Anaerobic Phase

In this phase, no terminal electron acceptor is present and the organisms are in an environment which is relatively high in easily biodegradable substrate. As a result of these conditions, the NADH/NAD ratio rises and no oxidative phosphorylation occurs. This leads to a decrease in the ATP/ADP ratio. These changes in the NADH/NAD and ATP/ADP ratios exert a regulatory effect on the tricarboxylic acid cycle and polyphosphate degradation.

The activation of acetate to acetyl-CoA which requires ATP, reduces the ATP/ADP level so that polyphosphate degradation and consequent ATP synthesis is stimulated. The high concentration of acetyl-CoA and high NADH/NAD, stimulates polyhydroxybutyrate synthesis, during which NADH is used to reduce the NADH/NAD ratio so that the tricarboxylic acid cycle remains operational.

A summary of this hypothesis is that the aerobe Acinetobacter takes up acetate under anaerobic conditions and converts it to polyhydroxybutyrate. Energy for activation of the acetate is derived from polyphosphate degradation. The tricarboxylic acid cycle is sequentially stimulated and inhibited under these conditions by the alteration in the NADH/NAD ratio.

These studies show that a positive relationship exists between polyphosphate degradation and acetate uptake under anaerobic conditions.

The hypothesis of Wentzel et al. (1986), includes the concept of metabolic feedback control. The electrophoretic studies clearly show that the anaerobic phase does not result in protein modification in the form of phosphorylation or alteration in enzyme synthesis.

β-hydroxybutyrate dehydrogenase catalyses the penultimate step in PHB degradation to acetyl-CoA. Inhibition of this reaction will thus limit PHB degradation. Inhibition of this key degradation enzyme by NADH ensures domination of the synthesis reaction under anaerobic conditions.

5.4.2 Aerobic Phase

Although a terminal electron acceptor is present in this phase, the organisms are in a carbon-limiting environment. The organisms however, contained accumulated polyhydroxybutyrate. The active electron transfer pathway ensures a continued reduction in the NADH/NAD ratio under these conditions and the partial inhibition of the tricarboxylic acid cycle is removed. At the same time, polyhydroxybutyrate degradation is initiated and oxidative phosphorylation continues to cause the ATP/ADP ratio to increase. Polyphosphate synthesis then occurs.

These studies confirm the synthesis of polyphosphate under aerobic conditions after anaerobic substrate uptake. Polyhydroxybutyrate degradation under aerobic conditions has also been confirmed. Under these conditions the nicotinamide adenine dinucleotide is oxidised, which leads to the cessation of the inhibition by NADH. β-hydroxybutyrate dehydrogenase is also inhibited by acetyl-CoA and oxaloacetate. Acetyl-CoA enters the tricarboxylic acid cycle by a condensation reaction with oxaloacetate. The inhibitory effect of these two metabolites on PHB degradation indicates that if the tricarboxylic acid cycle is operating maximally, the degradation of polyhydroxybutyrate is not necessary.

5.4.3 The Activated Sludge Process

For a specific organism to dominate a given environment, it is essential that the organism has the competitive ability

to adapt successfully to the environment. In the case of activated sludge, the dominant organism will not only have to make effective use of the available nutrients, but will also have to form flocs to remain in the system (Toerien et al. 1987).

Acinetobacter has already shown the latter ability thoroughly (Buchan, 1983; Lötter and Murphy, 1986; Lötter et al., 1987). Concerning the effective use of nutrients, these studies have shown that Acinetobacter possesses strategies to take up substrate under unfavourable (lack of oxygen) conditions, and to convert it to an accumulation product which can later be used in a second, and probably also unfavourable (lack of external carbon) situation, as energy source which can maintain the active uptake of other essential nutrients like phosphate. Metabolic control by feedback mechanisms provides this organism with a method of rapidly reacting to environmental change.

The results of this study also provide fundamental reasons for operational observations, which are already well-known.

The presence of nitrate in the anaerobic zone, provides certain organisms with a terminal electron acceptor. Acetate will thus be used for denitrification and will not be converted to polyhydroxybutyrate. Synthesis of this accumulation product requires acetyl-CoA and high NADH levels, both of which will not be available under denitrifying conditions. The absence of accumulated carbon and thus an energy source reduces the organism's ability as a result of the lack of an energy source to take up phosphate and accumulate polyphosphate under aerobic conditions.

Polyphosphate accumulation is also dependant on stimulation of polyphosphate kinase, which occurs by simultaneous phosphate release and substrate uptake under anaerobic conditions.

The correct aeration of the aerobic zone is fundamental to the success of this process. Without sufficient oxygen, the electron transfer path and oxidative phosphorylation to form ATP, will not function satisfactorily. Polyhydroxybutyrate will not be degraded and the anaerobic process of acetate uptake will be deleteriously affected. Excess ATP conversion to polyphosphate will also not occur.

5.5 Plant Applications

The importance of substrate quality, denitrification and aeration in the biological phosphate removal process, was simultaneously confirmed by Johannesburg researchers. Fermentation of primary sludge linked to denitrification of the return sludge entering the anaerobic zone, considerably improved the efficiency of the recently completed Bushkoppie works (Pitman et al., 1987).

Similar improvements were observed at the Johannesburg Northern Works, when the return sludge underwent endogenous denitrification. Optimisation of fermentation in the primary settling tanks, to form volatile fatty acids, was successfully applied (Nicholls et al., 1987).

Although considerable progress has been made in the underlying processes and full-scale success of biological phosphate removal has been achieved, further research is essential in this area, in order to maintain wastewater treatment in South Africa on the level which is essential for the future expansion of the country's industries.

5.6 Recommendations for Future Work

- Current biochemical and mathematical models developed by the University of Cape Town and the City of Johannesburg, should be continually updated in the light of new findings, in order to retain these models at the forefront of research in this area.

The theoretical data generated by these models should be linked to operational aspects and then consolidated into a cohesive whole.

- Particulate COD contains a biodegradable component which must be utilised during the process. No research has been carried out into the optimal use of this carbon source, or its role in various processes prevailing in an activated sludge system.

- The removal of non-biodegradable COD has, like the utilisation of particulate COD, received little or no attention. Effluent COD levels in excess of the effluent standard have been observed to arise as a result of high levels of non-biodegradable COD in the influent. Methods to reduce effluent levels to within the standard should be investigated.

CHAPTER SIX

References

ABBOTT, B.J. (1973). Ethanol inhibition of a bacterium Acinetobacter calcoaceticus in chemostat culture. J. Gen. Microbiol. 75, 383 - 389.

ABBOTT, B.J., LASKIN, A.I. and McCOY, C.J. (1973). Growth of Acinetobacter calcoaceticus on ethanol. 25, 787 - 792.

AHRENS, J. and SCHLEGEL, H.G. (1966). Zür regulation der NAD-abhangigen hydrogenase aktivitat. Archiv. Mikrobiologie 55, 257 - 265.

ALLEN, L.A. (1944). The bacteriology of activated sludge. J. Hygiene 43, 424 - 431.

ALLISON, N., O'DONNEL, M.J. and FURSON, C.A. (1985). Membrane-bound lactate dehydrogenases and mandelate dehydrogenases of Acinetobacter calcoaceticus. Biochem. J. 231, 407 - 416.

ALPER, R., LUNDGREN, D.E., MARCHESSAULT, R.H., and COTE, W.A. (1963).Properties of Poly-β-hydroxybutyrate. 1. General considerations concerning the naturally occurring polymer. Biopolymers 1, 545 -556.

AMERICAN PUBLIC HEALTH ASSOCIATION (1981). Standard methods for the examination of water and wastewater (15th edition). American Public Health Association, Washington DC.

ANALYTLAB PRODUCTS (1977). Analytical Profile Index : Enterobacteriaceae and other gram negative bacteria.

ANDREWS, A.T. (1981) Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications. Clarendon Press. Oxford.

ARVIN, E. (1985). Biological removal of phosphorus from wastewater. CRC Critical Rev. in Environ. Contr. 15, 25 - 64.

ARVIN, E., HENZE, M., HOLM KRISTEN, G. and PETERSEN, G. (1985). A model for biological phosphorus removal based on biological and chemical mechanisms. Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment, Lisbon.

ARVIN, E. and KRISTENSEN, G.H. (1985). Exchange of organics, phosphate and cations between sludge and water in biological phosphorus and nitrogen removal processes. Wat. Sci. Tech. 17, 147 - 162.

ASPERGER, O., NAUMANN, A. and KLEBER, H. (1984). Inducibility of cytochrome P-450 in Acinetobacter calcoaceticus by n-alkanes. Appl. Microbiol. Biotechnol. 19, 398 - 403.

ASPERGER, O., STUWER, B. and KLEBER, H. (1985). Aryl hydrocarbons as inducers of cytochrome P-450 in Acinetobacter calcoaceticus. Appl. Microbiol. Biotechnol. 21, 309 - 312

ATKINSON, D.E. (1968). The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochem. 7, 4030 - 4034.

ATKINSON, D.E. (1971). Adenine nucleotides as stoichiometric coupling agents in metabolism and as regulatory modifiers : the adenylate energy charge. Metabolic regulation V (ed) H J Vogel, Academic Press, New York.

BARNARD, J.L. (1973). Biological denitrification. J. Wat. Poll. Contr. 72, 705 -720.

BARNARD, J.L. (1974). Cut P and N without chemicals. Water & Wastes Engineering 11, 33 - 36.

BARNARD, (1975).Nutrient removal in biological systems. J. Inst. Wat. Poll. Contr. 74, 59 -73.

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

BAUMANN, P., DOUDOROFF, M. and STANIER, R.Y. (1968). A study of the Moraxella group. J. Bacteriol. 95, 1520 - 1541.

BAXTER, M. and JENSEN, T.H. (1980). Uptake of magnesium, strontium, barium and manganese by Plectonema boryanum (Cyanophyceae). Protoplasm 104, 81 - 89.

BECCARI, M., DI PINTA, A.C.I., RAMADORI, R and TANDOI, V. (1985). Enhanced biological phosphorus removal in single sludge systems. Proc. International Conference on Management Strategies for phosphorus in the Environment, Lisbon.

BENEDICT, R.G. and CARLSON, D.A. (1971). Aerobic heterotrophic bacteria in activated sludge. Water Res. 5, 1023 - 1030.

BENNETT, R.L. and MALAMEY, M.H. (1970). Arsenate resistant mutants of Escherichia coli and phosphate transport. Biochem. Biophys. Res. Commun 40, 496 - 503.

BERGMEYER, H.U., GAWEHN, K., KLOTSCH, H., KREBS, H.A. and WILLIAMSON, D.H. (1967). Purification and properties of crystalline 3-hydroxybutyrate dehydrogenase from Rhodopseudomonas spheroides. Biochem. J. 102, 423 - 431.

BORTHWICK, A.C., HOLMS, W.H. and NIMMO, H.G. (1984). Isolation of active and inactive forms of isocitrate dehydrogenase from Escherichia coli ML308. Eur. J. Biochem. 141, 393 - 400.

BOVRE, K. and HENRIKSEN, S.D. (1976). Minimal standards for description of taxa within the genera Moraxella and Acinetobacter : Proposal by the subcommittee on Moraxella and allied bacteria. Int. J. of Systematic Bact. 26, 92 - 96.

BOYER, P.D. (1977). Coupling mechanisms in capture, transmission and use of energy. Ann. Rev. Biochem. 46, 957 - 966.

BRADFORD, M.M. (1976). A rapid and sensible method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248 - 254.

BRAUNEGG, G. SONNLEITER, B. and LAFFERTY, R.M. (1978).A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. Biotechnol. 6, 29 - 30.

BRODIE, A.F., HIRATA, H., ASANO, A., COHEN, N.S., HINDS, T.R., AITHAL, H.N. and KALRA, V.K. (1972). The relationship of bacterial membrane orientation to oxidative phosphorylation and active transport. Membrane Res. (ed) C Fred Fox, Academic Press, New York.

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

BROWN, C.M., MAC DONALD-BROWN, D.S. and MEERS, J.L. (1974). Physiological aspects of microbial inorganic nitrogen metabolism. Adv. Microbiol. Physiol. 11, 1 - 52.

BRYAN, B.A., LINHARDT, R.J. and DANIELS, L. (1986). Variation in composition and yield of exopolysaccharides produced by Klebsiella sp strains K32 and Acinetobacter calcoaceticus BD4. Appl. and Environ. Microbiol. 51, 1304 - 1308.

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

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

BURNELL, J.N., JOHN, P. and WHATLEY, F.R. (1975). Phosphate transport in membrane vesicles of Paracoccus denitrificans. FEBS Lett. 58, 215 - 218.

CANELLI, E. and MITCHELL, D.G. (1975). A semi-automated procedure for the determination of phosphorus in water, wastewaters and particulates. Water Res. 9, 1093.

CANOVAS, J.L., WHEELIS, M.L. and STANIER, R.Y. (1968). Regulation of the enzymes of the ℓ-ketoadipate pathway in Moraxella calcoacetica. Eur. J. Biochem. 3, 293 - 304.

CANOVAS. J.L. and JOHNSON, B.F. (1968). Regulation of the enzymes of the ℓ-ketoadipate pathway in Moraxella calcoaceticus. Eur. J. Biochem. 3, 312 - 317.

CECH, J.S. and CHUDOBA, J. (1983). Influence of accumulation capacity of activated sludge micro-organisms on kinetics of glucose removal. Water Res. 17, 659 - 666.

CHEN, M. (1974). Kinetics of phosphorus absorption by Corynebacterium bovis. Microbial Ecology 1, 164 - 175.

CHIEF DIRECTOR: TECHNICAL SERVICES, JOHANNESBURG. (1986). Annual report.

CLOETE, T.E. STEYN, P.L. and BUCHAN, L. (1985). An aut-ecological study of Acinetobacter in activated sludge. Wat. Sci. Tech. 17,139 - 146.

COMEAU, Y., HALL, K.J., HANCOCK, R.E.W. and OLDHAM, W.K. (1985). Biochemical model for enhanced biological phosphorus removal. Proceedings of University of British Columbia Conference on New Directions and Research in Waste Treatment and Residuals Management, Vancouver, Canada.

CUPPOLETTI, J. and SEGEL, I.H. (1975). Kinetics of sulphate transport by Penicillium notatum. Interactions of sulphate, protons, and calcium. Biochemistry 14, 4712 - 4718.

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

DE CROMBRUGGHE, B. and PASTAN, I. (1973). Regulation of gene expression by cyclic AMP in Escherichia coli. In Rate Control of Biological Processes, Society for Experimental Biology, University Press, Cambridge.

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

DE VRIES, H.P., VAN LOOSDRECHT, M. and RENSINK, J.H. (1985). New developments in biological excess phosphorus removal. H_2O 18, 358 - 362.

DIAS, F.F. and BHAT, J.V. (1964). Microbial ecology of activated sludge 1. Dominant bacteria. Appl. Microbiol. 12, 412 - 417.

DOLD, P.L., EKAMA, G.A. and MARAIS, G v R. (1980). A general model for the activated sludge process. Prog. Wat. Tech. 12, 47.

DOLD, P.L. and MARAIS, G v R. (1986). Evaluation of the general activated sludge model proposed by the IAWPRC task group. Wat. Sci. Tech. 18, 63 - 89.

DOLIN, M.E. and JUNI, E. (1978). Utilization of oxaloacetate by Acinetobacter calcoaceticus. Evidence for coupling between malic enzyme and malic dehydrogenase. J. Bacteriol. 133, 786 - 793.

DUBERY, I.A. (1985). Aspekte van die regulatoriese en fisies-chemiese eienskappe van fenielalanienammoniakliase van die vrugweefsel van Citrus sinensis. Ph. D. Thesis. Rand Afrikaans University.

DUINE, J.A., FRANK, J. and VAN ZEELAND, J.K. (1979). Glucose dehydrogenase from Acinetobacter calcoaceticus. FEBS Lett. 108, 443 - 446.

DU PREEZ, J.C., TOERIEN, D.F.and LATEGAN, P.M. (1981). Growth parameters of Acinetobacter calcoaceticus on acetate and ethanol. Eur. J. Appl. Microbiol. Biotechnol. 13, 45 - 53.

DU PREEZ, J.C., LATEGAN, P.M. and TOERIEN, D.F. (1984). Influence of the growth rate on the macromolecular composition of Acinetobacter calcoaceticus in carbon limited chemostat cluture. FEMS Microbiol.Lett. 23, 71 - 75.

DZANDU, J.K., DEH, M.E., BARRATT, D.L. and WISE, G.E. (1984). Detection of erythrocyte membrane proteins, sialoglycoproteins and lipids in the same polyacrylamide gel using a double-staining technique. Proc. Natl. Acad. Sci. 81, 1733 - 1737.

EIGENER, U. and BOCK, E. (1972). Auf-und Abbau der polyphosphat-fraktion in Zellen von Nitrobacter winogradskyi Buch. Arch. Mikrobiol. 81, 367 - 378.

ENSLEY, B.D. and FINNERTY, W.R. (1980). Influences of growth substrates and oxygen on the electron transport system in Acinetobacter sp HOl-N. J. Bacteriol. 142, 859 - 868.

FEWSON, C.A. (1985). Growth yields and respiratory efficiency of Acinetobacter calcoaceticus. J. Gen. Microbiol. 131, 865 - 872.

FISCHER, B., CLAUS, R. and KLEVER, H.P. (1984). Lokalization der pyridennukleotide-abhangigen alkohol dehydrogenase und der NADP+ abhangigen aldehyd dehydrogenase in Acinetobacter calcoaceticus 69/V. Zeitschrift für Algemeine Mikrobiologie 24, 587 - 590.

FLORENTZ, M. and HARTEMANN, P.(1982). Enzymatic study of activated sludge in aerobic-anaerobic run. Env. Tech. Lett. 3, 345 - 350.

FOCHT, D.D. and CHANG, A.C. (1975). Nitrification and denitrification processes related to wastewater treatment. Appl Microbiol 19, 153 - 182.

FOUNDATION FOR RESEARCH DEVELOPMENT (1985). The limnology of Hartbeespoort Dam. SA National Scientific Programmes Report No 110.

FRICKE, B., BERGMAN, R., SORGER, H. and AURICH, H. (1982). Optimierung van Kulturbedingungen für Acinetobacter calcoaceticus buim wachstum aur n-alkanen in einen laborfermentor. Zeitschrift fur Algemeine Mikrobiol. 22, 365 - 372.

FRIEDBERG, I. and AVIGAD, G. (1968). Structures containing polyphosphate in Micrococcus lysodeikticus. J. Bacteriol. 96, 544 - 553.

FRIEDMAN, B.A., DUGAN, P.R., PFISTER, R.M. and REMSEN, C.C. (1969). Structure of exocellular polymers and their relationship to bacterial flocculation. J. Bacteriol. 98, 1328 - 1334.

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

FUKASE, T., SHIBATA, M. and MIYAJI, (1982). Studies on the mechanism of biological phosphorus removal. Japan J. Water Poll. Res. 5, 309 - 317.

GABRIEL, O. (1971). Locating enzymes on gels. Methods for Enzymology 22, 578 - 604.

GARNAK, M. and REEVES, H.C. (1979). Phosphorylation of isocitrate dehydrogenase of Escherichia coli. Science 203, 1111 - 1112.

GAUDY, A.F. and GAUDY, E.T. (1981). Microbiology for environmental scientists and engineers. Mc Graw-Hill, International Book Co, London.

GERBER, A. and WINTER, C.T. (1985). The influence of extended anaerobic retention time on the performance of Phoredox nutrient removal plants. Wat. Sci. Tech. 17 81 -92.

GERBER, A., MOSTERT, E.S., WINTER, C.T. and DE VILLIERS, R.H. (1986). The effect of acetate and other short chain carbon compounds on the kinetics of biological nutrient removal. Water SA 12, 7 - 12.

GERBER, A. MOSTERT, E.S. WINTER, C.T. DE VILLIERS, R.H. (1987). Interactions between phosphate, nitrate and organic substrate in biological nutrient removal processes. Wat. Sci. Tech. 19, 183 -194.

GOLDMAN, S., SHABTAI, Y., RUBINOVITZ, C., ROSENBERG, E and GUTNICK, D.L. (1982). Emulsan in Acinetobacter calcoaceticus RAG-1 : Distribution of cell free and cell associated cross reacting material. Appl. Environ. Microbiol. 44, 165 -170.

GOVERNMENT GAZETTE. (1956). Water Act. Notice R1567.

GOVERNMENT GAZETTE. (1980). Amendment of Standards for Industrial wastewaters and effluents. Notice R1567.

GRAY, C.T., WIMPENNY, J.W.T. and MOSSMAN, R. (1966). Regulation of metabolism in facultative bacteria. Biochim. Biophys. Acta 117, 33 - 41.

GROBLER, D.C. and SILBERBAUER, M J. (1984). Impact of eutrophication measures on the trophic status of South African impoundments. Report to the Water Research Commission of South Africa.

GURR, E. (1973). Biological staining methods. G D Searle (Pty) Ltd, Searle Diagnostic.

GUTOWSKI, S. and ROSENBERG, H. (1975). Succinate uptake and related proton movements in Escherichia coli K12. Biochem. J. 152, 647 - 654.

HARDY, G.A. and DAWES, E.A. (1985). Effect of oxygen concentration on the growth and respiratory efficiency of Acinetobacter calcoaceticus. J. Gen. Microbiol. 131, 855 - 864.

HAROLD, F.M. (1960). Accumulation of inorganic polyphosphate in mutants of Neorospora crassa. Biochim. Biophys.Acta 45, 172 - 188.

HAROLD, F.M. (1962).Depletion and replenishment of the inorganic phosphate pool in Neurospora crassa. J. Bacteriol. 83, 1047 - 1057.

HAROLD, F.M. (1963). Accumulation of inorganic polyphosphate in Aerobacter aerogenes. J. Bact. 86, 216 - 221.

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

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

HAROLD, F.M. (1974). Chemiosmotic interpretation of active transport in bacteria. Ann. N. Y. Acad. Sci. 227, 297 - 311.

HAROLD, F.M. (1977). Membranes and energy transduction in bacteria. Curr. Topics Bioenergetics 6, 83 - 149.

HAROLD, F.M., BAARDA, J.R. and PAVLASOVA, E. (1970). Extrusion of sodium and hydrogen ions as the primary process in potassium ion accumulation by Streptococcus faecalis. J. Bacteriol. 101, 152 - 159.

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

HAROLD, F.M. and PAPINEAU, D. (1972). Cation transport and electrogenesis by Streptococcus faecalis. J. Membrane Biol. 8, 45 - 62.

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

HAROLD, F.M. and SYLVAN, S. (1963). Accumulation of inorganic polyphosphate in Aerobacter aerogenes. J. Bacteriol. 86, 222 - 231.

HARRIS, R.H. and MITCHELL, R. (1973). The role of polymers in microbial aggregation. Ann. Rev. Microbiol. 27, 27 - 50.

HARRISON, D.E.F. (1972). Physiological effects of dissolved oxygen tension and redox potential on growing populations of micro-organisms. J. Appl. Chem. Biotechnol. 22, 417 - 440.

HART, R.C. and ALLANSON, B.R. (red). (1984). Limnological criteria for management of water quality in the southern hemisphere. SA National Scientific Programmes Report No 93.

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

HASCOET, M.C. and FLORENTZ, M. (1985). Influence of nitrates on biological phosphorus removal from wastewater. Water SA 11, 1 - 8.

HASSID,W.Z. (1970) Biosynthesis of sugars and polysaccharides. In "The Carbohydrates" (ed) W. Pigman and D. Horton. Academic Press. New York.

HAUGE, J.G. and HALBERG, P.A. (1956). Solubilization and properties of the structurally bound glucose dehydrogenase of B. Anitratum (A. calcoaceticus). Biochim. Biophys. Acta, 81, 251 - 256.

HAUGE, J.G. and MÜRER, E.J. (1964). Studies on the nature of the prosthetic group of glucose dehydrogenase of Bacterium anitratum. Biochim. Biophys. Acta 81, 244 - 250.

HEEFNER, D.L. and HAROLD, F.M. (1982). ATP-driven sodium pump in Streptococcus faecalis. Proc. Natl. Acad. Sci. 79, 2798 - 2802.

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

HENRIKSEN, S.D. (1976). Moraxella, Neisseria, Branhamella and Acinetobacter. Ann. Rev. Microbiol. 30, 63 - 83.

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

HILLS, C.S. and FEWSON, C.A. (1983). Regulation of expression of novel mandelate dehydrogenase in mutants of Acinetobacter calcoaceticus. J. Gen. Microbiol. 129, 2009 - 2015.

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

HOFMANN, F. (1982). Regulation of cellular functions by phosphorylation and dephosphorylation of proteins : An introduction. Cell Regulation by Intracellular Signals (ed) S Swillens & J E Dumont, Plenum Press, New York.

HORWITZ, W. (ED) (1980). Official methods of analysis. 13de uitgawe. Association of Official Analytical chemists.

HUGHES, D.E. and WIMPENNY, J.W.T. (1969). Oxygen metabolism by micro-organisms. Adv. Microbiol. Physiol. 3, 197 - 232.

INGLEDEW, W.M., TRESGUERRES, E.F. and CANOVAS, J.L. (1971). Regulation of enzymes of the hydroaromatic pathway in Acinetobacter calcoaceticus. J. Gen. Microbiol. 68, 273 - 282.

JAIN, M.K. and WAGNER, R.C. (1980). Passive facilitated diffusion. In : Introduction to Biological Membranes. John Wiley and Sons, New York.

JASEWICZ, L. and PORGES, N. (1956). Biochemical oxidation of dairy wastes. VI : Isolation and study of sludge micro-organisms. Sew. Ind. Wastes 28, 1130 - 1136.

JIRKA, A.M., CARTER, M.J., MAY, D. and FULLER, F.D. (1976). Ultra-micro semi-automated method for simultaneous determination of total phosphorus and total kjeldahl nitrogen in wastewaters. Environ. Sci. Technol. 10, 1038 - 1042.

JOHNSON, D.E. and HANSON, R.S. (1974). Bacterial citrate synthases purification, molecular weight and kinetic mechanism. Biochim. Biophys. Acta 350, 336 - 353.

JONES, P.H., TADWALKER, A. and HSU, C.L. (1985). Studies in the enhanced uptake of phosphorus by activated sludge. Proceedings of University of British Columbia, Conference on New Directions and Research in Waste Treatment and Residuals Management, Vancouver,Canada.

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

JUNI, E. (1984). Acinetobacter. In : Bergey's Manual of Systematic Bacteriology Vol 1, (ed) N R Krieg and J G Holt, Williams and Wilkins, Baltimore.

JYSSUM, K. and JONER, P.E. (1965). Regulation of the nitrogen assimilation from nitrate and nitrite in bacterium anitratum. Acta Path. Microbiol. Scand. 64, 387 - 397.

JYSSUM, K. and JONER, P.E. (1966). Hydroxylamine as a possible intermediate in nitrate reduction by Bacterium anitratum. Acta Path. Microbiol. Scand. 67, 139 - 148.

KABACK, H.R. (1968). The role of the phosphoenol pyruvate-phophotransferase system in the transport of sugars by isolated membrane preparations of Escherichia coli. J. Biol. Chem. 243, 3711 - 3724.

KALTWASSER, H. (1962). Die rolle der polyphosphate im phosphat stoffwechsel eines knallgasbacteriums (Hydrogenomonas Stamm 20). Archiv für Mikrobiol. 41, 282 - 306.

KATAGIRI, M. and WHEELIS, M.L. (1971). Comparison of the two iso-functional enol-lactone hydrolases from Acinetobacter calcoaceticus. J. Bacteriol. 106, 369 - 374.

KAY, W.W. (1972). Genetic control of the metabolism of propionate by Escherichia coli K12. Biochim. Biophys. Acta 264, 508 - 521.

KELL, D.B., PECK, M.W., RODGER, G. and MORRIS, J.G. (1981). On the permeability to weak acids and bases of the cytoplasmic membrane of Clostridium pasteurianum. Biochim. Biophys. Res. Commun. 99, 81 - 88.

KERDACHI, D.A. and ROBERTS, M.R. (1983). Further developments in the understanding of phosphate removal at Umhlatuzana. IMIESA Sept, 32 - 43.

KOBAYASHI, M., VAN BRUNT, J. and HAROLD, F.M. (1978). ATP-linked calcium transport in cells and membrane vesicles of Streptococcus faecalis. J. Biol. Chem. 253, 2085 - 2092.

KONINGS, W.N., HELLINGWERF, K.J. and ROBILLARD, G.T. (1981). Transport across bacterial membranes. In : Membrane Transport, (ed) S L Bonting and J J H de Pont, Elsevier, North Holland, Biomedical Press, Amsterdam.

KORNBERG, A., KORNBERG, S.R. and SIMMS, E.S. (1956). Metaphosphate synthesis by an enzyme from E. coli. Biochim. Biophys. Acta 20, 215 - 227.

KREBS, E.G. (1985). The phosphorylation of proteins : a major mechanism for biological regulation. Biochem. Soc. Trans. 13, 813 - 820.

KREBS, G. and BEAVO, J.A. (1979). Phosphorylation - dephosphorylation of enzymes. Ann. Rev. Biochem. 48, 923 - 959.

KULAEV, I.A. (1975). Biochemistry of inorganic polyphosphates. Rev. Physiol. Biochem Pharmacol. 73, 131 - 158.

KULAEV, I.S., BOBYK, M.A., NIKOLAEV, N.N.M SERGEEV, N.S. and URYSON, S.O. (1971). Polyphosphate synthesizing enzymes in some fungi and bacteria. Biokhimiya. 36, 943 - 949.

KULAEV, I.S. and VAGABOV, V.M. (1983). Polyphosphate metabolism in micro-organisms. Adv. in Microbial Physiology 24, 83 - 171.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227, 680 - 685.

LAIMINS, L.A., RHOADS, D.B., ALTENDORF, K. and EPSTEIN, W. (1978). Identification of the structured proteins of ATP-driven potassium transport system in Escherichia coli. Proc. Natl. Acad. Sci. 75, 3216 - 3219.

LAN, J.C., BENEFIELD, L. and RANDALL, C.W. (1983). Phosphorus removal in the activated sludge process. Wat. Res. 17, 1193 - 1200.

LANGLEY, W.D., DAVIS, W.B. and RICHARDS, P.A. (1968). The decomposition of aliphatic esters by aerobic micro-organisms. Proc 23rd Ind Waste Conf Purdue University, 229 - 234.

LARNER, J. (1971). Intermediary metabolism and its regulation. Foundation of Modern Biochemistry Series, Prentice-Hall Inc, New Jersey.

LASKEY, R.A. and MILLS, A.D. (1977). Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. FEBS Lett. 82, 314 - 316.

LAW, B.A., ANDREWS, A.T. and SHARPE, M.E. (1977). Gelation of ultra-high-temperature-sterilized milk by proteases from a strain of Pseudomonas fluorescens isolated from raw milk. J Dairy Res 44, 145 - 148.

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

LEVIN, G.V., TOPOL. G.J., TORNAY, A.G. and SAMWORTH, R.B. (1972). Pilot plant tests of a phosphate removal process. J. Wat. Poll Contr. Fed. 44, 1940 - 1954.

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

LEVINSON, S.L., JACOBS, L.H., KRULWICH, T.A. and LI, H.C. (1975). Purification and characterisation of a polyphosphate kinase from Arthrobacter atrocyaneus. J. Gen. Microbiol. 88, 65 - 74.

LI, H.C. and BROWN, G.G. (1973). Orthophosphate and histone dependent polyphosphate kinase from E. coli. Biochim. Biophys. Res. Commun. 53, 875 - 881.

LLOYD, D., PROTHEROE, R., WILLIAMS, T.N. and WILLIAMS, J.L. (1983). Adaptation of the respiratory system of Acanthamoeba castelloni to anaerobiosis. FEMS Microbiol. Lett. 17, 143 - 146.

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

LöTTER,L.H. (1987) The Biochemistry of the Bardenpho process. Proceedings of the Institute of Water Pollution Control Biennial Conference. Port Elizabeth.

LöTTER, L.H. and DUBERY, I.A. (1987). Metabolic control in polyphosphate accumulating bacteria and its role in enhanced biological phosphate removal. Adv. Wat. Poll. Contr. 7 - 14 (ed) R Ramadori IAWPRC Conference Series, Pergamon Press.London.

LöTTER, L.H. and MURPHY, M.(1987). Microscopic evaluation of carbon and phosphorus accumulation in nutrient removal activated sludge. Wat. Sci. Tech. 20 37 - 49.

LöTTER, L.H. and MURPHY, M. (1985). The identification of heterotrophic bacteria in an activated sludge plant with particular reference to polyphosphate accumulation. Water SA 11, 179 - 184.

LöTTER, L.H. and VAN DER MERWE, E.H.M. (1987). The activities of some fermentation enzymes in activated sludge and their relationship to enhanced phosphorus removal. Wat. Res. 21 1307 - 1310.

LöTTER, L.H., WENTZEL, M.C., EKAMA, G.A. and MARAIS, G v R. (1986). An investigation into the heterotrophic bacterial population of various activated sludge plants. Water SA Manuscript in preparation.

MCKINNEY, R.E. and HORWOOD, M.P. (1952). Fundamental approach to the activated sludge process. Sewage and Industrial Wastes 24, 117 - 123.

MACRAE, R.M. and WILKINSON, J.F. (1958). Poly-β-hydroxybutyrate metabolism in washed suspensions of Bacillus cereus and Bacillus megaterium. J. Gen. Microbiol. 19, 210 - 222.

MAHLER, H.R. and CORDES, E.H. (1971). Biological chemistry. Harper & Row, New York.

MALNOU, D., MEGANCK, M., FOUP, G.M. and DU ROSTU, M. (1984). Biological phosphorus removal : A study of the main parameters. Wat. Sci. Tech. 16, 173 - 185.

MARAEVA, O.B., KOLOT, M.N., NESMEYANOVA, M.A. and KULAEV, I.S. (1979). Interrelationships between metabolic and genetic regulation of alkaline phosphatase and poly- and pyrophosphate. Biokhimiya 44, 715 - 719.

MARAIS, G v R. and EKAMA, G.A. (1976). The activated sludge process. Part 1 : Steady state behaviour. Water SA 2, 163 - 200.

MARAIS, G.R. and EKAMA, G.A. (1982). Developments in the activated sludge process. IMIESA Nov 39 - 57.

MARAIS, G v R., LOEWENTHAL, R.E. and SIEBRITZ, I.P. (1983). Reviews : Observations supporting phosphorus removal by biological excess uptake. Wat. Sci. Technol. 15, 15 - 41.

MARR, J.J. and WEBER, M.M. (1969). Concerted inhibition of a NADP specific isocitrate dehydrogenase and the implications for metabolic regulation. Biochem. Biophys. Res. Commun. 35, 12 - 19.

MEDVECZKY, N. and ROSENBERG, H. (1971). Phosphate transport in Escherichia coli. Biochim. Biophys. Acta, 241, 404 - 506.

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

MENAR, A.B. and JENKINS, D. (1970). Fate of phosphate in waste treatment processes enhanced removal of phosphate by activated sludge. Environ. Sci. & Technol. 4, 1115 - 1121.

METCALF and EDDY INC. (1979). Wastewater engineering : Treatment, disposal, re-use. Mc Graw Hill Book Co, New York.

MILBURY, W.F., MC CAULEY, D. and HAWTHORNE, C.H. (1971). Operation of conventional activated sludge for maximum phophorus removal. J. Wat. Poll. Contr. Fed. 43, 1890 - 1901.

MINO, J., KAWAKAMI, T. and MATSUO, T. (1985). Behaviour of intracellular polyphosphate in the biological phosphate removal process. Wat. Sci. Tech. 17, 11 - 21.

MITCHELL, P. (1968). Chemiosmotic coupling and energy transduction. Glyn Research Ltd., Bodmin, England.

MITCHELL, P. (1977). A commentary on alternative hypotheses of protonic coupling in the membrane systems catalysing oxidative oxidative and photosynthetic phosphorylation. FEBS Lett. 78, 1 - 20.

MUDD, S., YOSHIDA, A. and KOIKE, M. (1958). Polyphosphate as accumulator enzyme from phosphorus and energy. J. Bacteriol. 75, 224 - 235.

MUHAMMED, A. (1961). Studies on biosynthesis of polymetaphosphate by an enzyme from Corynebacterium xerosis. Biochim. Biophys. Acta 54, 121 - 132.

MUHAMMED, A. RODGERS, A. and HUGHES, D.E. (1962). Purification and properties of polyphosphatase from Corynebacterium xerosis. J. Gen. Microbiol. 20, 482 - 496.

MUHLRADT, P.F. (1971). Synthesis of high molecular weight polyphosphate with a partially purified enzyme from Salmonella. J. Gen. Microbiol. 68, 115 - 122.

MUKKADA, A.J. and BELL, E.J. (1969). Partial purification and properties of the fructose; 1,6-diphosphatase of A lwoffi. Arch Biochem 142, 22 - 31.

MULLER, R.H. and BABEL, W. (1986). Glucose as an energy donor in acetate growing Acinetobacter calcoaceticus. Arch. of Microbiol. 144, 62 - 66.

MULLER, H., NAUMANN, A., CLAUS, R. and KLEBER, H.P. (1983). ntracytoplasmic membrane induction by hexadecane in Acinetobacter calcoaceticus. Zeitschrift für Algemeine. Mikrobiol. 23, 645 - 651.

MURATA, K., UCHIDA, T., TANI, D., KATO, J. and CHIBATA, I. (1980). Metaphosphate : A new phosphoryl donor for NAD phosphorylation. Agric. Biol. Chem. 44, 61 - 68.

MURPHY, M. and LöTTER, L.H. (1986). The effect of acetate and succinate on polyphosphate formation and degradation in activated sludge with particular reference to Acinetobacter calcoaceticus. Appl. Microbiol. Biotechnol. 24, 512 - 517.

NESMEYANOVA, M.A., DMITRIEV, A.D.and KULAEV, I.S. (1973). High molecular weight polyphosphates and enzymes of polyphosphate metabolism in the process of E. coli growth. Mikrobiologiya 42, 213 - 219.

NESMEYANOVA, M.A., DMITRIEV, A.D. and KULAEV, I.S. (1974). Regulation of the enzymes of phosphorus metabolism and the level of polyphosphate in E.coli K-12 by exogenous O-PO_4. Mikrobiologiya 43, 227 - 234.

MURATA, K., UCHIDA, T., TANI, D., KATO, J. and CHIBATA, I. (1980). Metaphosphate : A new phosphoryl donor for NAD phosphorylation. Agric. Biol. Chem. 44, 61 - 68.

MURPHY, M. and LöTTER, L.H. (1986). The effect of acetate and succinate on polyphosphate formation and degradation in activated sludge with particular reference to Acinetobacter calcoaceticus. Appl. Microbiol. Biotechnol. 24, 512 - 517.

NESMEYANOVA, M.A., DMITRIEV, A.D.and KULAEV, I.S. (1973). High molecular weight polyphosphates and enzymes of polyphosphate metabolism in the process of E. coli growth. Mikrobiologiya 42, 213 - 219.

NESMEYANOVA, M.A., DMITRIEV, A.D. and KULAEV, I.S. (1974). Regulation of the enzymes of phosphorus metabolism and the level of polyphosphate in E.coli K-12 by exogenous O-PO_4. Mikrobiologiya 43, 227 - 234.

NEUFELD, R.J., ZAJIC, J.E. and GERSON, D.F. (1983). Growth characteristics and cell partitioning of Acinetobacter on hydrocarbon substrates. J. Ferment. Technol. 61, 315 -321.

NEUFELD, R.J. and ZAJIC, J.E. (1984). The surface activity of Acinetobacter calcoaceticus sp 2CAZ. Biotechnol. Bioeng. 26, 1108 - 1113.

NICHOLLS, H.A. (1975). Full scale experimentation on the new Johannesburg extended aeration plants. Water SA 1, 121 - 132.

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

NICHOLLS, H.A., PITMAN, A.R. and OSBORN, D.W. (1985). The readily biodegradable fraction of sewage its influence on phosphorus removal and measurement. Wat. Sci. Tech. 17, 73 - 87.

NICHOLLS, H.A. OSBORN, D.W. and PITMAN, A.R. (1987). Improvement to the stability of the biological phosphorus removal process at the Johannesburg Northern Works. Adv. Wat. Poll. Contr. 261 - 272. (red) R. Ramadori. IAWPRC Conference Series. Pergamon Press. London.

NIMMO, G.A., BORTHWICK, A.C., HOLMS, W.H. and NIMMO, H.G. (1984). Partial purification and properties of isocitrate dehydrogenase kinase/ phosphatase from Escherichia coli ML308. Eur. J. Biochem. 141, 401 - 408.

NOEGEL, A. and GOTSCHLICH, E.C. (1983). Isolation of a high molecular weight polyphosphate from Neisseria gonorrhoeae. J. Exp.. Med. 157, 2049 - 2060.

OEDING, V. and SCHLEGEL, H.G. (1973). β-ketothiolase from Hydrogenomonas eutropha H16 and its significance in the regulation of poly-β-hydroxy-butyrate metabolism. Biochem. J. 134, 239 - 248.

OHTAKE, H., TAKAHASHI, K., TSUZUKI, Y. and TODA, K. (1984). Phosphorus release from a pure culture of Acinetobacter calcoaceticus under anaerobic conditions. Environ. Technol. Letters 5, 417 - 424.

OSBORN, D.W., LöTTER, L.H., PITMAN, A.R. and NICHOLLS, H.A. (1986). Enhancement of biological phosphate removal by altering process feed composition. Report to the Water Research Commission of South Africa : WRC 137/1/86.

OSBORN, D.W., LöTTER, L.H., PITMAN, A.R. and NICHOLLS, H.A. (1989). Enhancement of biological phosphate removal by altering process feed composition. (Plant and Laboratory Studies). Report to the Water Research Commission of South Africa : WRC 137/2/89.

OSBORN, D.W. and NICHOLLS, H.A. (1978). Optimisation of the activated sludge process for the biological removal of phosphorus. Prog. Wat. Tech. 10, 261 - 277.

OXENDER, D.L. (1972). Membrane transport. Ann. Rev. Biochem. 41, 777 - 814.

PACKETT, L.V. and MC CUNE, R.W. (1965). Determination of steam volatile organic acids in fermented media by gas liquid chromatography. Appl. Microbiol. 13, 22 - 27.

PADAN, E. ZILBERSTEIN,D. and SCHULDINER, S. (1981). pH Homeostasis in bacteria. Biochim. Biophys. Acta. 650, 151 - 166.

PAGEL, J.E. and SEYFRIED, P.L. (1976). Numerical taxonomy of aquatic Acinetobacter isolates. J. Gen. Microbiol. 95, 220 - 232.

PAINTER, H.A. (1970). A review of literature on inorganic nitrogen metabolism in micro-organisms. Wat. Res. 4, 393 - 450.

PARDIE, A.B.and PALMER, L.M. (1973). Regulation of transport systems : A means of controlling metabolic rates. Rate Control of Biological Processes, Society of Experimental Biology, University Press, Cambridge, 1973.

PARK, M.H. WONG, B.B. and LUSK, J.E. (1976). Mutants in three genes affecting transport of magnesium in Escherichia coli: genetics and physiology. J. Bacteriol. 126, 1096 - 1103.

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

PEPIN, C.A. and WOOD, H.G. (1986). Polyphosphate glucokinase from Propionibacterium shermanii. Kinetics and demonstration that the mechanism involves both processive and non-processive type reactions. J. Biol. Chem. 261, 4476 - 4480.

PIKE, E.B., CARRINGTON, E.G. and ASHBURNER, P.A. (1972). An evaluation of procedures for enumerating bacteria in activated sludge. J. Appl. Bacteriol. 35, 309 - 321.

PINES, O. and GUTNICK, D. (1986). Role of emulsan in growth of Acinetobacter calcoaceticus RAG-1 on crude oil. Appl. Environ. Microbiol. 51, 661 - 663.

PINES, O., BOYER, E.A. and GUTNICK, D.L. (1983).Localization of emulsan-like polymers associated with the cell surface of Acinetobacter calcoaceticus. J. Bacteriol. 154, 893 - 905.

PITMAN, A.R. (1983). Optimisation of the modified activated sludge process for nutrient removal. Project 61/1 Final Report to the Water Research Commission.

PITMAN, A.R., VENTER, S.L.V. and NICHOLLS, H.A. (1983). Practical experience with biological phosphorus removal plants in Johannesburg. Wat. Sci. Technol. 15, 233 - 259.

PITMAN, A.R TRIM, B.C. and VAN DALSEN, L. (1987). Operating experience with biological nutrient removal at the Johannesburg Bushkoppie Works. Wat. Sci. Tech. In Press.

POOLE, J.E.P. (1984). A study of the relationship between the mixed liquor fauna and plant performance for a variety of activated sludge sewage treatment works. Water Res. 18, 281 - 287.

POSTMA, P.W. and ROSEMAN, S. (1976). The bacterial phosphoenolpyruvate : sugar phophotransferase system. Biochim. Biophys. Acta 457, 213 - 257.

POTGIETER, D.J.J. and EVANS, B.W. (1983). Biochemical changes associated with luxury phosphate uptake in a modified phoredox activated sludge system. Wat. Sci. Tech. 15, 105 - 115.

PRAKASAM, T.B.S. and DONDERO, N.C. (1967). Aerobic heterotrophic bacterial populations of sewage and activated sludge : III Adaptation in a synthetic waste. Appl. Microbiol. 15, 1128 - 1137.

PRAKASAM, T.B.S. and DONDERO, N.C. (1970). Aerobic heterotrophic bacterial populations of sewage and activated sludge : V analysis of population structure and activity. Appl. Microbiol. 19, 671 - 680.

PROCOS, J. (1961). Modification of the spectrophotometric determination of ketone bodies in blood enabling the total recovery of beta-hydroxybutyric acid. Clinical Chem. 7, 97 - 106.

RABINOWITZ,C. GUTNICK, D.L. and ROSENBERG, E.(1982). Emulsan production by Acinetobacter calcoaceticus in the presence of Chloramphenicol. J. Bacteriol. 152, 126 - 132.

RABINOWITZ, B. and OLDHAM, W.K. (1985). The use of primary sludge fermentation in the enhanced biological phosphorus removal process. Proceedings of University of British Columbia Conference on New Directions and Research in Waste Treatment and Management, Vancouver, Canada.

RAMOS, S. and KABACK, H.R. (1977). The relationship between the electrochemical proton gradient and active transport in Escherichia coli membrane vesicles. Biochem. 16, 854 - 859.

RAO, N.N., ROBERTS, M.F. and TORRIANI, A. (1985). Amount and chain length of polyphosphates in Escherichia coli depend on cell growth conditions. J. Bacteriol. 162, 242 - 247.

REBER, H.H. (1982). Inducibility of benzoate oxidizing cell activities in Acinetobacter calcoaceticus strain BS5 by chlorobenzoates as influenced by the position of chlorine atoms and the inducer concentration. Eur. J. Microbiol. Biotechnol. 15, 138 - 140.

REBER, H.H. and THIERBACH, G. (1980). Physiological studies on the oxidation of 3-chlorobenzoate by Acinetobacter calcoaceticus strain BS5. Eur. J. Appl. Microbiol. Biotechnol. 10, 223 - 233.

REEVES, H.C., O'NEIL, S. and WEITZMAN, P.D.J. (1983). Modulation of isocitrate dehydrogenase activity in Acinetobacter calcoaceticus by acetate. FEBS Lett. 163, 265 - 268.

REEVES, H.C., O'NEIL, S. and WEITZMAN, P.D.J. (1986). Changes in NADP-isocitrate dehydrogenase isoenzyme levels in Acinetobacter calcoaceticus in response to acetate. FEMS Microbiol. Lett. 35, 229 - 232.

RENSINK, J.H. (1981). Biologische defosfatering en procesbepalende factoren. In Symp. Boek, Defosfatering Niewe Ontwikkelingen en Praktijkervaringen in Nederland en Zweden NVA Symp.

RENSINK, J.H., DONKER, H.J.G.W. and DE VRIES, H.P. (1981). Biological P-removal in domestic wastewater by the activated sludge process. Proceedings of 5th European Sewage and Refuse Symposium EAS Munich, Gesellschaft zür Fordeung der Abwassertechnik V St Augustin, 487 - 498.

RICKARD, M.D. and GAUDY, A.F. (1968). Effect of oxygen tension on O_2 uptake and sludge yield in completely mixed heterogeneous populations. Proceedings, 23rd Industrial Waste Conference, Purdue University 883 - 893.

RITCHIE, G.A.F., SENIOR, P.J. and DAWES, E.A. (1971). The purification and characterisation of acetoacetyl-coenzyme A reductase from Azotobacter beijerinckii. Biochem. J. 121, 309 - 316.

ROBINSON, N.A., GOSS, N.H. and WOOD, H.C. (1984). Polyphosphate kinase from Propionibacterium shermanii : Formation of an enzymatically active insoluble complex with basic proteins and characterisation of synthesized polyphosphate. Biochemistry International 8, 757 - 769.

ROBINSON, N.A. and WOOD, H.G. (1986). Polyphosphate kinase from Propionibacterium shermanii. Demonstration that the synthesis and utilisation of polyphosphate is by a processive mechanism. J. Biol. Chem. 261, 4481 - 4485.

ROSEN, B.P. and MC CLEES, J.S. (1974). Active transport of calcium in inverted membrane vesicles of Escherichia coli. Proc. Nat. Acad. Sci. 71, 5042 - 5046.s

ROSENBERG, H., MEDVECZKY, N. and LA NAUZE, J.M. (1969). Phosphate transport in Bacillus cereus. Biochim. Biophys. Acta 193, 159 - 167.

ROSENBERG, H., GERDES, R.G. and CHIGWIDDEN, K. (1977). Two systems for the uptake of phosphate in Escherichia coli. J. Bacteriol. 131, 505 - 511.

ROSENBERG, M., BAYER, E.A., DELAREA, J. and ROSENBERG, E. (1982). Role of thin fimbriae in adherence and growth of Acinetobacter calcoaceticus RAG-1 on hexadecane. Appl. Environ. Microbiol. 44, 929 - 937.

ROSENBERG, E., KAPLAN, N., PINES, O., ROSENBERG, M. and GUTNICK, D. (1983). Capsular polysaccharides interfere with adherance of Acinetobacter calcoaceticus T hydrocarbon. FEMS Microbiol. Lett. 17, 157 - 160.

SALANITRO, J.P. and WEGENER, W.S. (1971). Growth of Escherichia coli on short chain fatty acids : Nature of the uptake system. J. Bacteriol. 108, 893 - 901.

SAR, N. and ROSENBERG, E. (1983). Emulsifier production by Acinetobacter calcoaceticus strains. Current Microbiol. 9, 309 - 314.

SAVAGEAU, M.A., KOTRE, A.M. and SAKAMOTO, N. (1972). A possible role in regulation of primary amination for a complex of glutamine -ketoglutarate amidotransferase and glutamate dehydrogenase in Escherichia coli. Biochem. Biophys. Res. Commun. 48, 41 - 47.

SCHULDINER, S. and FISHKES, H. (1978). Sodium/proton antiport in isolated membrane vesicles of Escherichia coli. Biochemistry 17, 706 - 711.

SCHUTTE, C.E. (1987). The distribution and metabolic activity of Acinetobacter strains present in an anaerobic digester while treating a petrochemical effluent. M Sc Tesis, Universiteit van die Oranje-Vrystaat.

SCOTT, C.C.L. and FINNERTY, W.R. (1976). Characterization of intra cytoplasmic hydrocarbon inclusions from the hydrocarbon oxidizing Acinetobacter species HO-10. J. Bacteriol. 127, 481 - 489.

SEKIKAWA, Y., NISHIKAWA, S., OKAZAKI, M. and KATO, K. (1966). Release of soluble orthophosphate in the activated sludge process. J. Wat. Poll. Contr. Fed. 38, 364 - 365.

SELF, C.H. and WEITZMAN, P.D.J. (1970). Separation of isoenzymes by zonal centrifugation. Nature 225, 644 - 645.

SELF, C.H. and WEITZMAN, P.D.J. (1972). The isocitrate dehydrogenases of Acinetobacter lwoffi. Separation and properties of two nicotinamide-adenine dinucleotide phosphate-linked isoenzymes. Biochem. J. 130, 211 - 219.

SENIOR, P.J. and DAWES, E.A. (1971). Poly-β-hydroxybutyrate biosynthesis and the regulation of glucose metabolism in Azotobacter beijerinckii. Biochem. J. 125, 55 - 66.

SEVERIN, A.I., LUSTA, K.I., NESMEYANOVA, M.A. and KULAEV, I.S. (1975).Membrane bound polyphosphate of Escerichia coli. Biokhimiya, 41, 357 -362.

SHABTAI, Y, and GUTNICK, D.L. (1986).Enhanced emulsan production in mutants of Acinetobacter calcoaceticus RAG-1 selected for resistance to cetyltrimethylammonium bromide. Appl. Environ. Microbiol. 52, 146 - 151.

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

SHAPIRO, J., LEVIN, G.V. and ZEA, H. (1967). Anoxically induced release of phosphate in wastewater treatment. J. Wat. Poll. Contr. Fed. 39, 1810 - 1818.

SHEINTUCH, M., LEV, O., EINAV, P. and RUBIN, E. (1986). Role of exocellular polymer in the design of activated sludge. Biotechnol. Bioeng. 28, 1564 - 1576.

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

SIERRA, G. and GIBBONS, N.E. (1963). Production of poly-β-hydroxybutyric acid granules in Micrococcus halodenitrificans. Can. J. Microbiol. 8, 246 -253.

SILVER, S. (1978). Transport of cations and anions. Bacterial Transport (ed) B P Rosen, Microbiol. Series Vol 4, Marcel Webber Inc. New York.

SIMPSON,D.E. and HEMENS, J. (1973). Sugar mill effluent treatment with nutrient addition. J. Wat. Poll. Contr. Fed. 45, 2194 - 2198.

SKERMAN, V.B.D., MC GOWAN, V. and SNEATH, P.H.A. (1980). Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30, 225 - 420.

SKULACHEV, V.P. (1977). Transmembrane electrochemical H^+-Potential as a convertible energy source for the living cell. FEBS Letters 74, 1 - 9.

SKULACHEV, V.P. (1978). Membrane-linked energy buffering as the biological function of Na^+/K^+ gradient. FEBS Letters 87, 171 - 179.

SOCIETY OF AMERICAN BACTERIOLOGISTS (1957). Manual of microbiological methods. Mc Graw-Hill Book Co USA.

SRINATH, E.G., SASTRY, C.A. and PILLAY, S.C. (1959). Rapid removal of phosphorus from sewage by activated sludge. Water & Waste Treatment 11, 410 - 415.

STANIER, R.Y., ADELBERG, E.A. and INGRAHAM, J.L. (1980). General Microbiology. The Mac Millan Press, London.

STOCKDALE, H., RIBBONS, D.W. and DAWES, E.A. (1968). Occurrence of poly-ß-hydroxybutyrate in the Azotobacteriaceae. J. Bacteriol. 95, 1798 - 1803.

STURM, R.N., HERMAN, N.J. and BELL, E.J. (1970). Metabolic control in Acinetobacter species : Effect of C4 vs C2 substrates on -ketoglutarate dehydrogenase synthesis. Can. J. Microbiol. 16, 817 - 820.

SURESH, N., WARBURG, R., TIMMERMAN, M., WELLS, J., COCCIA, M., ROBERTS, M.F. and HALVORSON, H.O. (1985). New strategies for the isolation of micro-organisms responsible for phosphate accumulation. Wat. Sci. Tech. 17, 99 - 111.

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

SWANSTROM, R. and SHANK, P.R. (1978). X-ray intensifying screens greatly enhance the detection by autoradiography of the radioactive isotopes ^{32}P and ^{125}I. Anal. Biochem. 86, 184 - 192.

SWEDES, J.S., SEDO, R.J. and ATKINSON, D.E. (1975). Relation of growth and protein synthesis to the adenylate energy charge in an adenine-requiring mutant of Escherichia coli. J. Biol. Chem. 250, 6930 - 6938.

SYZMONA, M.and OSTROWSKI, W. (1964). Inorganic polyphosphate glucokinase of Mycobacterium phlei. Biochim. Biophys. Acta 85, 283 - 295.

SYZMONA, O. and SYZMONA, M. (1979). Polyphosphate and ATP-glucose phosphotransferase activities in Nocardia minima. Acta Microbiologica Polonica 28, 153 - 160.

SYZMONA, O., URYSON, S.O. and KULAEV, I.S. (1967). Detection of polyphosphate glucokinase in various micro-organisms. Biokhimiya 32, 495 - 503.

TAYLOR, R., BEST, H.J. and WIECHERS, H.N.S. (1984). The effluent phosphate standard in perspective. Part I : Impact, control and management of eutrophication. IMIESA October 43 - 56.

TAYLOR, R. and JUNI, E. (1961). Pathways for biosynthesis of a bacterial capsular polysaccharide. J. Bacteriol. 81, 688 - 693.

TERRY, K.R. and HOOPER, A.B. (1970). Polyphosphate and orthophosphate content of Nitrosomonas europaea as a function of growth. J. Bacteriol. 103,

THOMAS, A.D., DOELLLE, H.W. WESTWOOD, A.W. and GORDON, G.L. (1972). Effect of oxygen on several enzymes involved in the aerobic and anaerobic utilization of glucose in Escherichia coli. J. Bacteriol. 112, 1099 - 1105.

TOERIEN, D.F GERBER, A.T. LÖTTER, L.H. and CLOETE, T.E. (1987) Enhanced biological phosphorus removal in activated sludge systems. Adv. Microbial Ecol. Manuscript in preparation.

TOERIEN, D.F. HYMAN, K.L. and BRUMER, M.J. (1975). A preliminary trophic status classification of some South African impoundments. Water SA 1, 15 - 23.

T'SEYEN, J., MALNOU, D., BLOCK, J.C. and FAUP, G. (1985). Polyphosphate kinase activity during phosphate uptake by bacteria. Wat. Sci. Tech. 17, 43 - 56.

TSUCHIYA, T. and ROSEN, B.P. (1976).Characterization of an active transport system for calcium in inverted membrane vesicles of Escerichia coli. J. Biol. Chem. 251, 7687 -7692.

UNZ, R.F. and DONDERO, N.C. (1967). The predominant bacteria in natural Zoogleae colonies. Can. J. Microbiol. 13, 1671 - 1682.

VACKER, D., CONNELL, C.H. and WELLS,W.N. (1967). Phosphate removal through municipal wastewater treatment at San Antonio, Texas. J. Wat. Poll. Contr. 39, 750 - 771.

VAILLANCOURT, S., BEAUCHEMIN-NEWHOUSE, N. and CEDERGREN, R.J. (1978). Polyphosphate-deficient mutants of Anacystis nidulans. Can. J. Microbiol. 24, 112 - 116.

VAN GROENESTIJN, J.W. and DEINEMA, M.H. (1985). Effects of cultural conditions on phosphate accumulation and release by Acinetobacter strain 210A. Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment. Seeper Ltd, Londen.

VAN HAANDEL, A.C., DOLD, P.L. and MARAIS, G. v R. (1982). Optimization of nitrogen removal in the single stage activated sludge process. Wat. Sci.Tech. 14, 443 - 461.

VAN SCHIE, B.J., HELLINGWERF, K.J., VAN DIJKEN, J.P., ELFERENK, M.G.L., VAN DIJL, J.M., KEUNEN, G. and KONINGS, W.N. (1985). Energy transduction by electron transfer via a pyrrolo-quinoline, quinone dependent glucose dehydrogenase in Escherichia coli, Pseudomonas aeruginosa and Acinetobacter calcoaceticus var lwoffi. J. Bacteriol. 163, 493 - 499.

VAN SCHIE, B.J., VAN DIJKEN, J.P. and KUENEN, J.G. (1984). Non-coordinated synthesis of glucose dehydrogenase and its prosthetic group QQP in Acinetobacter and Pseudomonas spp. FEMS Microbiol. Lett. 24, 133 - 138.

VAN STEVENINK, J. and BOOIJ, H.L. (1964).The role of polyphosphates in the transport mechanism of glucose in yeast cells. J. Gen. Physiol. 48, 43 - 60.

VENTER, S.L., HALLIDAY, J. and PITMAN, A.R. (1978). Optimization of the Johannesburg Olifantsvlei extended aeration plant for phosphorus removal. Progr. Wat. Tech. 10, 279 - 292.

VILJOEN, F.C. (1984). The necessity of phosphate restrictions within the Vaal River barrage catchment. IMIESA September 11.

VISSER, A.S. and POSTMA, P.W. (1973). Permeability of Azotobacter vinelandii to cations and anions. Biochim. Biophys. Acta 298, 333 - 340.

VOELZ, H., VOELZ, U. and ORTIGOCA, R.O. (1966). The polyphosphate overplus pheomenon in Myxococcus xanthus and its influence on the architecture of the cell. Archiv. für Mikrobiol. 53, 371 - 388.

WANG, J.Y.J. and KOSHLAND, D.E. (1978). Evidence for protein kinase activities in the procaryote Salmonella typhimurium. J. Biol. Chem. 253, 7605 - 7608.

WALSH, K. and KOSHLAND, JR., D.E. (1985). Branch point control by the phosphorylation state of isocitrate dehydrogenase. J. Biol. Chem. 260, 8430 - 8437.

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

WATSON, S.W. (1974). Nitrobacteriaceae. In Bergey's Manual of Determinative Bacteriology. 8 th Edition. Williams en Wilkens. Baltimore.

WEBER, K. and OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406 - 4412.

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

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

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

WELLS, W.N. (1969). Differences in phosphate uptake rates exhibited by activated sludges. J. Wat. Poll. Cont. Fed. 41, 765 - 771.

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

WENTZEL, M.C., LöTTER, L.H., LOEWENTHAL, R.E. and MARAIS, G v R. (1986). Metabolic behaviour of Acinetobacter spp in enhanced biological phosphorus removal : A biochemical model. Water SA 12, 209 - 224.

WEST, I.C. and MITCHELL, P. (1974). Proton/sodium antiport in Escherichia coli. Biochem. J. 144, 87 - 90.

WHITTAKER, P.A. (1971). Terminal respiration in Moraxella lwoffi. Microbiol. 4, 65 - 70.

WIECHERS, H.N.S. (1987). Sewage purification in South Africa - quo vadis. Proceedings of the biennial conference of the Institute of Water Pollution Control. Port Elizabeth.

WIECHERS, H.N.S. and BEST, H.J. (1985). Environmental phosphorus management in South Africa. Proceedings of the International Conference on Management Strategies for Phosphorus in the Environment, Lisbon.

WIECHERS, H.N.S., TAYLOR, R.and BEST, H.J. (1984). The effluent phosphate standard in perspective. Part II : State of the art of technology for limiting phosphate discharge to the water environment. IMIESA, November, 27 - 38.

WILLIAMSON, D.H. and MELLANBY, J. (1974). D-(-)-3-hydroxybutyrate. Methods of Enzymic Analysis 4, (ed) H Bergmeyer, Verlag Chemie, Academic Press, New York.

WILSON, D.B. (1978). Cellular transport mechanisms. Ann. Rev. Biochem. 47, 933 - 965.

WIMPENNY, J.W.T. and NECKLEN, D.K. (1971). The redox environment and microbial physiology. I : The transition from anaerobiosis to aerobiosis in continuous cultures of facultative anaerobes. Biochim. Biophys. Acta 253, 352 - 359.

WINDER, F.G. and DENNENY, J.M. (1957). The metabolism of inorganic polyphosphate in Mycobacteria. J. Gen. Microbiol. 17, 573 - 585.

WINKLER, M.A. and THOMAS, A. (1978). Biological treatment of aqueous wastes. Topics in enzyme and fermentation biotechnology. (ed) A. Wiseman. John Wiley and Sons. New York.

WONG, P.P. and EVANS, H.J. (1971). Poly- -hydroxybutyrate utilization by soybean (Glycine max. mer) nodules and assessment of its role in maintenance of nitrogenase activity. Plant Physiol. 47, 750 - 755.

WOOD, H.G. and GOSS, N.H. (1985). Phosphorylation enzymes of the propionic acid bacteria and the roles of ATP, inorganic pyrophosphate and polyphosphates. Proc. Natl. Acad. Sci. 82, 312 - 315.

WOODSIDE, E.E. and KWAPENSKI, J.B.G. (1974). Microbial polysaccharides. In Molecular Biology. John Wiley and Sons. New York.

YAGIL, E. (1975). Derepression of polyphosphate in Escherichia coli by starvation for inorganic phosphate. FEBS Lett. 55, 124 - 127.

YALL, I., BOUGHTON, W.H., KNUDSEN, R.C. and SINCLAIR, N.A. (1970). Biological uptake of phosphorus by activated sludge. Appl. Microbiol. 20, 145 - 150.

YAMAMOTO, Y. (1966). Variation of nictoinamide adenine dinucleotide phosphate level in bean hypocotyls in relation to O_2 concentration. Plant Physiol. 44, 519 - 522.

YAMAMOTO, Y. and TEZUKA, T. (1972). Regulation of NAD Kinase by phytochrome and control of metabolism by variation of NADP level. Phytochrome (ed) K Mitrakos and W Shropshire, Academic Press, London.

ZAITSEVA, G.N. and BELOZERSKII, A.N. (1960). Formation and utilisation of polyphosphates catalyzed by an enzyme isolated from Azotobacter vinelandii. Dokl. Akad. Nauk., SSR, 132, 950 - 953.