THE AEROBIC TO ANAEROBIC SWITCH IN ESCHERICHIA .COLI K_{12}

BY

PETER MAURICE CLARKE

A dissertation submitted to the University of London in candidature for the degree of Doctor of Philosophy.

September 1984

Department of Biochemistry
Imperial College
University of London
London SW7 2AZ

Dedicated to Christine

with many thanks

PREFACE

I should like to take this opportunity to thank my supervisor, Professor Brian Hartley, for his help in the form of discussions and encouragement.

Thanks are also due to:

Dr M Payton for introducing me to the field of microbial physiology.

Mr Jim Brannigan for help with the proof reading of this dissertation.

Miss Christine Hope for typing this dissertation.

The staff of Imperial Biotechnology Ltd. for the use of continuous culture apparatus and many useful suggestions.

Finally I would like to thank my parents for their continuous support and encouragement.

The work described in this disseration was performed at Imperial college and was supported by an M.R.C. grant.

ABSTRACT

This dissertation describes an investigation of the switch in physiology which occurs in E.coli K12 between aerobic and anaerobic growth; in particular that area of physiology concerned with the various fates of carbon from glucose.

Fermentation balances, both aerobic and anaerobic, of cultures of available mutant strains led to further strain construction by P1Kc transduction and HfrH conjugation to assemble the genetic lesions in an isogenic background. Further batch culture fermentation balances led to the selection of three strains for study in a glucose limited chemostat.

Steady states were set up under different aeration conditions by alteration of the N_2 to air ratio in the influent gases. At these steady states metabolic intermediates of glucose metabolism were assayed by a combination of gas, liquid chromatography, infra-red spectroscopy and enzymatic assay, whereas the cells themselves were assayed for enzyme levels, dry weight and internal redox potential (as determined by the ratio, NAD+ /NADH).

From the batch culture results the accumulation of lactate was found to be unnecessary for the anaerobic growth of E. coli K12, and the production of lactate was found to be more of a response to high carbon flux than to redox pressure. It was also determined from batch culture experiments that E. coli K12 with a lesion in the acetate producing pathway would not grow anaerobically but would exhibit glucolysis, mainly to lactate. This was attributed to the inability of these mutants to recycle acetyl CoA to free co-enzyme A.

From the continuous culture results a description of the switch in wild type E.coli K12 has been obtained and an argument has been presented which attributes the aerobic accumulation of acetate to the requirement to maintain a free co-enzyme A pool.

From both batch and continuous cultures the possibility that internal redox potential was an effector of the switch was discounted. Instead evidence is presented that the switch is mediated by a component associated with the succinate dehydrogenase portion of the electron transport chain.

CONTENTS

Chapter 2 contd.

Chapter 2 contd.

Chapter 2 contd.

Chapter 3 contd.

Chapter 3 contd.

Chapter 4 contd.

ABBREVIATIONS

Most are standard, as given by the Biochemical Journal (1983) 209, 1-27. Attention is however, drawn to the following:-

Ace	Acetate
ADH	Alcohol dehydrogenase
ADP	Adenosine diphosphate
APAD	3-Acetylpyridine adenine dinucleotide
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CoA-SH	Free co-enzyme A
DCPIP	Dichlorophenolindolphenol
DEGA	Diethylglycoladipate
DNA	Deoxyribose nucleic acid
DOT	Dissolved oxygen tension
EDTA	Ethyl diamine tetra acetate
eop	Efficiency of plating
EtOH	Ethanol
EID	Flame ionisation detector
F_p( )	Flavoprotein oxidised/reduced form
GDP	Guanosine diphosphate
GLC	Gas liquid chromatography
GLUC	Glucose
GTP	Guanosine triphosphate

IR	Infra red
LB	Luria broth
Mntl	Mannitol
MTT	3-(4,5-Dimethylthiazoyl-2) 2,5 diphenyltetrazolium bromide
NAD^+/H	Nicotinamide adenine dinucleotide oxidised or reduced form
NADHox	NADHoxidase (ie. NADH dehydrogenase complex)
$NADP^+/H$	Nicotinamide odenine dinucleotide phosphate oxidised or reduced form
NBT	Nitro blue tetrazolium
NTG	N-Methyl-N'-nitro-N-nitrosoguanide
OD_x	Optical density at wavelength Xnm
2OGDH	2 oxoglutarate dehydrogenase
pCO_2	Partial pressure of CO_2
PEP	Phosphoenpyruvate
PES	Phenazine ethosulphate
pfu	Plaque forming unit
Pi	Inorganic phosphate
PMS	Phenozine methosulphate
RNA	Ribose nucleic acid
Succ	Succinate
TCD	Thermal conductivity detector
TIM	Tetrzolium indicator medium

Abbreviations contd.

tRNA	Transfer RNA
w/t	Wild type
X^-	No growth on substrate X
X^+	Growth on substrate X
Y_x	Yield in terms of X
+ve	Positive response
-ve	Negative response

All genetic characters are abbreviated as shown in Bachmann and Low (1980).

NB. The genotype of those strains constructed by HfrH conjugation is uncertain as only those lesions directly concerned with the conjugation (ie. selection and counter-selection markers) and the lesions in glucose catabolism were tested for.

Table 1 BACTERIAL STRAINS

STRAIN	ORIGIN	RELEVANT GENOTYPE
CSH3	J.Miller (1974)	F^-, *thi* , *trp* , *rpsL*
CSH3B	Spontaneous mutant to rifampicin resistance of CSH3	F^-, *thi* , *trp* , *rpsL*, *rpoB*
CSH57	J.Miller (1974)	F^-, *thi* , *trp* , *rpsL*, *pyrC*
CSH59C	Spontaneous mutant to ampicillin resistance of CSH59	F^-, *thi* , *trp* , *rpsL*, *ampA*, *pyrC*
CSH59B	Spontaneous mutant to rifampicin resistance of CSH59	F^-, *thi* , *trp* , *rpsL*, *pyrC*, *rpoB*
CSH62	J.Miller (1974)	HfrH, *thi*
CSH62B	Spontaneous mutant to ampicillin resistance of CSH62	HfrH, *thi* , *ampA*
IY12	Young & Wallace(1976)	F^-, *his* , *ilv* , *trp* , *rpsL*, $P1^r$, *ndh*
IY13	Young & Wallace(1976)	F^-, *his* , *ilv* , *trp* , *rpsL*, $P1^r$
IY13B	Sponaneous mutant to rifampicin resistance of IY13	F^-, *his* , *ilv* , *trp* , *rpsL*, $P1^r$, *rpoB*
AN385	Young et al (1972)	F^-, *thi* , *rpsL*, *ubiA*
HΔ13	Langley & Guest(1977)	F^-, *thiA*, *aroP*, *aceE*
6161	C.G.S.C.(a)	F^-, *pfl*
DC272	Clark & Cronan(1980)	F^-, *adhR*

(Table 1 contd.)

STRAIN	ORIGIN	RELEVANT GENOTYPE
LCB900	Casse et al (1976)	F⁻,anal,thr ,leu ,lacY,rpsL, supE,tonA
LCB901	Casse et al (1976)	F⁻,ana2,thr ,leu ,lacY,rpsL supE,tonA
JRG1061	Guest (1979)	F⁻,ack ,trpR,rpsL
JRG1078	Guest (1979)	F⁻,facA,gyrB,trpR,rpsL
JRG1086	Guest (1979)	F⁻,pta ,purF,trpR
CC904	Exconjugant CSH62B x AN385	HfrH,ubiA,ampA,thi ,rpsL
CC1006	Exconjugant CC904 x CSH59B	HfrH,thi ,pyrC,trp ,rpsL, rpoB,ampA
CC1103	Extransductant P1kc(DC272) x CSH59B	F⁻,adhR,thi ,pyrC,rpsL,rpoB
CC1202	Extransductant P1kc (LCB900)xCSH59B	F⁻,anal,thi ,pyrC,rpsL,rpoB
CC1307	Extransductant P1kc (LCB901)xCSH59B	F⁻,ana2,thi ,pyrC,rpsL,rpoB
CC1410	Exconjugant CSH62BxIY12	HfrH,ndh ,his ,ilv ,trp ,rpsL, ampA
CC2807	Extransductant P1kc(DC272)xCSH3B	F⁻,thi ,rpsL,rpoB,adhR
CC2913	Extransductant P1kc(LCB900)xCSH3B	F⁻,thi ,rpsL,rpoB,anal
CC3005	Extransductant p1kc(LCB901)xCSH3B	F⁻,thi ,rpsL,rpoB,ana2

(Table 1 contd.)

STRAIN	ORIGIN	RELEVANT GENOTYPE
CC3108	Extransductant	F^-,thi ,trp ,rpsL,rpoB,aroP,
	Plkc (H 13)xCSH3B	aceE
CC3310	Exconjugant	HfrH,thi ,trp ,rpsL,rpoB,ampA
	CC904 x CSH3B	ubiA
CC3502	Exconjugant	thi ,rpsL,rpoB,ampA,ubiA
	CC904 x CC2807	adhR
CC3703	Exconjugant	thi ,rpsL,rpoB,ampA,anal,ubiA
	CC904 xCC2913	
CC3805	Exconjugant	thi ,rpsL,rpoB,ampA,ana2,ubiA
	CC904 x CC3005	
CC4009	Exconjugant	thi ,rpsL,rpoB ampA,ubiA,his ,
	CC904 x IY13B	ilv ,trp ,Pl^r

Nomenclature - Bachmann and Low (1980)

(a) CGSC indicates the strain was obtained from the 'Coli Genetic Stock Centre' through the kindness of the curator Barbara Bachmann

Table 2 Strain Phenotypes

Relevant Genotypes	Phenotype
ubiA	Aerobic Gluc$^+$;aerobic Succ$^-$
ndh$^-$	Aerobic Gluc$^+$;aerobic Mntl$^-$
adhR	Aerobic EtOH$^+$;red colonies on T.I.M.
anal	Aerobic Gluc$^+$;anaerobic Gluc$^-$
ana2	"
aceF	Aerobic Gluc$^-$;aerobic Gluc+Ace$^+$
ack$^-$	Anaerobic gluc$^-$;low levels Ace when growing aerobically on Gluc.
pta$^-$	Anaerobic Gluc$^-$;low levels Ace when growing aerobically on Gluc.
facA	Anaerobic Gluc$^-$;low levels Ace when growing aerobically on Gluc.
pfl$^-$	Anaerobic Gluc poor;anaerobic Gluc+ Ace$^+$
w/t	Aerobic Gluc$^+$, Succ$^+$, Mntl$^+$,EtOH$^-$ white colonies on T.I.M., anaerobic Gluc$^+$

Table 3

PHAGE LIST

PHAGE	ORIGIN
P1 *vir*	J Miller (1974)
P1*kc*	Prof. Atkinson (Porton Down)
R17	J Miller (1974)

LIST OF FIGURES

List of Figures Contd.

LEGENDS TO FIGURES

LIST OF TABLES

List of tables contd.

LIST OF PHOTOGRAPHS

CHAPTER 1

INTRODUCTION

The properties of any microbial cell are ultimately determined by the characteristics of its genome. This carries all the information necessary for an organism to become a structural and functional unit, and also gives it the potential to respond to changes in its environment. This latter property is closely associated with the life-style of microbes. These organisms only have a limited capacity to control their environment and therefore, they almost invariably respond to environmental change by changing their physiology. In principle this can happen in two ways, by changes in their genetic constitution or by phenotypic adaptation. In this work the former of these two processes was not considered, the investigation being confined to the phenotypic response of E.coli K_{12} to one aspect of environmental change, namely oxygen availability.

The wide variety of phenotypic responses encountered within a specific genome clearly shows that none of the microbial species studied so far expresses all its genome under any given set of conditions (Koch, 1976). Instead microbes generally express only that part of their genome that enables them to adjust to a particular environment. This ability has led to the appreciation of the wide range of physiologies that confer a great degree of versatility. Thus an organism of a given genotype is very much a product of it's

environment, so much so, that "it is virtually meaningless to speak of the chemical composition of a micro-organism, without at the same time specifying the environmental conditions that produced it" (Herbert and Kornberg,1976). Essentially the same applies to the functional properties of many organisms (Tempest and Neijssel, 1978).

The effects of oxygen limitation in facultative anaerobes have been widely investigated (e.g. Moss, 1956; Harrison and Pirt, 1967) and responses may be placed into two categories. Primarily the induction of new enzyme systems and secondly the modulation of existing enzyme systems. Comparison of figures 1 and 3 indicates that the response of E.coli K_{12} to oxygen stress consists mainly of the induction of new enzyme systems, although those systems themselves are capable of modulation (Reichelt and Doelle, 1971). Although there are many changes in carbon flux (Model and Rittenberg, 1967) and enzyme levels (Smith and Neidhardt, 1983) throughout the glucolytic reactions of E.coli K_{12}, the greatest variation occurs in the fates of pyruvate. It is with those reactions that this work is most concerned, as it is by the accumulation of the end products of those reactions whereby E.coli K_{12} adapts it's physiology to allow growth without an exogenous electron acceptor.

The glucolytic metabolism of E.coli K_{12} differs greatly during aerobiosis and anaerobiosis and so as to provide a framework for the examination of the switch between these two states, a description of the metabolism during aerobic and anaerobic growth was required. The descriptions of the

glucolytic metabolism was mostly obtained from a text book (Metzler, 1977) and agreed with results obtained in this work.

Major Glucolytic Pathways of Aerobically grown E.coli K_{12}

(see Fig. 1)

Entry of Glucose

Aerobically, glucose enters E.coli K_{12} primarily by an active transport mechanism (a specific permease) which is capable of transporting glucose against a concentration gradient with the hydrolysis of ATP (Cohen and Monod, 1957; Kaback, 1968). This mechanism results in no chemical change in the glucose. A second transport system is also present aerobically in E.coli K_{12}, but plays a lesser rôle (Dills et al, 1980). This is a group translocation system which accumulates phosphorylated glucose inside the cell; the phosphotransferase system (Kundig et al, 1966). This system uses energy derived from the hydrolysis of phosphoenol-pyruvate to effect transport (Romano et al, 1970).

Glucose dissimilation to Pyruvate

Once inside the cell, the glucose is phosphorylated by hexokinase or a specific glucokinase to glucose-6-phosphate. At this point the phosphorylated glucose can enter either the pentose phosphate pathway (P.P.P.) or the Embden-Meyerhof pathway (E.M.P.). The pentose phosphate pathway provides NADPH and by a series of transaldolase and transketolase reactions, phosphorylated sugars for anabolic processes. The flux of

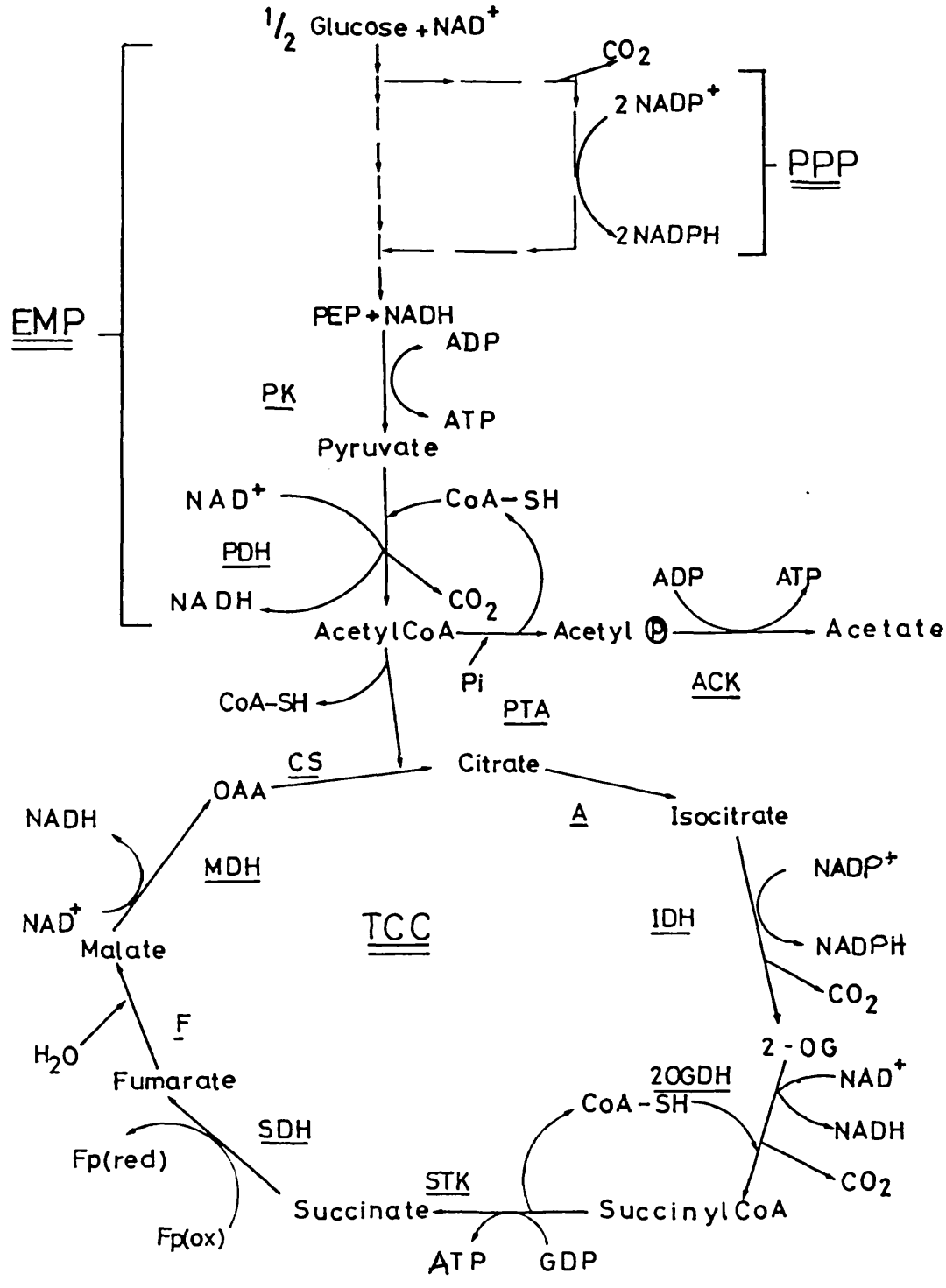


Figure 1

MAJOR GLUCOLYTIC PATHWAY IN AEROBICALLY GROWN E.COLI K_{12}

P.T.O. for legend

Legend to Figure 1

PPP	Pentose phosphate pathway
EMP	Embden-Meyerhoff pathway
TCC	Tricarboxylic acid cycle
PK	Pyruvate kinase
PDH	Pyruvate dehydrogenase
PTA	Phosphotransacetylase
ACK	Acetate kinase
CS	Citrate Synthase
A	Aconitase
IDH	Isocitrate dehydrogenase
2OGDH	2 oxoglutarate dehydrogenase
STK	Succinate thiokinase
SDH	Succinate dehydrogenase
F	Fumarase
MDH	Malate dehydrogenase

carbon through this pathway aerobically can be as high as 25% of the total carbon flux (Model and Rittenberg, 1967), and eventually rejoins the Embden-Meyerhof pathway at the triose level.

The Embden-Meyerhof pathway is the major pathway of glucose degradation in E.coli K_{12} (Doelle, 1975) and takes glucose-6-phosphate to the triose level via glucose-6-isomerase, phosphofructokinase and aldolase (see Metzler, 1977). From the triose level, carbon from the pentose phosphate pathway is metabolised by the Embden-Meyerhoff pathway to pyruvate. The dissimilation of glucose to pyruvate by the Embden-Meyerhoff pathway in E.coli K_{12} has the overall reaction:-

$$2Pi + 2ADP + Glucose + 2NAD^{+} \longrightarrow 2Pyruvate + 2NADH + 2ATP$$

Thus there is an overall net gain of 2ATP and 2NADH.

Recent results show that most micro-organisms metabolise glucose by more than one pathway. As illustrated and discussed by Wood and Kelly in some detail (1980), even the old concept that micro-organisms possess one major pathway and one minor pathway of glucose metabolism is certainly an over simplification. It appears, on the contrary that most microbes can not only vary the flux through the two parallel pathways, but can probably induce a third or even fourth pathway. For example, it is frequently documented that E.coli K_{12} possesses two pathways for glucose dissimilation, the EMP and the PPP (Doelle, 1975). However, E.coli K_{12} actually possesses an inducible Entner-Doudoroff pathway (Entner and Doudoroff, 1952), which allows gluconate grown cells to metabolise glucose and gluconate separately independently via the EMP and the Entner-Doudoroff pathway

(Eisenberg and Dobrogosz, 1967). However, for the present work, this third pathway may be ignored as gluconate is not used as a carbon source.

Aerobic Fates of Pyruvate

Although E.coli posseses a pyruvate oxidase of unknown physiological function which directly decarboxylates pyruvate to acetate (Mather et al, 1982), the most common fate of pyruvate during aerobic glucose metabolism is its conversion to acetyl CoA by the pyruvate dehydrogenase complex (PDH). This multi-enzyme complex consists of three sub-units (Koike et al, 1963), pyruvate dehydrogenase (E_1), dihydrolipoyl acetyltransferase (E_2) and the flavoprotein, dihydrolipoyl dehydrogenase (E_3). The E_3 component, which contains a bound lipoyl group (Tanaka et al, 1974), is also found in oxoglutarate dehydrogenase (Langley and Guest, 1977), a key regulatory enzyme in the tricarboxylic acid cycle. Another possible fate of pyruvate in aerobically grown E.coli K_{12} is the conversion to D-lactate by lactate dehydrogenase, probably more accurately termed pyruvate reductase as it operates unidirectionally (pyruvate→lactate) in vivo (Tarmy and Kaplan, 1968). NADH-linked lactate dehydrogenase in E.coli K_{12} is constituitively produced, though formed at higher levels anaerobically (Doelle et al, 1981) and can produce lactate during aerobic growth under appropriate conditions such as growth on high glucose concentrations or in mutants of E.coli K_{12} deficient in pyruvate dehydrogenase activity (Clarke and Payton, unpublished data).

Acetyl CoA, formed by the PDH complex may then either enter the tricarboxylic acid cycle by the action of citrate synthase, or be converted to acetate by the action of phosphotransacetylase and acetate kinase. The aerobic formation of acetate is as a response to an inhibition of the tricarboxylic acid cycle, causing a fall in the free co-enzyme A pool as acetyl CoA builds up. Co-enzyme A levels are then raised by the production of acetyl phosphate (and then acetate) from acetyl CoA which results in the release of free co-enzyme A (Pascal et al, 1981).

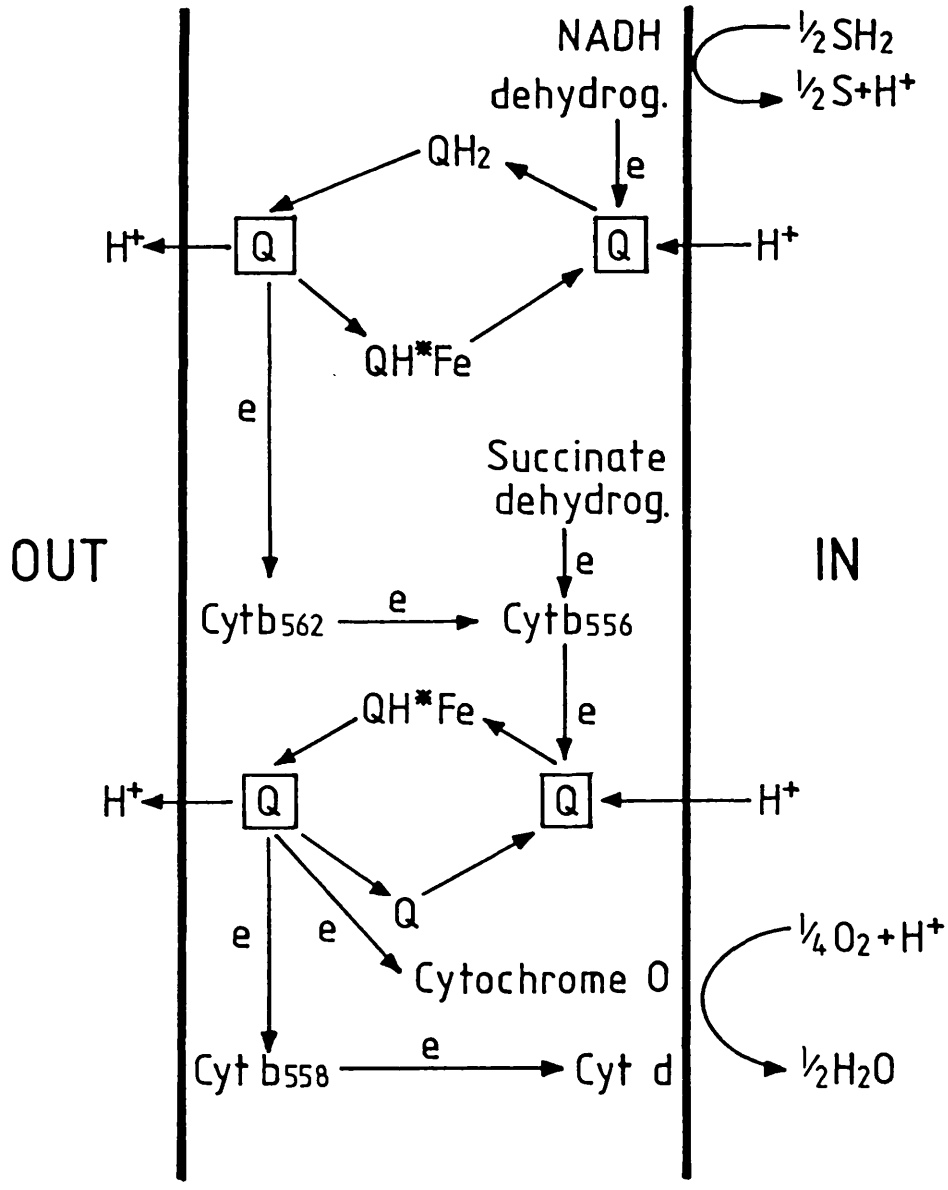
The flux of acetyl CoA into the tricarboxylic acid cycle is controlled by the activity of citrate synthase, which is inhibited by NADH (Flechtner and Hanson, 1970). Further control points are at 2 oxoglutarate dehydrogenase, isocitrate dehydrogenase and aconitase, all of which are repressed by anaerobiosis (Amarasingham and Davis, 1965; Hespell, 1976; Wimpenny and Cole, 1967). A review of the complex controls of the tricarboxylic acid cycle may be found elsewhere (Stouthamer, 1975), however, this work has indicated that the control of the cycle in a semi-anoxic environment is exerted predominately at the level of succinate dehydrogenase (see page 170).

Under fully aerobic conditions the tricarboxylic acid cycle can function to oxidise all the acetyl CoA from pyruvate to carbon dioxide. Under these conditions and assuming the presence of an electron transport chain that generates 2ATP per NADH reoxidised (West and Mitchell, 1974), the maximum ATP yield from the dissimilation of glucose to CO_2 in E.coli K_{12}

is 26 moles ATP per mole glucose.

Aerobic electron transport chain (see Fig. 2)

E.coli K_{12} synthesises a variety of redox carriers which comprise the electron transport chain in response to varying growth conditions (Ashcroft and Haddock, 1975; Jones, 1977) and these number at least nine. Respiration driven proton translocation is coupled to the oxidation of NAD(P)H or reduced flavins in aerobically grown E.coli K_{12}. Stoichiometric measurements of H^+/O ratios provide levels of 4 for NADH(P)H reoxidation, and 2 for flavin re-oxidation (Lawford and Haddock, 1973). Assuming that $2H^+$ are required for the synthesis of 1ATP by ATPase (West and Mitchell, 1974), the electron transport chain is considered to be organised into two equivalent conservation segments (Brice et al, 1974; Jones et al, 1975); one that is specifically associated with the NADH dehydrogenase region and the other with the cytochrome region of the respiratory chain (Haddock and Schairer, 1973). Since each of these segments transolocate 2 protons per NAD(P)H re-oxidised, these results indirectly confirm that ATP synthesis via the reversible, proton translocating ATPase occurs with a H^+/ATP ratio of two. These results are illustrated in Fig 3 and take into account both the proposed electron transport chain system (Cox et al, 1970) and Mitchell's proposed protonmotive ubiquinone cycle (Mitchell, 1975).



Electron transport chain of E.Coli K_{12} Figure 2

P.T.O for legend

Legend to Figure 2

Cyt	Cytochrome
NAD/H	Nicotinomide adenine dinucleotide
Q	Ubiquinone
QH*	Ubisemiquinone
QH_2	Ubiquinol
S	Substrate, oxidised
SH_2	Substrate, reduced

Under oxygen limitation E.coli K_{12} is able to synthesise a novel terminal cytochrome oxidase namely cytochrome d (Moss, 1952). This cytochrome, which has a lower Km for oxygen than cytochrome o (Haddock and Jones, 1977), is the terminal oxidase of a second pathway of equal efficiency to the cytochrome o pathway (Rice and Hempfling, 1978) and allows the electron transport chain to operate at lower oxygen concentrations.

Major Glucolytic Pathways of Anaerobically grown E.coli K_{12}

(see Fig 3)

Entry of Glucose

As in aerobiosis, E.coli K_{12} possesses two systems for concentrating glucose against a concentration gradient; the ATP hydrolysing specific permease system, and the group translocating phosphotransferase system (see page 3). However, during anaerobiosis the phosphtransferase system is the major uptake system (Dills et al, 1980). This method of glucose uptake allows for the conservation of ATP during anaerobiosis, as the energy to drive the transport is generated by the hydrolysis of phosphoenolpyruvate to pyruvate and not the hydrolysis of ATP. It has also been suggested that the phosphotransferase system is the flux generating step of glucose dissimilation and is therefore responsible for the increase in the flux of glucose anaerobically (Hunter and Kornberg, 1979).

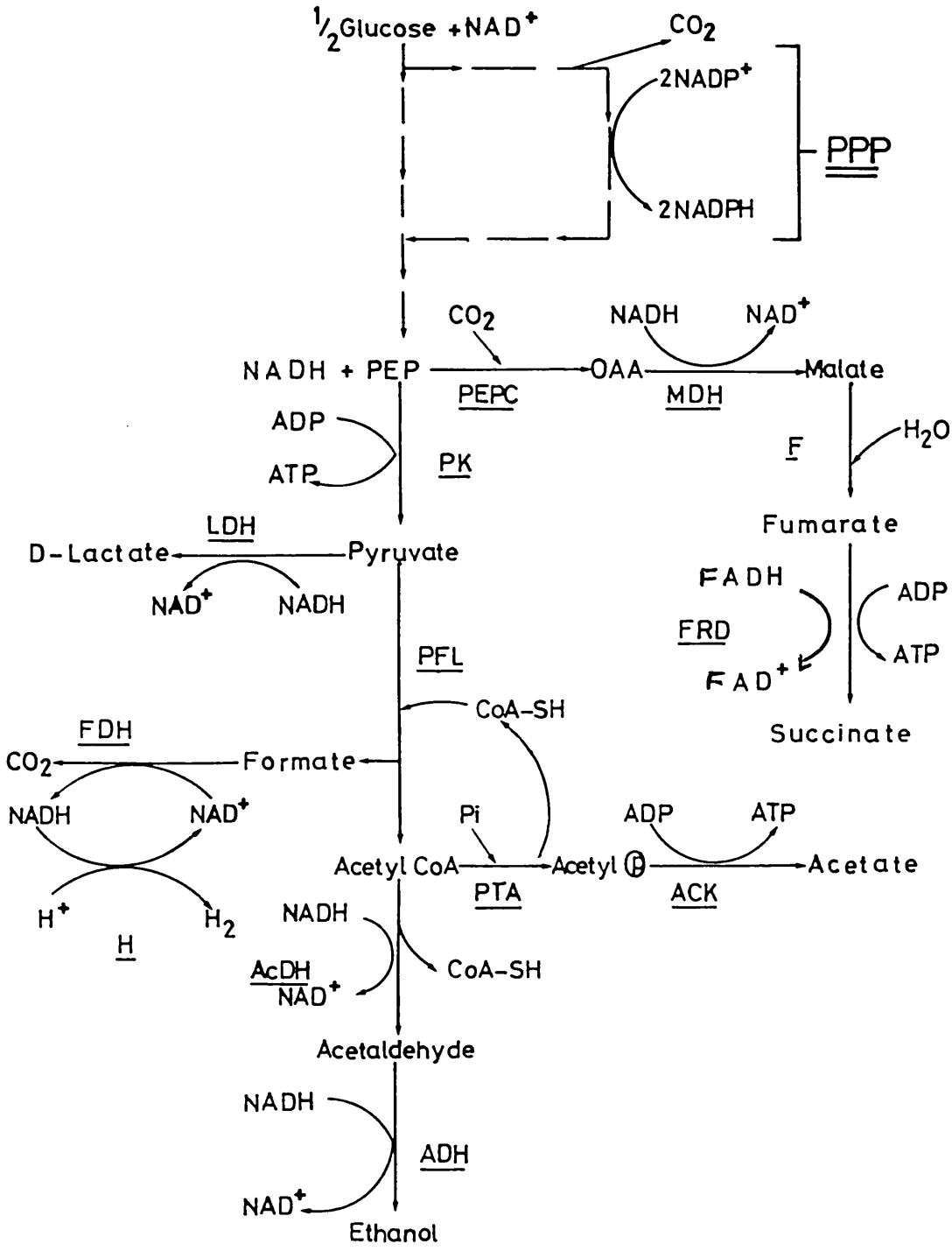


Figure 3

MAJOR GLUCOLYTIC PATHWAYS OF ANAEROBICALLY GROWN E.COLI K_{12}

P.T.O. for Legend

Legend to Figure 3

PPD	Pentose phosphate pathway
PK	Pyruvate kinase
PFL	Pyruvate formate lyase
AcDH	Acetaldehyde dehydrogenase
ADH	Alcohol dehydrogenase
PTA	Phosphotransacetylase
ACK	Acetate kinase
FDH	Formate dehydrogenase
H	Hydrogenase
FRD	Fumarate reductase
F	Fumarase
MDH	Malate dehydrogenase
PEPC	PEP carboxylase
LDH	Lactate dehydrogenase

Glucose dissimilation to pyruvate

Although E.coli K_{12} still possesses a pentose phosphate pathway anaerobically, the flux of glucose through this pathway falls from about 25% under aerobic conditions to about 7% under anaerobiosis (Model and Rittenberg, 1967), and the Embden-Meyerhoff pathway becomes the basis of fermentative glucose catabolism (ie. without an exogenous electron acceptor). This is brought about by the inhibition of glucose-6-phosphate dehydrogenase (the first enzyme in the pentose phosphate pathway) by raised NADH levels (Sanwal, 1970) and an increase in the enzyme activites of the Embden-Meyerhoff pathway, especially glyceraldehyde-3-phosphate dehydrogenase which increases in activity by 100 fold (Doelle and McIvor, 1980).

Therefore, glucose is predominantly dissimilated to pyruvate by the Embden-Meyerhoff pathway although there is a small flux of glucose through the pentose phosphate pathway to allow the generation of phosphorylated sugars and NADPH for anabolic reactions.

Anaerobic Fates of Pyruvate

The induction of enzyme systems under anaerobiosis means that the anaerobic fate of pyruvate is much more diverse than that under aerobic conditions. The one fate of pyruvate most commonly quoted is the conversion to D-lactate by lactate dehydrogenase, which was thought to be the major method whereby NADH was re-oxidised in E.coli K_{12} (Metzler, 1977).

However, this work indicated that lactate accumulation was not necessary for the production of a redox balance (see page 162), as E.coli K_{12} grew fermentatively without the accumulation of D-lactate.

During anaerobiosis the PDH complex is repressed, and inhibited by NADH (Hansen and Henning, 1966), but acetyl CoA may be formed from pyruvate by the action of pyruvate formate lyase (PFL). PFL exists in an inactive and active form (Knappe et al, 1969). The inactive form appears to be synthesised constituitively (Smith and Neidhardt, 1983a), and may undergo post translational modification by the incorporation of an organic free radical by a specific Fe2+ dependant activating enzyme (Knappe et al, 1984). The inactive form of the enzyme is stable, but the active form, upon contact with oxygen irreversibly loses its catalytic activity (Knappe et al, 1974). Therefore, during aerobiosis PFL is inactive and acetyl CoA formation is by the PDH complex whereas anaerobically, the PDH complex is inactive and PFL forms acetyl CoA from pyruvate.

Acetaldehyde dehydrogenase and alcohol dehydrogenase activites are induced under anaerobiosis (Sokatch, 1969), and therefore, the acetyl CoA formed by PFL may be reduced to ethanol with the concomitant oxidation of 2NADH for every ethanol formed. Recently, there has been evidence that E.coli K12 synthesises two alcohol dehydrogenase enzymes, one aerobically and a separate one anaerobically (Po-Keung Wong and Barrett, 1983). Presumably, the aerobic enzyme is responsible for growth on ethanol, whilst the other is

concerned with the maintenance of a redox balance by the production of ethanol from acetyl CoA, but no evidence has been presented to support this hypothesis.

Phosphotransacetylase and acetate kinase are also induced above their aerobic levels by anaerobiosis (Smith and Neidhardt, 1983a). This allows the formation of acetate from acetyl CoA and allows the cell to synthesise ATP if redox conditions allow. This third site of substrate level phosphorylation (the other two being pyruvate kinase and phosphoglycerate kinase in the Embden-Meyerhoff pathway) allows a maximum production of 3moles ATP per mole glucose dissimilated during anaerobiosis.

The formation of acetyl CoA from pyruvate by PFL also results in the formation of formate. The production of formate allows the formation of acetyl CoA without the concomitant production of NADH (which occurs in the reaction mediated by the PDH complex), and therefore eases the pressure on the organism to maintain a redox balance. The formate thus formed may then be converted to hydrogen and carbon dioxide by an anaerobically induced formate hydrogen lyase (FHL) activity (Pascal et al, 1975; Haddock and Jones, 1977). FHL in E.coli K_{12} consists of two components, NAD^+-linked formate dehydrogenase moiety which forms CO_2 and NADH from formate (Linnane and Wrigley, 1963) and a hydrogenase moiety, which reoxidises the NADH formed by formate dehydrogenase by producing molecular hydrogen from H^+ (Oltmann and Stouthamer, 1975). The formate dehydrogenase and hydrogenase moieties of

FHL may be uncoupled under certain physiological conditions. In the presence of low levels of oxygen (but those which allow the induction of the FHL system), hydrogenase activity is inhibited (Back et al, 1946) and the formate deydrogenase can donate reducing equivalents to the electron transport chain or for the reduction of fumarate to succinate (Haddock and Jones, 1977; Rosenberg et al, 1975). Whilst the hydrogenase moiety can oxidise reducing equivalents from sources other than formate dehydrogenase such as amino acid biosynthesis (Krebs, 1972).

Succinate is required anaerobically for certain biosynthetic reactions (Creaghan and Guest, 1978). As certain key enzymes of the cycle are repressed under anaerobiosis (e.g. 2-oxoglutarate dehydrogenase [Amarasingham and Davis, 1965]), the production of succinate by the tricarboxylic acid cycle is not possible. Therefore, succinate is formed anaerobically by the action of certain tricarboxylic acid cycle enzymes acting in reverse (compared to their aerobic activity), and the induction of a fumarate reductase activity distinct from the aerobic succinate dehydrogenase activity (Hirsch et al, 1963). Phosphoenolpyruvate (PEP) is carboxylated to oxaloacetate by PEP carboxylase (Doelle, 1975), an enzyme activated by acetyl CoA (Izui et al, 1970). Oxaloacetate is then converted to fumarate by the action of malate dehydrogenase and fumarase, resulting in the oxidation of one NADH molecule per fumarate molecule formed. Fumarate can then be reduced by a variety of reducing equivalents (e.g. protoporphyrinogen, Jacobs and Jacobs, 1978; formate/NADH,

Oltmann and Stouthamer, 1975) to succinate by the action of fumarate reductase. The fumarate reductase activity has a menaquinone containing electron transport chain, that facilitates the production of 1 mole ATP per mole fumarate reduced (Guest, 1979; Kroger, 1978). Therefore, in terms of reducing equivalents oxidised and ATP generated, the production of succinate and ethanol from phosphenolpyruvate is identical.

Electron Transport Chains

E.coli K_{12} is capable of synthesising a variety of inducible anaerobic electron carriers (Hackett and Bragg, 1983). However, for this work, where no exogenous electron acceptors were present under anaerobiosis, the only systems of physiological importance are those associated with the fumarate reductase and formate hydrogen lyase activities, which both use internally generated electron acceptors (ie. fumarate and H^+). The electron carriers for these systems are not as well characterised as the aerobic electron transport chain, but it is known that the fumarate dehydrogenase moiety of FHL uses cytochrome b and is not quinone mediated (Linnane and Wrigley, 1963), whereas the ATP generating electron transport chain of fumarate reductase is known to require menaquinone (Guest, 1979). For a discussion of the anaerobic electron transport chains of E.coli K_{12} see the reviews by Haddock and Jones (1977) and Hackett and Bragg (1983).

Scope of this Project

The first observation of a switch in physiology in a facultative aerobe growing in aerobic and anaerobic environments was made by Pasteur (1861), who noted that the addition of oxygen reduced the rate of product formation and glucose utilization. In later years (Doelle, 1975), it was found that anaerobic growth proceeded with a much smaller energy yield compared with that obtained during respiratory metabolism of glucose to carbon dioxide and water. In association with the difference in energy formation per mole of glucose degraded, the cell utilized glucose at a slower rate aerobically in comparison to that consumed during anaerobic growth. This interplay between energy availability and glucose uptake was termed the 'Pasteur Effect' and attempts have been made to account for its regulation, but no-one has been able to account for the effect in its entirety. The examination of the switch between aerobiosis and anaerobiosis has been attempted many times before, but either because of the use of a non-stable environment (ie. experiments carried out in batch culture) or the examination of one specific part of metabolism during the switch, no overall picture of the physiological changes which occured during the transition to anaerobiosis has been obtained.

Most studies have been concerned with the role of the enzyme phosphofructokinase as the regulator of the Embden-Meyerhoff pathway, which is reflected in the extensive reviews given to this enzyme (eg. Bloxham and Lardy, 1973; Uyeda, 1979). However, this attempt to explain the 'Pasteur

Effect' in strict terms of allostery paid no attention to the contribution to the switch, by enzyme induction and repression; and later work has indicated that phosphofructokinase plays only a limited role in the regulation of the switch (Doelle, 1981). The high resolution of proteins isolated from total cell extracts of aerobically and anerobically grown cells (Smith and Neidhardt, 1983; Smith and Neidhardt, 1983a) obtained from 2-D gel work, indicated that to a large extent, the 'Pasteur Effect' is mediated by the induction and repression of specific proteins.

Many regulators of the induction/repression of protein synthesis have been suggested; internal redox potential (Showe and DeMoss, 1968; Wimpenny, 1969; McPhedran et al, 1961), attenuation control (Buck and Ames, 1984), redox potential of a component of the respiratory chain (DeGroot and Stouthamer, 1970) or a hierachy of control proteins (Clark and Cronan, 1980). The possibility that oxygen itself might directly mediate the switch may be discounted, as it has been shown (Lilius, 1978) that synthesis of anaerobic proteins can take place in the presence of oxygen. Also the possibility that ATP levels may control the synthesis of proteins is unlikely, due to the large fluctuations in the level capable in aerobic growth without the induction of anaerobic systems, and the fact that there is no connection between fluctuations in ATP levels and protein induction during the transition to anaerobiosis (Cole, Wimpenny and Hughes, 1967). But apart from these two exceptions, no evidence has appeared to narrow down the field of possible effectors.

Control of the switch by internal redox balance is the most obvious of control mechanisms, as the change in internal redox potential, as described by the NAD^+/NADH ratio, is a major effect of anaerobiosis (Wimpenny and Firth, 1972). This method of control has been favoured in the past, but recently with the increasing awareness of the complexity of the switch and the discovery of 'anaerobic control proteins' such as *adhR* and *fnr* gene products (Clark and Cronan, 1980; Shaw and Guest, 1982) which are responsible for the induction of anaerobic enzyme systems, has led to the development of new theories on the control of the switch. One of these is the attenuation control of DNA transcription (Yanofsky, 1981) by modified nucleotides on tRNA molecules (Buck and Ames, 1984). In this hypothesis it is theorised that the oxidation state of an unknown component of the electron transport chain determines the form of a specific nucleotide on the tRNA, and that the state of this residue (modified or unmodified) determines whether the transcription of specific genes is attenuated. It was outside the scope of this work to prove or disprove these theories, but it was hoped that the immediate effector of the switch could be identified.

Therefore, the aims of this work were to provide an overall description of the switch in physiology from aerobiosis to anaerobisis in *E.coli* K_{12} using carbon flux as a surrogate of enzyme activity, and by the use of mutant strains with lesions in pertinent pathways to ascribe an effector to the induction/repression of protein biosynthesis observed during the transition to anaerobic metabolism.

To achieve this it was decided that a strictly defined environment was required, as pH (Wood, 1961), media composition (Wimpenny and Firth, 1972) and growth rate (Teixera de Mattos and Tempest, 1983) all can effect the physiology of microbes. To obtain the required definition of environment a glucose limiting minimal medium was used in the chemostat culture of the test organism. The use of chemostat culture enabled the maintenance of a stable environment (Herbert, Ellsworth and Telling, 1956), and the use of a minimal medium ensured a consistancy of media preparation not possible with rich media.

The facultative aerobe Escherichia coli K_{12} was chosen as a test organism as it was genetically and biochemically well characterised, and strains already existed which contained the genetic lesions of interest (Bachmann and Low, 1980).

The level of aeration was followed by noting the ratio of air to N_2 in the effluent gases, rather than monitoring redox potential or the DOT of the medium. This was because over large ranges of oxygen availability redox potential of cultures varies very little (Wimpenny and Necklen, 1971), also as the composition of the culture medium changes due to the accumulation of fermentation end products, the response of the redox electrode would change (Wimpenney and Necklen, 1971). DOT was deemed unsuitable to measure the supply of O_2 to the medium because near anaerobiosis the DOT falls to zero, even though O_2 is still being supplied (Wimpenney and Necklen,

1971). Furthermore, both redox potential and DOT record the amount of oxygen in the medium rather than the amount of 02 supplied. Therefore, this would allow different DOT or redox potential readings from cultures supplied with the same amount of oxygen, depending upon their affinity for oxygen.
The relationship between oxygen supply rate and the oxygen tension in the influent gases may be expressed by the following equations:-

Let C = concentration of dissolved oxygen

C_S = Saturating concentration of dissolved oxygen

t = time

$(dc/dt)_S$ = oxygen solution rate (ie. the rate of oxygen transfer from the gas to the liquid phase)

A = area of interface between gas and liquid

For the oxygen solution rate or supply rate (Finn, 1954)

$$\left(\frac{dc}{dt}\right)_S = 0\ A(C_S - C) \qquad (1)$$

Where 0 = a constant, dependent upon aeration conditions

Let T_L and TG (mm.Hg) be the oxygen tensions in the liquid and gas phases respectively.

Then $T_L = FC$ (2)

and $T_G = FC_S$ (3)

where F is a constant for a given medium

From (1), (2) and (3)

$$\left(\frac{dc}{dt}\right)_s = K(T_G - T_L) \qquad (4)$$

where $K = OA/F$

It follows from equation (4) that as long as K remains constant (ie. constant temperature, constant agitation and constant gas flow rate), the oxygen supply rate is dependent upon the oxygen tension in the gas phase of the influent gases.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Tryptone, yeast extract and 'Bacto' agar were from Difco Laboratories, West Molesey, Surrey.

D-Glucose and Folin-Ciocalteau reagent were obtained from Fisons Ltd., Loughborough, Leics.

Inorganic chemicals and solvents were obtained from B.D.H. Ltd., (Poole, Dorset), Fisons Ltd. (Loughborough, Leics.) or James Burrough Ltd. (London).

[^{14}C]-Leucine was purchased from Amersham International, Bucks.

Acetate enzyme assay kits were obtained from Boehringer Corporation, Lewes.

All other amino acids, dyes, antibiotics, bovine serum albumin, enzymes, co-enzymes and all other biochemicals were purchased from Sigma (London) Ltd., Poole, Dorset.

Gas chromotaography columns and packing materials were obtained from Phase-Sep Ltd., Chester, Cheshire.

'Aquasure' scintillant was purchased from New England Nuclear, Boston, Mass., USA.

Carbon dioxide and Hydrogen generators, palladium catalyst, anaerobic indicators and anaerobic jars were all obtained from B.B.L., Microbiology Systems, Cockeysville, MD. USA.

All gases were supplied by BOC limited.

Model 8310 gas chromatograph was supplied by Perkin-Elmer Ltd., Beaconsfield, Bucks.

The 104 gas chromatograph was supplied by Pye-Unicam Ltd., Cambridge, as was the CDP1 integrating computer.
'Meterate' flow meters were obtained from Glass Precision Engineering Ltd. of Hemel Hempstead, Herts.

Peristaltic pumps were the 12,000 varioplex from LKB (Sweden) or the 501 of Watson and Marlow (Falmouth).

The infra-red CO_2 spectrophotometer was from The Analytical Development Co. Ltd., Hoddeson.

The fermentor apparatus was by New Brunswick Scientific Inc., New Jersey, USA.

GENERAL TECHNIQUES

Spectrophotometry

A Cary 210 dual beam recording spectrophotometer was used routinely for assays coupled to changes in optical density. The temperature of the cuvette compartment was controlled by circulating water from a Grant SE15 water bath. Quartz cuvettes of 1cm light path and 1ml working volume, or disposable plastic cuvettes of 1cm light path and 4ml working volume were used.

Determination of pH

The pH values of solutions were routinely determined using a radiometer type PHM26pH meter, fitted with a GK2302 combined electrode and reference cell.

Sterilisation

Inorganic salts and carbon sources were heat sterilised in an autoclave or, for the chemostat basal medium by live steam. Where the heat sterilisation of a mixture may have resulted in

precipitation of, or degradation of a salt or carbon source, these were sterilised separately and added back to the mixture aseptically, after cooling.

All biochemicals and heat labile compounds were sterilised by filtration.

All glassware was heat sterilised and dried *in situ* in the autoclave.

Scintillation Counting of ^{14}C

An Intertechnique SL-30 liquid scintillation spectrometer was used, employing the pre-set window for ^{14}C counting. The scintillation fluid used for samples was 'Aquasure'. Cells were pelleted at 6000g for 10 minutes and then the pellet transfered to plastic scintillation vials and covered with 5ml scintillation fluid.

Counting efficiency was determined by adding a known amount of the [^{14}C]-Leucine to 5ml of scintillation fluid and comparing the expected to actual counts per minute.

CO_2 Analysis

Analysis was performed using an infra-red spectrophotometer. Baseline was set using technical grade O_2 free N_2 and the meter calibrated using a technical grade CO_2/N_2 mixture containing 0.5% CO_2.

MICROBIOLOGICAL TECHNIQUES

Media

L.B. (per litre)	Tryptone	10g
	Yeast extract	5g
	NaCl	8g

The pH was adjusted to 7.4 prior to autoclaving.

M9 (per litre)	Na_2HPO_4	6g
	KH_2PO_4	3g
	NaCl	0.5g
	NH_4Cl	1g

1ml of Sterile 0.1M $MgSO_4$ was added after autoclaving.

Both L.B. and M9 media were solidified by the addition of 1.8% 'Bacto' agar before autoclaving. Where required amino acids, carbon sources, antibiotics and co-factors were sterilised beforehand and added aseptically to the cooling, autoclaved medium.

Concentration of amino acids was 20µg/ml for the L Form or 40µg/ml for the DL form.

Tetrazolium indicator medium was prepared as described by Bochner and Savageau (1977):-

(Per litre)	Buffer	100ml
	TTC	25mg
	'Bacto' Agar	18g
	Tryptone	1.25g

0.5% of absolute alcohol was added as carbon source after autoclaving.

The Composition of the T.I.M. buffer was:-

(Per litre)	K_2HPO_4	70g
	KH_2PO_4	30g
	$MgSO_4$	1g

R Medium (per litre)	Tryptone	10g
	Yeast extract	1g
	NaCl	8g
	'Bacto' Agar	18g

2ml of sterile 1M $CaCl_2$ and 5ml of sterile 20% D-glucose was added after autoclaving.

R-Top medium was prepared as R medium but using only 8g/litre of 'Bacto' agar.

F-Top (per litre)	NaCl	8g
	Agar	8g

Media for chemostat experiments were made up in 60L volumes and stored at about pH 6.0 in a chilled container. The composition of the media per 60 litres was:-

M9	60L
$MgSO_4$	14.8g
L-Tryptophan	1.2g
Streptomycin	6.0g
Thiamin	60 mg
D-Glucose	210 g

This media was constructed to be glucose limiting for both aerobic and anaerobic growth.

The M9 base was sterilised separately at pH6.0 by live steam. The pH was dropped to prevent loss of ammonium salts as ammonia. The other components of the mixture were sterilised separately and added to the M9 base after cooling.

M.C.Buffer 0.1M $MgSO_4$

5.0mM $CaCl_2$

Culture Conditions

Small scale (i.e. <15ml) aerobic batch cultures were grown with vigorous aeration in bubbler tubes (i.e. boiling tubes through which sterile air may be passed). Small scale anaerobic batch cultures were grown in sealed universal tubes filled to the brim with freshly prepared media.

Plates were grown anaerobically by placing them inside a B.B.L. anaerobic jar, after activating the H_2 and CO_2 generator with 10ml of water. Anaerobic conditions were present when the indicator strip changed colour from blue to white. Aerobic conditions for plate cultures were maintained quite adequately by incubating them in air.

Large scale aerobic cultures were grown in ribbed conical flasks with vigorous shaking and were harvested no later than mid-log phase. Large scale anaerobic batch cultures were grown in conical flasks under a N_2 atmosphere.

All cultures were incubated at 37°C.

For chemostat culture conditions please see page 45.

Maintenance of Strains

Parental stocks of E.coli K_{12} strains were routinely held at 4°C, after overnight aerobic growth at 37°C on L.B. slopes.

From these stocks single colonies were isolated and tested for the parental phenotype before plating on L.B. for regular use.

Preparation and storage of P1 lysates

Lysates were prepared as described in 'Experiments in Molecular Genetics' (ed. J.H.Miller, 1972). One drop of an overnight culture of a strain of E.coli was subcultured into 5ml of L.B. containing 5mM$CaCl_2$. The cells were grown aerobically at 37°C until early log phage. Then approximately 10^7 P1 phage was added to 1ml of the culture and incubated at 37°C for 20 minutes. 2.5ml of molten R-top agar (kept at 45°C) was then added and the whole mixture immediately plated on fresh R medium.

The plates were incubated overnight at 37°C. At the end of this time the soft agar layer was scraped into a small centrifuge tube. The remaining agar surface was washed with 1ml L.B., and the wash also added to the centrifuge tube. A few drops of chloroform were added and the mixture vortexed vigorously for 30 seconds. The tube was then centrifuged at 3000 r.p.m for 5 minutes and the supernatant, which contained the lysate, was collected and stored at 4°C in a glass bijou bottle over a few drops of chloroform.

Titration of P1 lysates

This was peformed as described in 'Preparation and storage of P1 lysates', except serial dilutions of lysate were added to the early log phase culture in L.B.$CaCl_2$. After overnight incubation at 37°C plaques were clearly visible, and as 1 P1 phage generated 1 plaque the number of P1 phage (p.f.u.) per ml lysate was determined.

Transduction with P1 lysates

5ml of an overnight culture of the strain to be transduced was resuspended in 5ml of M.C. buffer and aerated vigorously at 37°C for 15 minutes. 0.1ml of the suspended cells were added to 5 small test tubes and 0.1ml of a series of lysate dilutions (in the range 10^{10}-10^{7}p.f.u/ml) were added to 4 of the tubes. To the fifth tube no phage was added, and to a sixth tube only phage was added; these last two tubes being used as controls. The phage were preadsorbed for 20 minutes at 37°C before 0.2ml of 1M sodium citrate was added to each tube. 2.5ml of molten F-top agar was added to each tube before immediately plating onto a selection plate. The plates were incubated for 24 hours at 37°C before single colonies were picked off and tested for the required phenotype.

Determination of sex by phage sensitivity

Phage R17 has its attachment site on the male pili of E.coli K_{12}. Therefore, any strains that form plaques with R17 are Hfr, F^+ or F'.

0.1ml of an overnight culture of the strain to be tested was resuspended in 1ml of L.B. containing 5mM $CaCl_2$. 2.5ml of molten R-top agar was then added to the suspended cells and immediately plated onto fresh R medium. After allowing the agar to set, a drop of R17 lysate was dried onto the plate. After overnight incubation at 37°C, sensitive strains showed an area of lysis where the phage was spotted.

Selection of spontaneous mutations

Overnight cultures of the strain to be mutated were resuspended in 10ml of L.B. and grown aerobically at 37°C to mid log phase. The cells were then spun down at 3000 rpm for 5 minutes and resuspended in 0.5ml L.B. 0.2ml of the suspended cells were then spread out and dried onto selection plates, before overnight incubation at 37°C. After this time, single colonies were picked off and replated onto the selection medium, and after overnight incubation, single colonies from these plates were used to prepare stocks of the mutant strain.

Hfr Conjugation

Overnight cultures of the male and female strain were resuspended in L.B. and grown aerobically at 37°C to mid log phase. The cells were then mixed in the ratio 1:20::Hfr:F^- and 2ml of this mixture placed in a sterile 75ml conical flask. The mixture was incubated at 37°C with gentle shaking for 3 hours, followed by centrifugation at 3000 r.p.m. for 5 minutes and resuspension of the cells in 0.4ml of sterile, distilled water. Four freshly prepared selection plates were then taken, and the male and female parental strains were spread out on two separate plates, whilst 0.2ml of the resuspended cells were spread onto the remaining plates. After the cells had dried onto the agar, they were incubated at 37°C overnight.

If the conjugation was successful the plates containing the parental strains would show no growth, whereas the other two plates would show single colonies of exconjugants. The single colonies were then picked off and re-isolated on the selective medium before preparing a stock of the exconjugant strain.

Culture density determination

Culture turbidities (apparent absorbances due to light scattering) were determined against a medium blank at 550nm.

Culture dry weight determination

Duplicate samples of 25ml were removed from exponentially growing samples and centrifuged for 10 minutes at 6,000g in pre-weighed centrifuge tubes. The pellet was resuspended and washed twice in distilled water before drying at 100°C for 24 hours. After cooling in a desiccator the tube were weighed and the increment in weight due to the cell pellet calculated. Comparison of dry weight with the culture density at 550nm allowed the construction of a dry weight vs. culture absorbance standard curve.

Preparation of crude cell extracts

Bacteria were harvested in mid-exponential phase in a sorvall RC-5 centrifuge (I.Sorvall Inc., Conneticut, USA) fitted with a 6 x 250ml angle head at 6,000g for 10 minutes at 4°C. Cell pellets were resuspended in 40mM potassium phosphate buffer, pH 7.4 by shaking with one quarter the original volume. Resuspended pellets were pooled and the washing procedure repeated once. Cell pellets were either stored at -20°C or used immediately for the preparation of crude cell extracts. Cells were disrupted using an M.S.E. Model 150 ultrasonic disintegrator fitted with a 1cm diameter probe. The cell suspension (maximum 5ml) was contained in a

10ml or 25ml beaker in broken ice. The suspension was sonicated at full power for 5 x 1 minute periods with 1.5 minute cooling periods between each minute of sonication.

The crude cell extract produced after sonication was assayed immediately for its enzyme activities.

CHEMOSTAT TECHNIQUES

Description of Apparatus

Media Supply (see fig. 4)

The basal M9 medium was batched up and sterilised by live steam in the 60 litre holding vessel, supplements were added and after thorough mixing chilled to 4°C. Media were passed down into a 20 litre aspirator by the opening of a steam sealed valve. From the aspirator the media was pumped, at a rate monitored by the burettes into the chemostats via the feed lines.

Gas supply

Influent gases were sterilised by filtration and supplied to the chemostat by two separate lines and spargers (Photo 2; A). The air line was a permanent fixture of stainless steel, whereas the N_2 line and sparger were improvised from silicone tubing. The temporary nature of the N_2 supply apparatus was necessary as the chemostats would return to their previous usage after this project had ended.

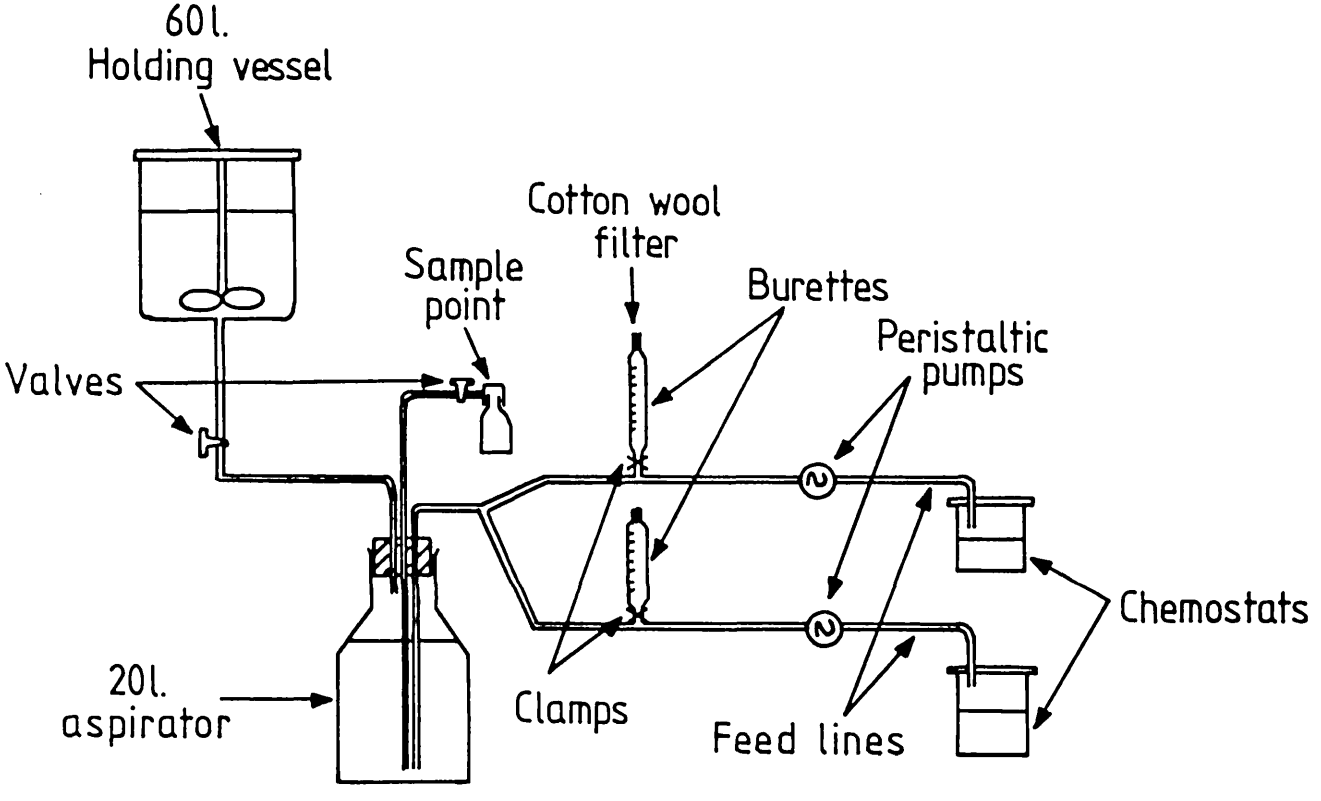


Diagram of Continuous Culture Apparatus

Figure 4

The N_2 sparging system proved to be stable within a run, but variations between runs were observed.

Agitation was provided by the single 9cm diameter impeller (Photo. 2;B) and baffles (Photo. 2;C).

Chemostat Headplate Configuration (see photograph 1)

The chemostat headplate and base were made from stainless steel, the head plate and glass walls of the chemostat were separated by a rubber 'O' ring, providing an air-tight seal.

Apertures (A) and (B) were for pH and DOT probes respectively. Aperture (C) was a sealed tube which held a thermometer to monitor the culture temperature. (D) was the inoculation port. (E) and (F) were for the influent gases, whilst (G) was for effluent gases. Line (H) was the medium overflow line and line (I) was the tubing which led to the rapid sampling port. Line (K) was for 10% NaOH for the maintainance of pH stasis. Line (L) was sealed. Line (M) was the medium feed line, and (N) was the stirrer shaft attachment.

Inoculation

The inoculum was 100ml of an overnight batch culture, grown aerobically at 37°C in medium identical to the chemostat medium. The inoculum was allowed to grow aerobically at 37°C as a batch culture for 5 hours before the media pumps were switched on and continuous culture started.

Determination of apparatus integrity

The integrity of the continuous culture apparatus was assessed each day by withdrawing samples from the aspirator and the chemostats. The aspirator sample was checked for sterility by passing it through a sterile 0.22 micron nitrocellulose filter and incubating it overnight on an L.B. plate. The chemostat samples were checked for the required phenotype by incubation on selective media.

Sampling from Culture apparatus

Samples were withdrawn from the aspirator at the sampling port indicated in photograph No.2.

Samples from the chemostats for assay of internal $NAD^+/NADH$ ratios were taken from the rapid sampling port (Figure 5). A sterile sample bottle was attached to the port and then stood in liquid N_2. A positive pressure from a syringe applied to point (A) on the sampling port (figure 5) caused the evacuation from the teflon tubing of any medium. A negative pressure then applied by the syringe caused a small volume ($<$1ml) of medium to enter the sample bottle, where it was immediately frozen.

All other samples from the chemostats were taken from the sampling ports in the overflow lines.

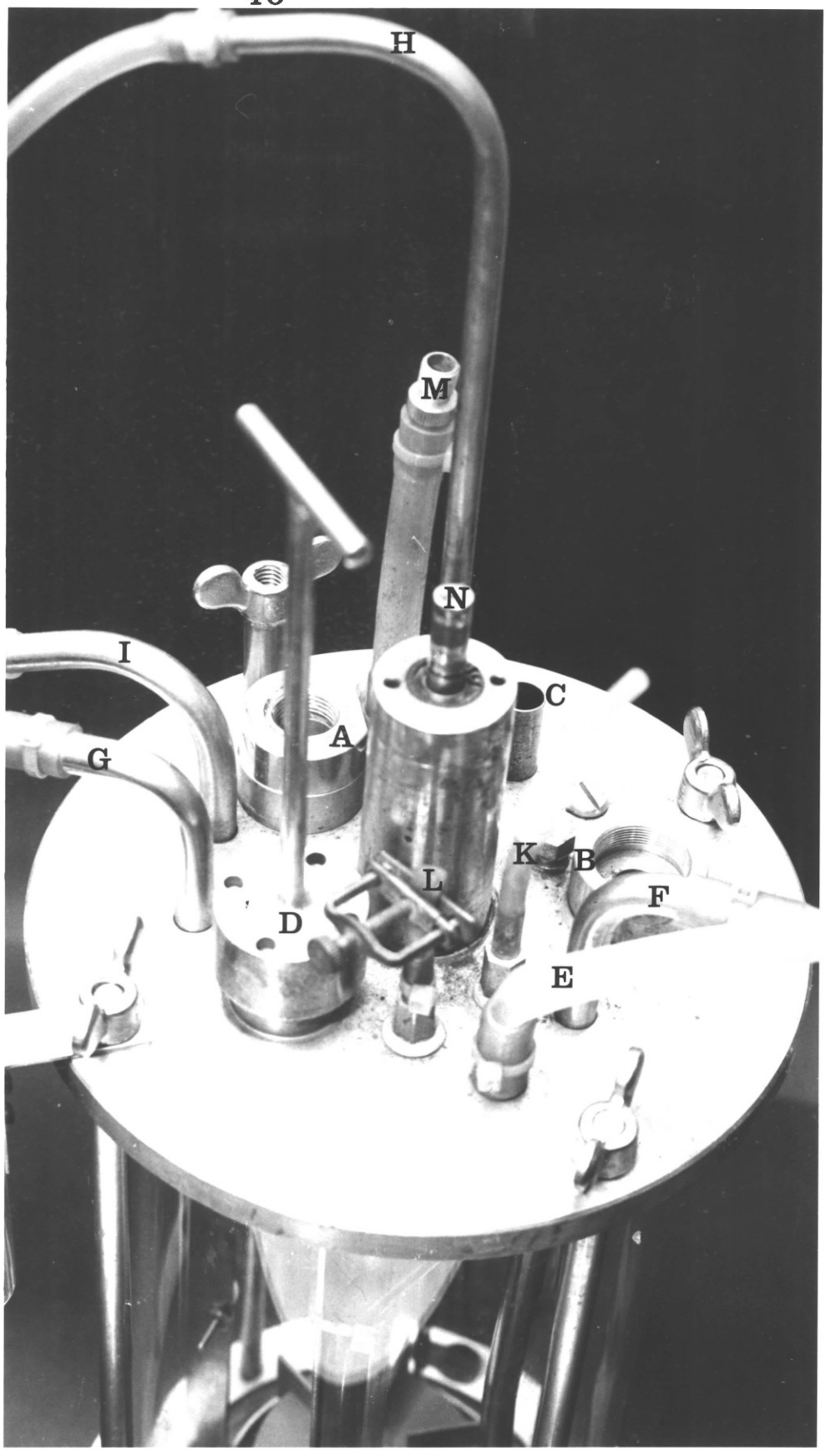


Photo 1

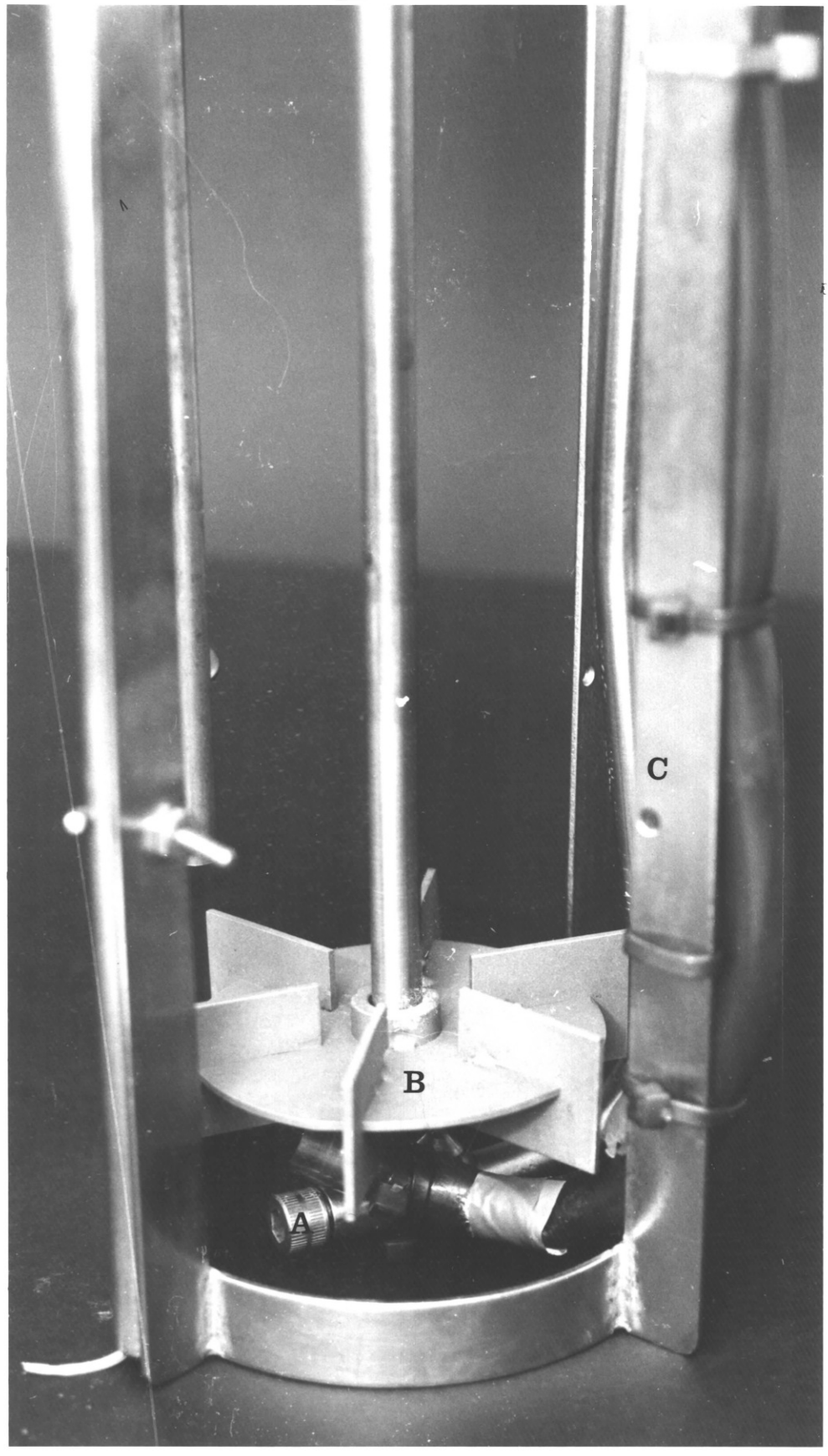


Photo 2

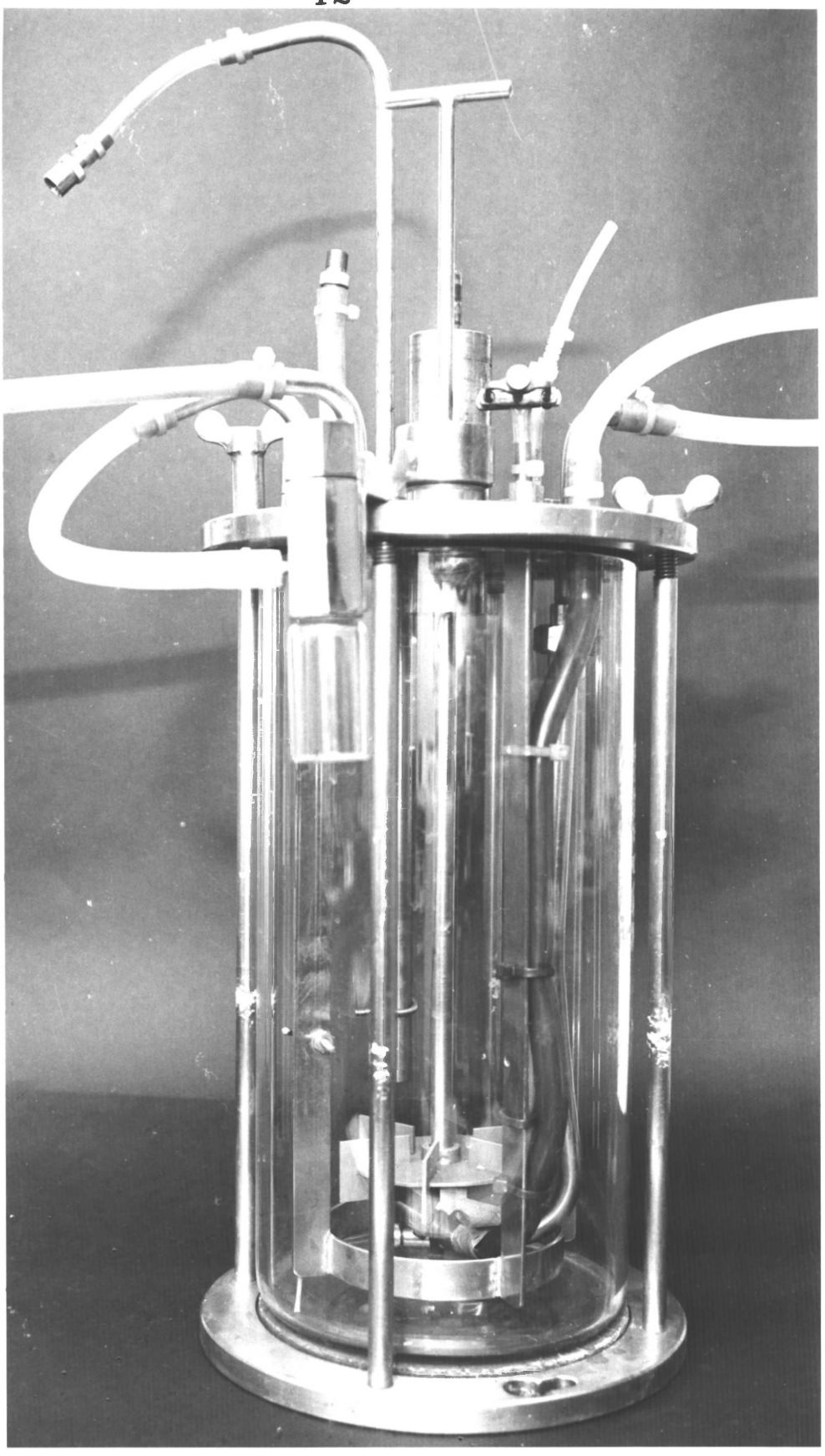


Photo 3

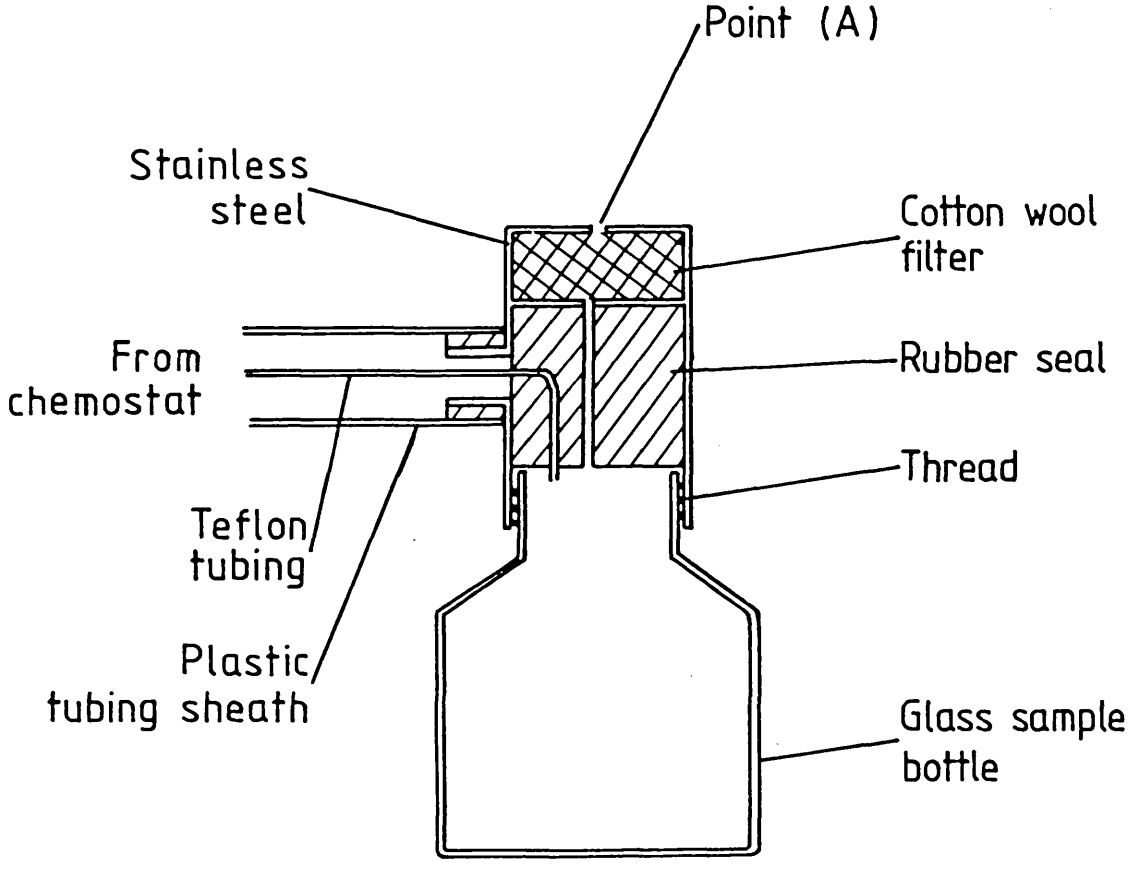


Diagram of Rapid Sampling Apparatus Figure 5

Determination of flow rate

The burettes in the feed line from the aspirator (Fig. 4) were allowed to fill up with medium and then the line to the aspirator clamped off. The time in which a fixed volume of medium was withdrawn from the burettes by the pump was measured, and from this figure the flow rate was calculated in litres/hr

pH Control

The pH was controlled automatically to within 0.05 of a pH unit by Pye-Unicam (Cambridge) probes (model 465-35-90) filled with 'Viscolyte B' attached to a Kent Electronic Instruments (Surrey) control system. When the pH dropped, a pump switched on and delivered 10% NaOH to the chemostat, until the required pH was reached. As the medium and the fermentation end products were acidic, no system was required to correct increases in pH.

Aeration

Fully aerobic conditions were described as 100% air in the influent gas, sparging underneath the impeller revolving at 200 r.p.m.

Fully anaerobic conditions were described as 100% N_2 in the influent gas sparging underneath the impeller revolving at 200 r.p.m.

Any aeration states between these two extremes were described by the ratio of N_2 to air in the influent gases supplied at a total flow rate of 1.25l/min.

Gas flow control

Gas flows were controlled by 'meterate' gas flow meters to within 5% of the set flow rate.

Chemostat culture conditions

The chemostat culture conditions were:-

Media flow rate(F)	0.25 lhr^{-1}
Culture volume(V)	2.5 l
Set pH	7.0
Agitation	200 r.p.m.
Temperature	37°C
Gas flow rate	1.25 $lmin^{-1}$
Growth rate(μ)	0.1 hr^{-1}

Temperature Control

Temperature was held at 37°C thermostatically by the New Brunswick water bath.

BIOCHEMICAL TECHNIQUES

Enzyme Units

One enzyme unit is that amount of enzyme which catalyses the transformation of 1μmol of substrate per minute (or the formation of 1μmol product per minute) under the assay conditions described. The molar extinction co-efficients used to calculate enzyme units are given with each assay of enzyme activity described.

Preparation of spent bacterial culture of supernatants

Spent bacterial culture supernatants were prepared by centrifugation of cultures at 6000g for 10 minutes. The supernatant was collected and stored at -20°C or assayed immediately.

D-Glucose assay

These assays were performed by the ortho-toluidine method of Ceriotti and Frank (1969). The assay measures the increase in absorbance at 630nm.

1ml of sample was thoroughly mixed with 3ml of 6% (v/v) ortho-toluidine in glacial acetic acid in a test tube and placed in a boiling water bath for 10 minutes. After this time the absorbance was measured against a reagent blank at 630nm and compared with a standard curve obtained from a series of standards in the range 0.1-0.4 mg D-Glucose/ml.

D-Lactate assay

These assays were performed as described by Gawehn and Bergmeyer (1974), and measured the increase in absorbance at 340nm due to the reduction of NAD^+ in the presence of D-lactate dehydrogenase and D-lactate.

$$\text{D-lactate} + NAD^+ \longrightarrow NADH + \text{pyruvate}$$

The assay mixture contained, 3.0ml glycine buffer (0.5M glycine, 0.4M hydrazine) pH 9.0, 0.2ml 41mM NAD^+ and 0.1ml of a spent culture medium sample. The absorbance at 340nM was

read and the reaction started by the addition of 0.01ml D-lactate dehydrogenase (1,500u/ml). After incubation at room temperature for 30 minutes the absorbance at 340nm was read and the change in absorbance calculated. A standard curve of absorbance at 340nm against D-lactate concentration was constructed.

Pyruvate assay

Pyruvate was assayed by the method of Von Korff (1969). The assay measured the decrease in absorbance at 340nm due to the oxidation of NADH by pyruvate in the presence of lactate dehydrogenase.

$$\text{Pyruvate} + \text{NADH} \longrightarrow \text{Lactate} + \text{NAD}^+$$

The assay mixture contained in 1ml final volume: Potassium phosphate buffer, pH 7.4, 10μmol; NADH 0.15μmol; pyruvate standard solution or sample, <0.1μmol. The absorbance at 340nm was read, and the reaction started by the addition of lactate dehydrogenase (5,000 units). The absorbance at340nm was read after 3 minutes when the value was constant and the change in absorbance 340nm calculated. A standard curve of $O.D._{340nm}$ against pyruvate concentration was constructed.

Ethanol assay

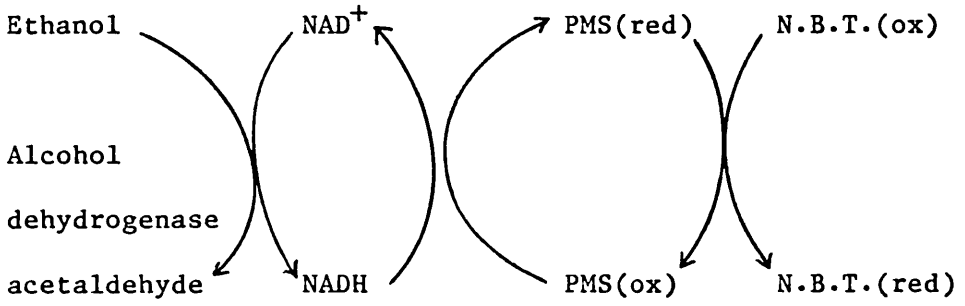
Ethanol was estimated as described by Bernt and Gutmann (1974) and was measured by noting the increase in $O.D._{340nm}$ that takes place due to the reduction of NAD^+ by ethanol in the presence of alcohol dehydrogenase.

$$\text{Ethanol} + \text{NAD}^+ \longrightarrow \text{Acetaldehyde} + \text{NADH}$$

The assay mixture contained, 3.0ml buffer (75mM tetrasodium pyrophosphate, 7.4mM semicarbazide and 21mM glycine) pH 8.7, 0.1ml 24mM NAD^+ and 0.2ml spent culture supernatant sample or ethanol standard solution, in the range of 0.05 - 0.2 ml/L ethanol. The $O.D._{340nm}$ was read and the reaction started by the addition of 0.02ml of alcohol dehydrogenase (9,000 u/ml). The $O.D._{340nm}$ was read after 30 minutes of incubation at 37°C when the value was constant and the change in $O.D._{340nm}$ calculated. A standard curve of $O.D._{340nm}$ against ethanol concentration was constructed.

Rapid Ethanol Test

This was an adaption of an alcohol dehydrogenase rapid test devised by Dr M.Payton (Pers. Comm.) and indicated the presence or absence of ethanol in a spent culture supernatant by the development of a blue colour from reduced nitro blue tetrazolium.



To 2ml of a spent bacterial culture supernatant was added 0.2ml, 24mM NAD^+, 0.2ml alcohol dehydrogenase (9,000u/ml) and 2ml PMS/NBT solution (0.2mg PMS, 2.0mgNBT in 50mM potassium phosphate buffer pH 7.0). After 5 minutes incubation in the dark at 37°C a blue colouration indicated presence of ethanol whereas a yellow colouration indicated absence of ethanol.

Acetate Asssay

An assay was devised for the estimation of acetate in spent bacterial supernatants by Clarke and Payton (1982), based on the acetate kinase assay of H.U.Bergmeyer and H.Môllering (1974). See Results Section, 'Acetate assay'.

Assay of Enzyme Activites

Cuvettes were pre-warmed to 30°C in the constant temperature water jacketed cuvette holder in the spectrophometer. All assays were performed at 30°C. Reagents were held at 30°C in the water bath where possible, or stored on ice; the enzyme extract was kept on ice from the time of its preparation until addition to the reaction mixture.

The initial linear rate of change of extinction which was the maximal rate of change of extinction in these assays was used to measure enzyme activity. Activity was measured with extracts diluted such that the initial rate of change of extinction was proportional to the enzyme concentration in the reaction mixture.

Lowry Protein Estimation

Protein concentration was measured by the modified Lowry method of Miller (1959). Stock solutions were prepared containing:-

a) 0.5% w/v $CuSO_4 . 7H_2O$ in 1% w/v potassium tartrate

b) 10% w/v Na_2CO_3 in 0.5M NaOH

The stock solutions were mixed immediately prior to use in the ratio 10A:1B and 1ml of this mixture added to a 1ml sample of neutral protein solution containing between 0.04 and 0.2mg protein. After standing for 1 minute at room temperature, 3ml of a 1:10 dilution of Folin-Ciocalteu phenol reagent was added and the sample mixed thoroughly by vortexing. After a further 30 minutes at room temperature the $O.D._{560nm}$ of the solution was determined against a reagent blank. Standard curves were prepared for each set of assays, using a standard solution of bovine serum albumin.

Alcohol Deydrogenase Assay

Alcohol dehydrogenase was assayed by a modification of the method described by Racker (1955). The reaction was followed by measuring the increase in OD_{340nm} due to the reduction of NAD^+ by ethanol in the presence of alcohol dehydrogenase.

The reaction mixture contained 0.1ml 24mM NAD^+; 0.75ml Buffer (75mM tetrasodium pyrophosphate, 7.5mM semicarbazide, 21mM glycine) pH 8.7 and 0.1ml of crude cell extract. The reaction was started by the addition of 0.2ml absolute alcohol after three minutes pre-incubation, and the increase in OD_{340nm} measured. The molar extinction coefficient of NADH is $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ at 340nm.

2-Oxoglutarate Dehydrogenase Assay

Total 2-oxoglutarate dehydrogenase activities were measured by the method of Guest and Creaghan (1973), but using

NAD^+ instead of APAD (3-acetylpyridine adenine dinucleotide) as cofactor. The reaction was followed by measuring the increase in OD_{340nm} due to the reduction of NAD^+ by 2-oxoglutarate in the presence of 2-oxoglutarate dehydrogenase.

The reaction mixture contained in a total volume of 1ml; Tris HCl buffer, pH 8.5, 120μmol;, L-Cysteine HCl, 3.0μmol; Coenzyme A, 0.08μmol; Thiamine pyrophosphate, 0.20μmol; NAD^+, 0.8μmol and crude cell extract. Sodium 2-oxoglutarate was added after three minutes pre-incubation to start the reaction and the increase in OD_{340nm} was measured.

NADH Oxidase Assay

The assay was based on that described by Young and Wallace (1976), but using potassium phosphate buffer instead of Sucrose-Tris-Mg acetate buffer and using a crude cell extract instead of a membrane preparation. The reaction was followed by measuring the decrease in the OD_{600nm} due to the reduction of DCPIP by NADH in the presence of NADH oxidase.

The reaction mixture contained 2.5 ml, 0.1M Potassium phosphate buffer pH 7.5; 0.05ml, 10mM DCPIP and 0.1ml crude cell extract. After three minutes preincubation, the reaction was started by addition of 0.02ml, 0.1M NADH and the decrease in OD_{600nm} was measured. The molar extinction coefficient of DCPIP is 21 x 10^3 M^{-1} cm^{-1} at 600nm.

Lactate Dehydrogenase Assay

The assay was performed as described by Bergmeyer (1974). The reaction was followed by measuring the decrease in absorbance at 340nm.

due to the oxidation of NADH by pyruvate in the presence of lactate dehydrogenase.

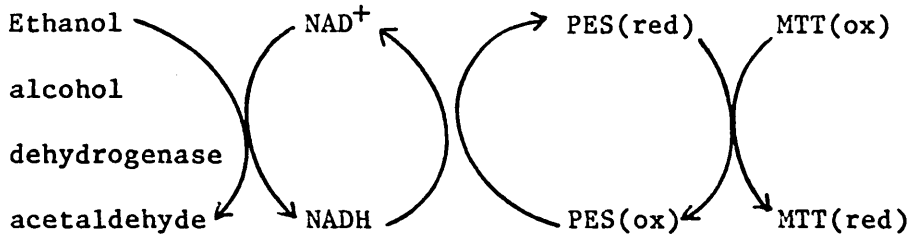
The reaction mixture contained 2.8ml, Buffer (0.05M triethanolomine, 5mM EDTA) pH 7.6; 0.04ml, 38mM NADH and 0.1ml Crude Cell extract. After 3 minutes preincubation, the reaction was started by the addition of 0.04ml, 15mM pyruvate and the decrease in OD_{340nm} was measured.

NAD^+ and NADH Assays

Samples withdrawn from the rapid sampling port were treated by a modification of the dinucleotide extraction method described by Heber and Santarius (1965).

Frozen samples of culture in preweighed bottles were taken out of the liquid N_2 bath and weighed. From the increase in weight thus determined the volume of the sample could be calculated and an equal volume of 0.2M HCl (for NAD^+ extraction) or 0.2M NaOH (for NADH extraction) added to the sample. Extraction of the specific dinucleotide took place by placing the sample bottles in a sand bath at 100°C for 10 minutes. Cell debris was then spun down at 6000g for 5 minutes and the supernatant containing the extracted dinucleotides were stored on ice.

The extracts containing their specific dinucleotide species were then assayed in duplicate by the method of Bernofsky and Swan (1973). This method utilized a cycling mixture composed of MTT, PES, ethanol and alcohol dehydrogenase. The reaction was followed by measuring the increase in aborbance at OD_{570nm} due to the reduction of MTT.



The rate of reduction of MTT is proportional to the concentration of co-enzyme. The reaction mixture contained: 0.1ml, 1.0M Bicine-NaOH buffer, pH 8.0; 0.25ml acid/alkaline extract; 0.25ml neutralising acid (0.1M HCl)/alkali (0.1M NaOH); 0.1ml, 16.6mM PES; 0.1ml 4.2mM MTT; 0.1ml absolute alcohol; and0.1ml 40mM EDTA. The mixture was preincubated for 3 minutes before the reaction was started by adding 0.02ml alcohol dehydrogenase (500u/ml) and the increase in absorbance at 570nm was measured. A standard curve in the range of 1.0 - 0.2μM of co-enzyme was constructed.

As the extraction system was not designed for bacterial culture, a series of tests were run on this assay system. For results see page 65.

GLC TECHNIQUES

Packing and Conditioning of Columns

All columns were packed in the laboratory by plugging one end of the column with glass wool, applying a negative pressure at that end and pouring the packing materials in at the other end. Even packing was ensured by gently vibrating the column until no more settling of packing material was visible. The other end was then plugged with glass wool.

Columns were conditioned by placing them in the gas chromatographs, but leaving the detector ends free. The oven temperature was then increased slowly to 10°C above the maximum operating temperature and left for 24 hours, whilst a stream of carrier gas was passed through the columns.

Derivitisation of spent culture medium supernatants

Spent culture medium supernatants were methylated prior to extraction into chloroform. 2ml of methanol and 0.75ml 50% (v/v) H_2SO_4 was added to 2ml of a standard or sample and incubated at 50°C for 30 minutes in a sealed glass universal bottle. After cooling on ice, 0.5ml chloroform was added, the methylated compounds were then extracted into the chloroform layer by vigorous shaking for 5 minutes.

GLC Parameters for Methylester analysis

For the analysis of methyl formate, methyl acetate, methyl pyruvate, methyl lactate and methyl succinate operating parameters of the Pye 104 series G.L.C. were set as suggested by Drummond and Shama (1982):-

Sample Volume	1µl
Carrier gas	N_2
Carrier gas flow rate	40ml/min
Detector temperature	150°C
Injector temperature	maximum
Oven temperature range	40°C 130°C

Ramp rate	16°C/min
Initial Lag time	1 minute
Detector	Dual F.I.D.
Columns	Dual, 2 Metre, glass
Packing Material	Diatomite C, 10% DEGA, 2% H_3PO_4

The assays were performed by comparing the peak areas (calculated by the CDP1 integrator) of standards to those of the samples.

GLC Parameters for H_2 analysis

Analysis of H_2 in the effluent gas stream from the chemostats was performed on a series 8310 GLC, fitted with T.C.D. and a gas sampling valve. Chromatograph parameters were set in the following manner:-

Carrier gas	N_2
Carrier gas flow rate	40ml/min
Sample volume	0.5ml
Oven temperature	40°C
Detector temperature	80°C
Dectector current	Setting 2
Columns	Dual, 1 metre, glass lined metal
Packing material	Porapak R

Assays were performed by measuring peak heights and comparing them to the peak height produced from a H_2/N_2 analytical grade mixture containing 0.25% H_2.

CHAPTER 3

RESULTS

Acetate Assay

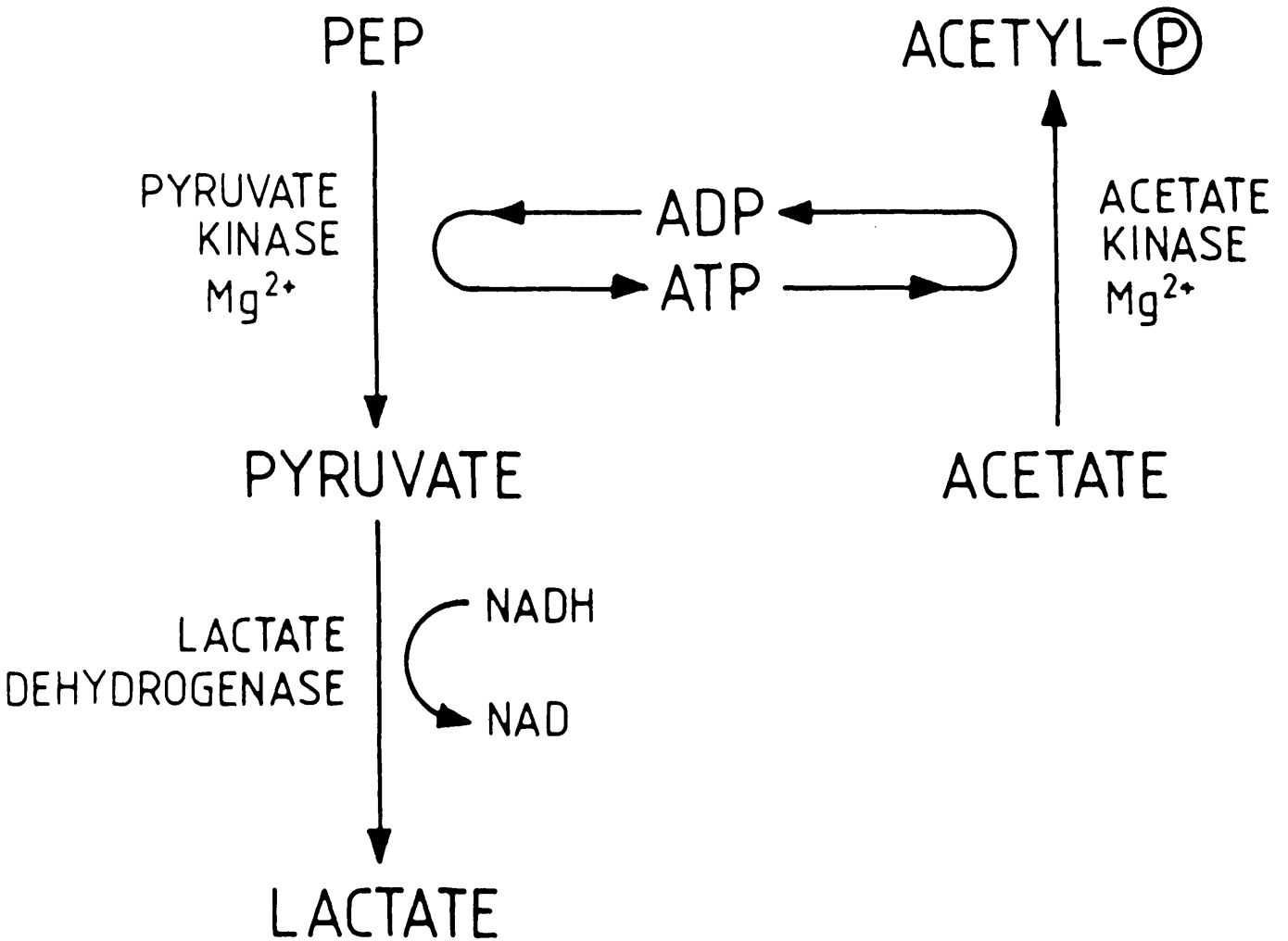
Before the acquisition of the PYE-104 GLC, a method for assaying acetate in spent bacterial culture supernatants enzymatically was required. It was found that previously published methods and commercially available enzymic assay kits were either unsuitable or too expensive. It was, therefore, decided to develop an alternative acetate assay based on a previously reported assay for acetate kinase. The assay involved the conversion of acetate to acetylphosphate using acetate kinase and ATP, and the measurement of the ADP produced by a pyruvate kinase/lactate dehydrogenase couple, which facilitated the direct spectrophotomeric assay at 365nm or 340nm of NADH disappearance (Fig 6).

This assay was cheaper and more convenient than currently available assays and unlike the preceding indicator reaction (Bergmeyer and Môllering,1966) produced a linear standard plot.

Assay procedure

The assay mixture contained the following in a final volume of 3.02 ml (final concentration in parentheses): Potassium phosphate buffer, pH 7.6 (152mM); Magnesium chloride (20mM); PEP (0.5mM); NADH (0.5mM); ATP (5.0mM); Pyruvate kinase/lactate dehydrogenase mixture (4.0 and 5.7 U/m, respectively); Sample (8 -16μM acetate).

BASIS OF ACETATE ASSAY Figure 6



The reagents were mixed and allowed to equilibrate for 10 minutes at 37°C. After noting the absorbance at 365nm (A_1), 20µl of acetate kinase were added to give a final concentration of 1.3 U/ml. The reagents were again mixed and incubated at 37°C for 60 minutes before noting the final absorbance at 365nm (A_2). The change in absorbance (= $A_1 - A_2$) was then calculated and related to concentration by reading from a standard plot.

Effect of pH upon linearity of response (Fig. 7)

The assay was performed at four pH values, 8.0, 7.6, 7.0 and 6.5 to ascertain the pH which gave the maximum and most linear response to acetate concentration. The optimum pH range was found to be fairly broad with little difference exhibited between pH values 8.0 to 7.0. However, at pH 6.5 a marked decline in sensitivity and linearity was observed.

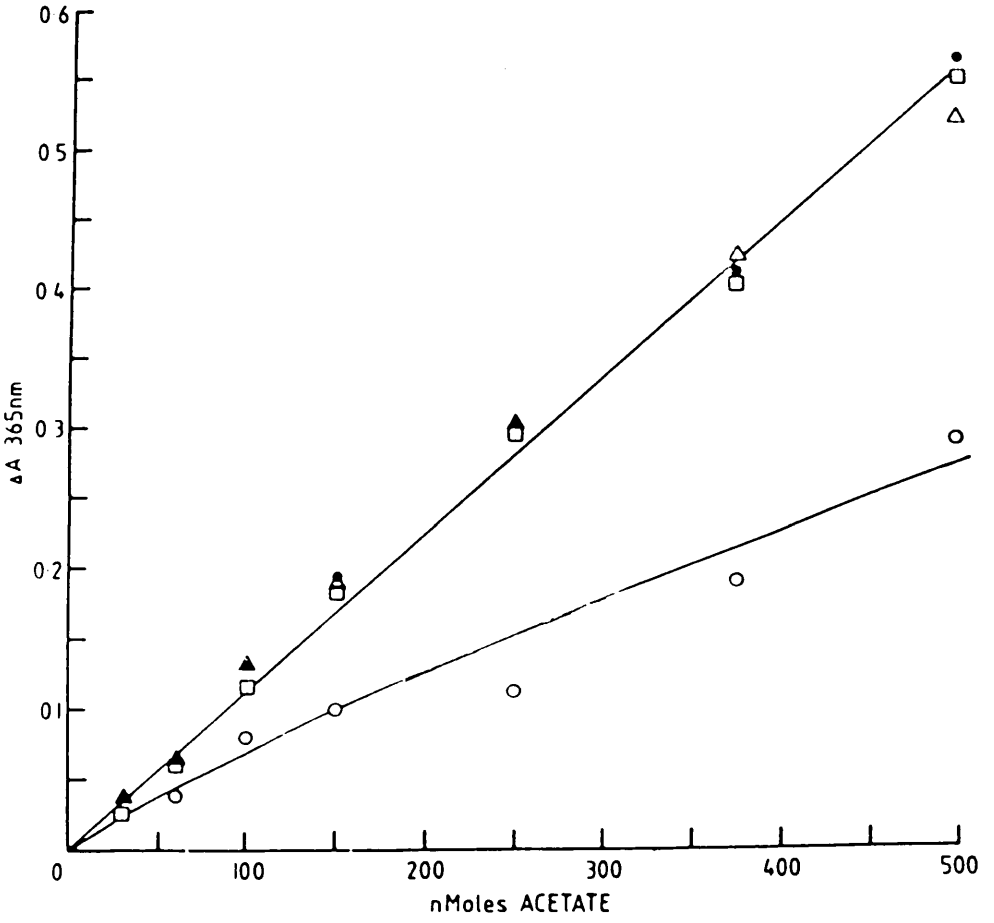
An optimum pH of 7.6 was chosen as it lay in the middle of the pH range which permitted both the reaction to proceed to completion (within the reaction time of 60 minutes) and the production of a reproducibly linear standard plot. At this pH the assay was sufficiently sensitive easily to detect acetate levels as low at 50µM.

Interference with the assay

The assay was found to be very specific the only reported side specificity of this acetate kinase from E.coli is towards propionate which exhibits only 10% of the activity with acetate (Rose et al, 1954). However, it was noted that high

DETERMINATION OF pH OPTIMUM FOR ACETATE ASSAY Figure 7

● pH 8.0
□ pH 7.6
△ pH 7.0
○ pH 6.5



levels of pyruvate in spent bacterial culture supernatants interfered with the assay by reducing the levels of NADH in the reaction mixture. The inclusion of a reaction blank which lacked acetate kinase indicated pyruvate levels and changes in absorbance due to this were taken into account. If pyruvate levels were exceptionally high however, (ie. those levels resulting in an initial $O.D._{365nm}$ below 0.80), the addition of higher concentrations of NADH was necessary to maintain NADH in excess in the cuvette.

Fig. 8 shows the effect of the addition of pyruvate (1.5mM final conc.) to a set of acetate standards. It was seen that in this example at higher acetate concentrations (above 250nmol/per cuvette) there was a deviation from the normal linear plot due to depletion of NADH, resulting in insufficient amounts of NADH to allow the coupled acetate-dependant reaction to proceed to completion. This was compensated for however, by the addition of more NADH; Fig 8 shows the effect of the addition of doubled the amount of NADH. Alternatively, the problem of 'pyruvate interference' was both indicated and overcome by routine assay of appropriate dilutions of the unknown sample.

Comparison of Assay with other methods

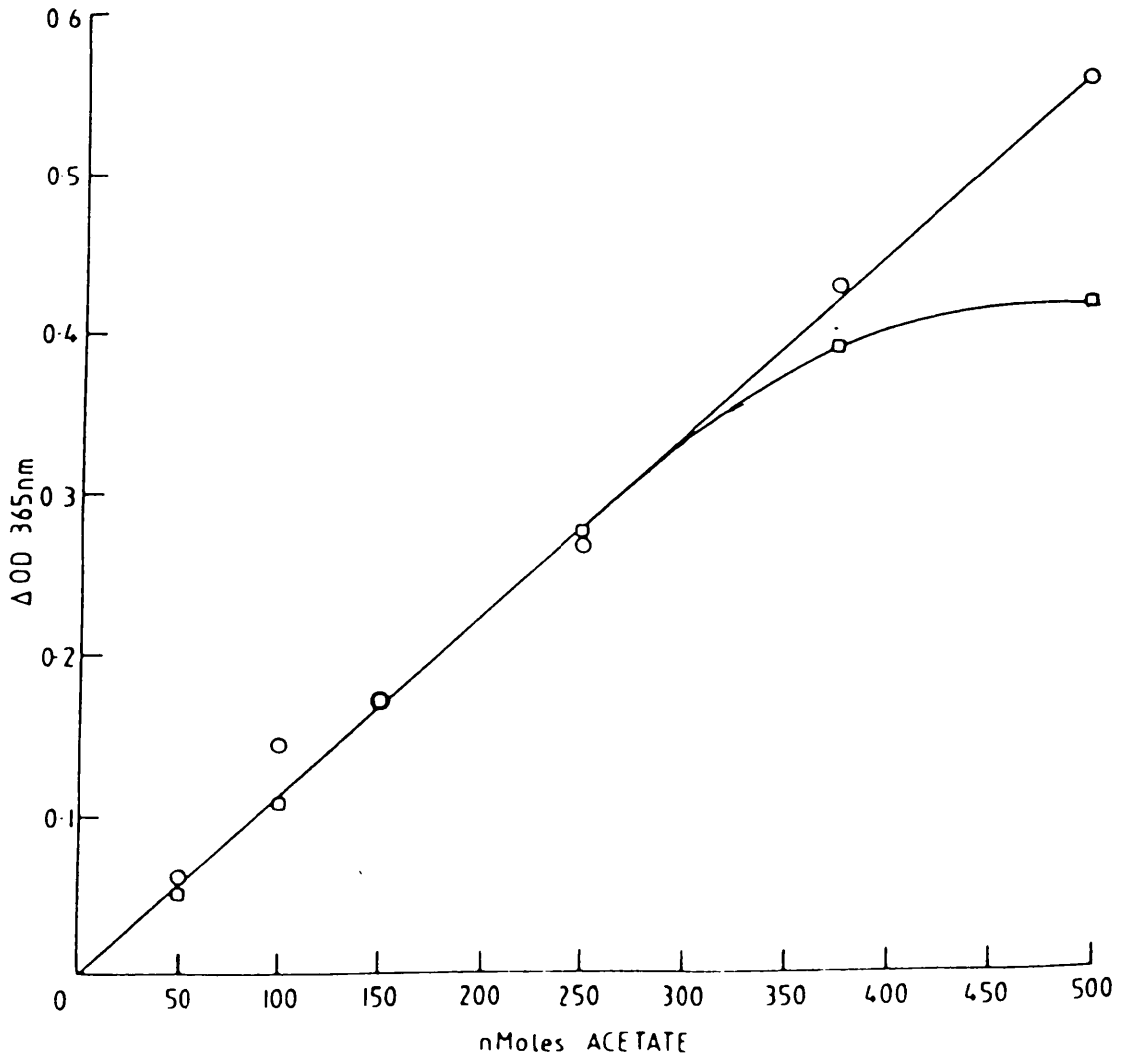
In order to compare the results of this assay with other available methods, samples of spent culture supernatants were prepared from overnight anaerobic cultures of E.coli IY13 and assayed by three different methods; the assay described here, gas chromatography by the method of Drummond and Shama (1982) and the 'preceding indicator reaction' marketed by Boehringer-

EFFECT OF 1.5mM PYRUVATE IN ACETATE STANDARDS UPON THE LINEARITY OF THE STANDARD PLOT

Figure 8

□ Normal NADH concentration

○ Double NADH concentration



Mannheim. The results shown in table 4 indicated that the assay described here was as accurate as both the other assays.

Assay of w/t and Ace$^-$ strains

Determinations of acetate levels in spent culture supernatants of strains harbouring mutations in those genes responsible for growth on, and formation of acetate were also undertaken. The consequences of these mutations were that acetate kinase (ack$^-$)-deficient strains (eg. JRG1061) showed reduced levels of acetate when grown aerobically on glucose (0.2%) minimal medium, whilst phosphotransacetylase (pta$^-$)-deficient strains and facA strains deficient in both acetate kinase and phosphotransacetylase activities (eg. JRG1086 and JRG1078, respectively) showed little or no acetate production when grown aerobically on glucose minimal medium (Brown et al, 1977).

Samples of spent culture supernatants were prepared from overnight aerobic cultures of a wild-type strain of E.coli K_{12} CSH62, and three mutant E.coli K_{12} strains, JRG1061 (ack$^-$), JRG1078 (facA) and JRG1086 (pta$^-$), and were assayed by the method described here. The results in Table 5 showed levels of acetate predicted by the strain's phenotype. JRG1061 (ack$^-$ showed levels of acetate lower than those of the wildtype CSH62 culture, whereas JRG1078 and JRG1086 (facA and pta$^-$, respectively) showed little or no acetate production.

Table 4 Apparent levels of Acetate in the Spent Culture Medium of E.coli IY13 determined by Three Different Methods

Sample	Assay Method	Apparent acetate Concentration (mM)
IY13	This work	26
IY13	Gas Chromatography	23
IY13	Boehringer-Mannheim assay kit	26

Table 5 Apparent levels of Acetate in the Spent Culture Medium of mutant strains of E.coli K_{12}

Strain	Relevant Genotype	Acetate Concentration(mM)
CSH62	w/t	15.0
JRG1061	*ack*$^-$	9.0
JRG1078	*facA*	1.0
JRG1086	*pta*$^-$	0.0

Discussion

This assay has been routinely used in the laboratory to determine acetate production by many strains of E.coli K_{12} and other organisms (results not shown), consistently giving values identical with those obtained by other assay proceedures, but more rapidly and at a lower cost.

Although the description of the assay has been confined to the determination of acetate in spent culture supernatants, the assay is valid for a wide range of biological fluids and in the food, brewing and dairy industries.

Experiments into suitability of NAD^+ and NADH assay

The accurate assay of internal metabolic pool sizes is determined by two factors, the rapid harvesting and inactivation of the sample and the specificity and sensitivity of the assay system. As NAD^+ and NADH pool sizes were so small (Wimpenny and Firth, 1972), the cycling assay of Bernofsky and Swan (1973) was adopted (see 'Materials and Methods'). However this assay was not devised for bacterial cultures where the harvesting of sufficient biomass in a short period of time (Harrison et al (1974) have shown that NAD^+ and NADH pool sizes in bacteria responded rapidly to alterations in dissolved oxygen concentration) is a problem. Therefore, a rapid 'freeze-clamp' method of sampling was developed (page 52) along with a modified extraction procedure (see 'Materials and Methods').

This section describes experiments to assess the efficiency of the combination of rapid sampling, extraction and cycling assay systems in the determination of internal levels of NAD^+ and NADH in E.coli K_{12}.

Specificity of extraction

Standard solutions of 10μM NAD^+ and NADH underwent the extraction procedure for the other dinucleotide (ie. NAD^+ was treated with hot 0.2M NaOH, whilst NADH was treated with hot 0.2M HCl), and the solutions were then assayed. The results shown in Table 6 indicated that no dinucleotide was found in either sample after treatment.

Therefore, the extraction of dinucleotide species by the methods described is specific for those dinucleotide species.

Reproducibility of assay

Two samples taken from a mid-log phase culture of CSH3B grown aerobically in L.B. were extracted and assayed along with standard solutions of NAD^+ and NADH as described. It was seen from the results in Table 6 that NAD^+ and NADH standards responded equally well to the assay, and therefore, that only one dinucleotide species was required to draw a standard curve. It was also shown that the levels of dinucleotide in the samples (Table 6) closely agreed with each other.

Table 6

Reproducibility and specificity of the NAD^+ and NADH assay

Sample	Treatment	O.D./min
10μM NAD^+	Alkali	0.00
10μM NADH	Acid	0.00
1.00μM NAD^+	Acid	3.92×10^{-2}
0.75μM NAD^+	"	2.88×10^{-2}
0.50μM NAD^+	"	1.72×10^{-2}
0.30μM NAD^+	"	1.31×10^{-2}
0.20μM NAD^+	"	0.71×10^{-2}
1.00μM NADH	Alkali	4.09×10^{-2}
0.75μM NADH	"	2.93×10^{-2}
0.50μM NADH	"	1.85×10^{-2}
0.30μM NADH	"	1.04×10^{-2}
0.20μM NADH	"	0.75×10^{-2}
Sample(A) NAD^+	Acid	0.160×10^{-2}
Sample(B) NAD^+	"	0.154×10^{-2}
Sample(A) NADH	Alkali	0.051×10^{-2}
Sample(B) NADH	"	0.049×10^{-2}

Therefore, it was deduced that the reproducibility of the assay was sufficient for use in the determination of *in vivo* levels of NAD^+ and NADH.

Determination of Chemostat parameters

Dilution rate

To maintain a chemostat culture the dilution rate must be below the culture's maximum growth rate on that medium (Herbert, Ellsworth and Telling 1956). Therefore the growth rate of a series of strains of *E.coli* (among them the slowest growing lesion, *ubiA*) was determined in batch culture growing on the chemostat medium. Although batch and continuous cultures behave differently, the exponential growth rate, in batch, gives an indication of maximum growth rate in continuous culture.

Overnight aerobic cultures of the sample strains grown in chemostat medium were resuspended in that medium and incubated either aerobically or anaerobically as described in materials and methods. Growth was followed by aseptically withdrawing aliquots of the cultures and measuring their optical density at 550nm. From these results growth curves were drawn on semi-log paper and the doubling time during the exponential phase of growth determined. As :-

$$\mu = \frac{\ln 2}{td} \quad \text{.....} \quad \text{where } \mu = \text{Growth rate; } hr^{-1}, \; td = \text{Doubling time; hr}$$

(Pirt, 1975)

The exponential growth rates were calculated and are shown in Table 7. From these results a dilution rate of 0.1/hr was chosen as being well inside maximum growth rates.

Gas flow rate

A chemostat culture of CSH3B growing in the stated glucose limiting chemostat medium and at a gas flow rate of 2.5 l/min was found to 'wash-out' under anaerobic conditions at a dilution rate of 0.1/hr. As CSH3B had been demonstrated to grow both aerobically and anerobically on this medium in batch culture (see Table 7) it was deduced that the failure to maintain a culture anaerobically was due to factors in continuous culture not present in batch culture, namely dependence upon growth rate and gas flow.

It had already been shown that the set dilution rate of 0.1 l/hr was within the organisms maximum growth rate (see Table 7), therefore the gas flow was examined. The requirement of E.coli for CO_2 had long been established (Rockwell and Highberger, 1927), however, in most cultures CO_2 is provided by catabolism of glucose (see Figure 3), but in this case, as glucose levels were so low (ie. 0.4% w/v) the sparging of N_2 could have reduced pCO_2 levels below the 3×10^{-5} Atm.required for growth (Lwoff and Monod, 1947).

This hypothesis was examined by growing a culture of CSH3B aerobically in a chemostat set up as described in 'Materials and Methods' (except a gas flow rate of 2.5 l/min was employed) and observing the OD_{550nm} as a surrogate for

Table 7 Exponential growth rates of batch Cultures of E.coli K_{12}

Strain	Relevant genotype	Growth condition	Growth rate/hr
CSH3B	w/t	Anaerobic	0.25
CSH3B	w/t	Aerobic	0.63
CC3310	*ubiA*	Aerobic	0.29
CC2807B	*adhR*	Aerobic	0.51

biomass, as the gas stream was changed from 100% air, to 100% N_2, to 100% CO_2, to 4% CO_2 in N_2.

The results obtained (Figure 9) showed that the supply of 4% CO_2 allowed the maintenance of an anerobic culture at a dilution rate of 0.1/hr.

As CO_2 in effluent gases was to be measured, the incorporation of CO_2 in the influent gases was to be avoided, lest it swamp the small amounts of metabolically generated CO_2. The problem of maintenance of adequate pCO_2 levels was finally overcome simply by reducing the influent gas flow rate to 1.25 l/min which allowed the anaerobic, chemostat culture of E.coli K_{12}.

Fermentation Balances of Mutant Strains

Mutant strains of E.coli K_{12} collected in the laboratory were grown aerobically overnight in L.B. and subcultured into a small volume of minimal M9 medium (containing 0.2% glucose, 0.02% acetate and supplemented with the required amino acids and co-factors as described in 'Materials and Methods') and grown aerobically and anaerobically. Mid-log phase cultures were spun down at 6000g for 5 minutes and the resulting spent culture supernatant assayed for metabolic intemediates by a combination of gas chromatography and enzyme assay.

The results (in Tables 8 and 9) showed the carbon flux from glucose to end products, and indicated the pathways operating under those conditions. Because of the differences in carbon flux in the wild-type strains it was concluded that unreported genetic lesions were present and that to overcome this, constructions of mutant phenotypes in an isogenic

Effect of N_2 and CO_2 on the anaerobic growth of CSH3B

Figure 9

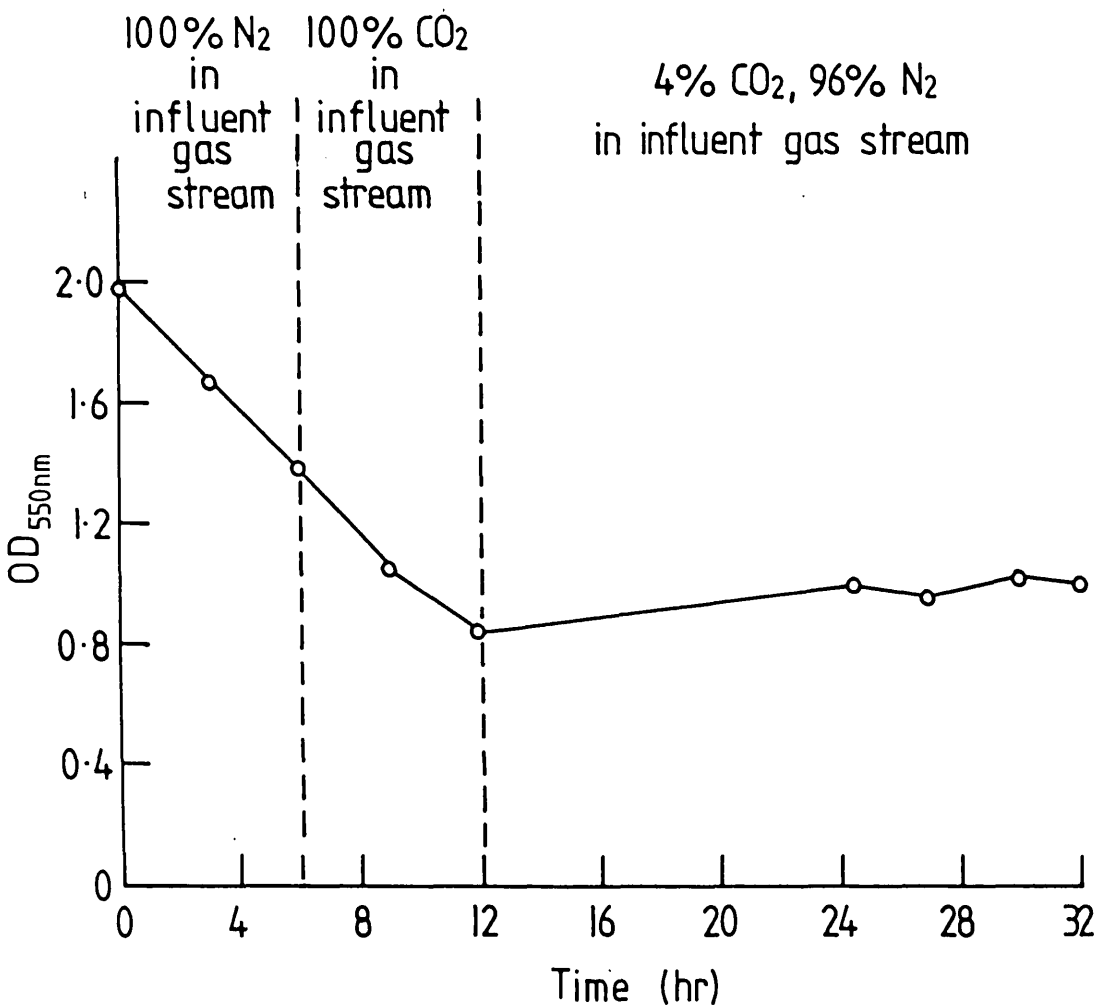


Table 8 Results of fermentation balances of spent culture supernatants of Aerobically grown cells

Strain	Formate (μM)	Acetate (mM)	Pyruvate (mM)	Lactate (mM)	Succinate (μM)	Ethanol (mM)	Glucose (mg/ml)
IY13 (W/t)	102	3.8	N.D.[a]	N.D.	N.D.	N.D.	0.12
CSH3B (W/t)	N.D.	2.9	N.D.	N.D.	N.D.	N.D.	0.04
6161B (pfl^-)	N.D.	3.1	N.D.	N.D.	N.D.	N.D.	0.05
LCB900 (ana1)	N.D.	3.47	N.D.	N.D.	N.D.	N.D.	0.06
LCB901 (ana2)	N.D.	8.75	N.D.	N.D.	N.D.	N.D.	0.02
HΔ13 (aceE)	N.D.	N.D.	7.96	4.4	N.D.	N.D.	0.10
IY12 (ndh^-)	N.D.	2.3	N.D.	6.6	N.D.	N.D.	0.12
AN385 (ubiA)	1222	9.6	0.18	2.5	93	0.33	0.02
DC272 (adhE)	1075	5.4	N.D.	N.D.	N.D.	0.59	0.06
JRG1086 (pta^-)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.08
JRG1061 (ack)	N.D.	1.0	N.D.	N.D.	N.D.	N.D.	0.08

(a) N.D. = Not Detected

Table 9 Results of Fermentation balance of Spent Culture Supernatants of Anaerobically grown cells

Strain	Formate (mM)	Acetate (mM)	Pyruvate (mM)	Lactate (mM)	Succinate (mM)	Ethanol (mM)	Glucose (mg/ml)
IY13 (wlt)	14.7	11.2	N.D[a]	2.75	0.9	7.6	N.D.
CSH3B (wlt)	20.4	4.3	N.D.	N.D.	0.9	6.2	N.D.
6161B (pfl⁻)							
LCB900 (ana1)			NO GROWTH				
LCB901 (ana2)							
HΔ13 (aceE)	16.5	9.2	N.D.	4.34	2.26	5.27	N.D.
IY12 (ndh⁻)	8.4	6.7	N.D.	8.76	0.9	5.2	N.D.
AN385 (ubiA)	18.5	14.5	N.D.	1.35	1.35	8.5	N.D.
DC272 (adhE)	11.6	11.1	N.D.	0.45	1.3	9.9	N.D.
JRG1061 (ack⁻)							
JRG1086 (pta⁻)			NO GROWTH				

(a) N.D. = Not Detected

NB. Throughout strain construction it was assumed that only one recombinant event would occur in HfrH conjugations. However, this need not be the case, and much larger amounts of donor DNA may have recombined into the host chromosone than was at first thought. Therefore the attempts to transfer lesions into an isogenic background by conjugation cannot have been successful.

background were to be undertaken.

Strain Construction

Rationale of Strain Construction

Strains were constructed either by P1*kc* transduction or HfrH conjugation or a combination of both methods. Transduction and conjugation of genetic characters both relied on the positive selction of that character in the extransductant/exconjugant. Where the character to be transferred did not exhibit a positively selectable phenotype in the extransductant/exconjugant, a co-transferable selectable character (ie. a character lying within 2 minutes of the desired character for transduction, or 5 minutes for conjugation) was used, and single colony isolates of this phenotype were assayed for the required co-transfered phenotype. The percentage of colonies containing the co-transfered phenotype was dependant upon the distance between the two chromosomal characters (Miller, 1974).

Construction in E.coli IY13

IY13 was chosen as a host for strain construction because, as a revertant of IY12, the *ndh*$^-$genotype was already in that background, also it was *Trp*$^-$ which allowed the cotransduction of *adhR*, *anal* and *ana2* genotypes with *Trp*$^+$. Initial problems with the transduction of genetic characters into the IY13 background were attributed to the use of a P1*vir* phage, which gave an efficiency of plating (e.o.p.) of

1.0 - 0.1 on certain strains of E.coli B, C and W. However, with E.coli K_{12} the e.o.p. dropped as low as 10^{-7} with certain strains (Lennox, 1955). This, linked with the fairly low titres (10^8 - 10^9) produced from strains carrying the pertinent lesion, was thought to explain the unsuccessful transductions.

Therefore it was decided to use a P1kc phage, which produced an e.o.p. of 1.0 on E.coli K_{12} (Lennox, 1955) obtained from Porton Down. Lysates of strains containing those lesions to be transduced were prepared with P1kc as described, and titred at values ranging from 10^9 - 10^{11} pfu/ml dependent upon the strain. These lysates were then used in an attempt to transduce the required lesions into an IY13 background. Again, no success was recorded. After many further unsuccessful attempts where the transduction method was altered slightly (eg. increased preadsorption times; alteration of the pfu/ml in the transduction mixture; increased sodium citrate concentration etc.), the sensitivity of IY13 to phage P1kc was examined.

In the genotype provided by Dr I.Young for this strain, no mention of P1 resistance was given, therefore IY13 was assumed to be P1 sensitive. However, upon the attempt to produce plaques in a confluent lysis plate (Swanstrom and Adams, 1951) it became clear that IY13 was resistant to P1kc as no plaques formed after overnight incubation.

As P1kc was a large part of the attempt to construct strains, strain IY13 was abandoned as a host strain for these constructions.

Construction in E.coli CSH59B

CSH59B was chosen as host for strain construction as it was *pyrC*$^-$, that is it required uracil for growth (Beckwith *et al*, 1962). This lesion was 1 minute distant from *ndh* on the chromosome (Bachmann and Low,1980) and allowed the co-conjugation of *ndh*$^-$ and *pyrC*$^+$. Also the strain was *trp*$^-$ which allowed the cotransduction of *adhR*, *anal* and *ana2* with *trp*$^+$.

Construction of CC904

CC9 series strains were constructed by conjugation between CSH62B and AN385 as described in 'Materials and Methods' to provide an HfrH *ubiA ampA* strain for use in further strain constructions.

CSH62B (HfrH, *thi* , *ampA*) x AN385 (F^-, *thi* , *ubiA*, *rpsL*)

↓

CC9 Series

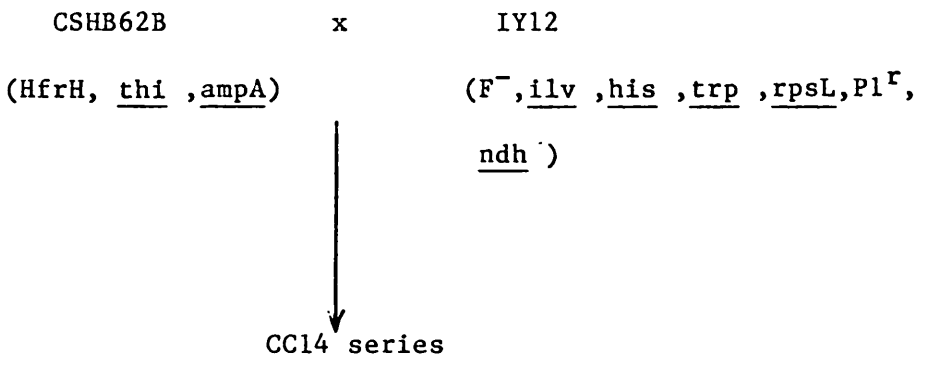
Inital selection of exconjugants was on L.B. ampicillin, streptomycin plates, selecting for ampicillin resistance with streptomycin resistance as the counter-selection.

Single colony isolates of exconjugants were then tested for sex by phage R17 sensitivity. As *ampA* at 94 minutes is close to the point of origin of HfrH, a high proportion (ie. 70%) of single colony isolates were found to be male.

The ubiA phenotype of the male exconjugants was then tested by growth tests on minimal M9 plates supplemented with either 0.2% glucose or 0.2% succinate. After incubating aerobically overnight those strains with the phenotype Glu^+, $Succ^-$ were purified and then stored on L.B. slopes. As ampA was only 3 minutes away from ubiA (minute 91) only 30% of the male exconjugants retained an ubiA phenotype; strain CC904 was selected for further use.

Construction of CC1410

CC14 series strains were constructed by conjugation between CSH62B and IY12 as described in 'Materials and Methods' to provide an Hfr ndh^- strain for further strain construction.



Inital selection of exconjugants was on L.B. ampicillin, streptomycin plates, selecting for ampicillin resistance and counter-selecting with streptomycin.

Single colony isolates of exconjugants were then tested for sex by phage R17 sensitivity. As ampA at 94 minutes is close to the point of origin of HfrH at 97 minutes, a high proportion (ie. 60%) of single colony isolates were found to be male.

The *ndh*$^-$ phenotype of the male exconjugants was then tested by overnight aerobic growth on minimal M9 plates supplemented with either glucose or mannitol. Those strains with the phenotype, Glu$^+$ Mntl$^-$ were purified and stored as slopes on L.B. medium. CC1410 was selected for further use.

Construction of strain CC1202

CC12 series strains were constructed by the transduction of a P1*kc* lysate of LCB900 into CSH59B as described in 'Materials and Methods'.

P1*kc*(LCB900) x CSH59B

(F^-, *thi* , *trp* , *pyrC*, *rpsL*, *rpoB*)

↓

CC12 series

As the *anal* phenotype, growth on glucose aerobically, but not anaerobically, is difficult to select for, the closely linked *trp*$^+$ marker of LCB900 (*anal* = minute 27; *trp*$^+$ = minute 27.5) was used to co-transduce the *anal* phenotype.

Initial selection of extransductants was on minimal M9 plates supplemented with thiamin, uracil and streptomycin. As *trp*$^+$ and *anal* were so close together, the *anal* phenotype of aerobically Glu$^+$; anaerobically Glu$^-$ was found in 50% of single colony isolates of the extransductants. CC1202 was stored on L.B. slopes and used for further study.

Construction of strain CC1307

CC13 series strains were constructed as for CC12 series strains, but using a P1*kc* lysate from LCB901 instead of lysate from LCB900. Co-transduction frequencies of *trp*$^+$ and *ana2* were the same as for *trp*$^+$ and *anal* (ie. 50% of single colony isolates of extransductants had the phenotype, aerobically, Glu$^+$; anaerobically, Glu$^-$). Extransductants with the *ana2* phenotype were stored on L.B. slopes. Strain CC1307 was selected for further study.

Construction of CC1103

CC11 series strains were constructed by the transduction of a P1*kc* lysate of DC272 into CSH59B.

P1*kc* (DC272) x CSH59B

(F^-, *thi* , *trp* , *rpsL*, *rpoB* *pyrC*)

↓

CC11 series

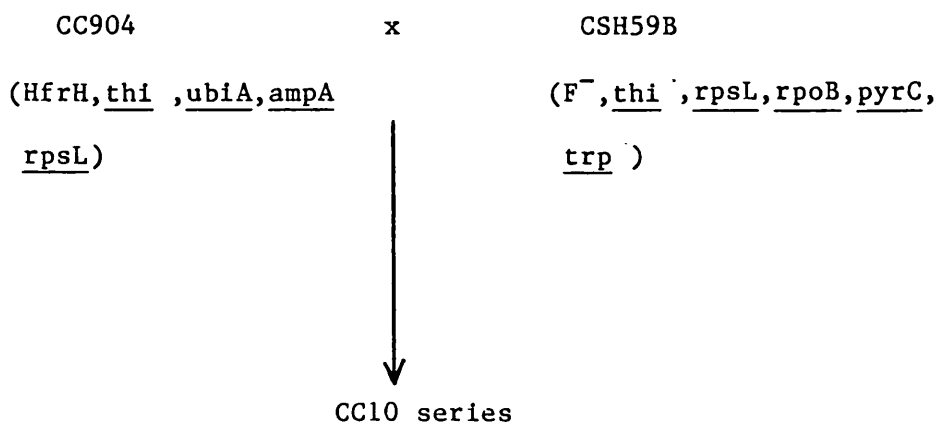
The adhR phenotype of DC272 was cotransduced into CSH59B with trp^+. The inital selection was on minimal M9 plates supplemented with thiamin, uracil and streptomycin with 0.2% Glucose as carbon source.

Single colony isolates of extransducants were tested for adhR phenotype by the ability to produce ethanol aerobically. Overnight aerobic cultures of the isolates were resuspended in 5ml of L.B. supplemented with 0.2% Glucose. The cells were incubated at 37°C with vigorous aeration and harvested in mid log phase to produce spent culture supernatants. The rapid ethanol assay was then used to determine those isolates which produced ethanol.

The cotransduction frequency for trp^+ (minute 27.5) and adhR (minute 27) was found to be 50%. Extransductants with the adhR phenotype were stored on L.B. slopes. Strain CC1103 was chosen for further study.

Construction of strain CC1006

CC10 series strains were constructed by the conjugation of CC904 with CSH59B.



Initial selection was on L.B. plates supplemented with ampicillin and rifampicin. Selection was for ampicillin resistance with rifampicin resistance as counter selection.

Single colony isolates of the exconjugants were then tested for ubiA character on minimal M9 media plates supplemented with thiamin, tryptophan, uracil, ampicillin and with either 0.2% Glucose or 0.2% succinate as carbon source. 100% of the extransductants were found to have the ubiA phenotype on this medium. Exconjugant CC1006 was chosen for further study. It was found later, that all CC10 strains lost their ubiA phenotype if tested on minimal medium lacking ampicillin. From this it was deduced that the host strain CSH59B had a partial lesion in the electron transport chain (Etc^-), unnoticed during growth without ampicillin, but accentuated during growth with this antibiotic.

To test this hypothesis spontaneous mutants of CSH59 to ampicillin resistance were obtained (CSH59C) and grown aerobically along with CC904 and CSH62B on minimal M9 plates supplemented with thiamin, tryptophan, uracil, ampicllin and with either 0.2% Glucose or 0.2% succinate as carbon source. The results (Table 10) showed the hypothesis to be correct.

As the genotype of the lesion was unknown it was necessary to abandon the strains constructed in CSH59B and commence another round of constructions in another strain.

Table 10 Results of aerobic growth of CC904, CSH59C and CSH62B, on ubiA selection plates supplemented with ampicillin

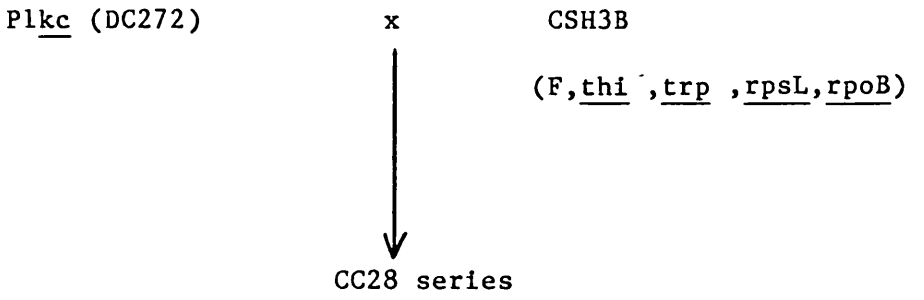
Strain	Relevant genotype	Carbon Source Glucose	Carbon Source Succinate
CC904	ubiA	+ve	-ve
CSH59C	unknown	+ve	-ve
CSH62B	w/t	+ve	+ve

Construction in E.coli CSH3B

CSH3B, a spontaneous revertant to rifampicin resistance of CSH3, was chosen as host for strain construction because it was Trp^-, and allowed the contransduction of adhR, anal and ana2 with Trp^+; it was Plkc sensitive and it grew well on succinate and glucose aerobically, indicating that it was 'wild-type' for glucose and succinate metabolism.

Construction of CC2807

CC28 series strains were constructed by Plkc transduction of a DC272 lysate into CSH3B.



The adhR phenotype was cotransduced into CSH3B with trp^+. The inital selection was on Minimal M9 plates supplemented with thiamin, streptomycin and 0.2% glucose.

Single colony isolates of extransductants were tested for adhR phenotype by the ability to produce ethanol aerobically. Overnight aerobic cultures of the isolates were resuspended in 5ml L.B. supplemented with 0.2% glucose. The cells were incubated at 37°C with vigorous aeration and harvested in mid

log phase to produce spent culture supernatants. The rapid ethanol assay was then used to determine those isolates which produced ethanol. The cotransduction frequency for *trp*$^+$ (27.5 minutes) and *adhR* (27 minutes) was found to be 50%. Extransductants with the *adhR* phenotype were stored on L.B. slopes. CC2807 was selected for further study.

Construction of CC2913

CC29 series strains were constructed by P1*kc* transduction of a LCB900 lysate into CSH3B.

P1*kc* (LCB900) x CSH3B

(F^-,*trp* ,*thi* ,*rpsL*,*rpoB*)

↓

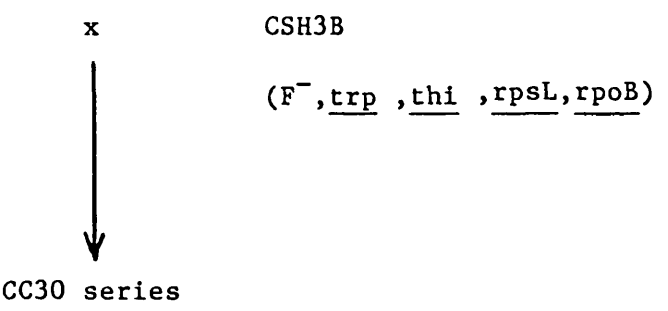
CC29 series

The *anal* phenotype was cotransduced with *trp*$^+$. The inital selction was on minimal M9 medium plates supplemented with thiamin, streptomycin, and 0.2% glucose.

Single colony isolates of extransductants were tested for *anal* phenotype by growth on minimal M9 plates supplemented with thiamin, streptomycin and 0.2% glucose. Those single colony isolates with the phenotype; aerobic growth on glucose, anaerobic no growth on glucose were stored on L.B. slopes. CC2913 was selected for further study. Cotransduction frequency of *trp*$^+$ and *anal* was 30%.

Construction of CC3005

CC30 series strains were constructed by P1kc transduction of a LCB901 lysate into CSH3B.

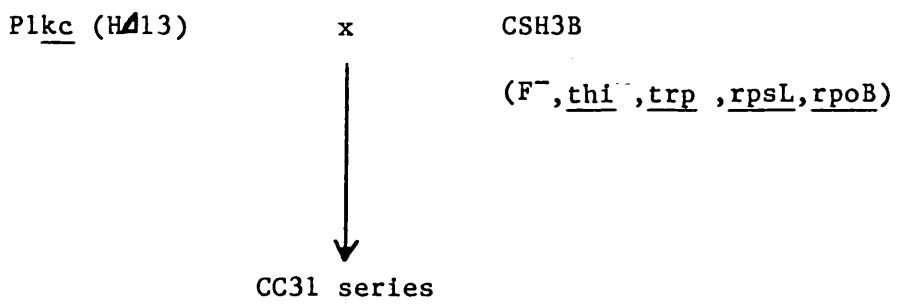


The ana2 phenotype was cotransduced with trp^+. The initial selection was on minimal M9 plates supplemented with thiamin, streptomycin and 0.2% glucose.

Single colony isolates of extransductants were tested for the ana2 phenotype by their ability to grow on minimal M9 plates supplemented with thiamin, streptomycin and 0.2% glucose aerobically, but not anaerobically. Cotransduction frequency of trp^+ (minute 27.5) and ana2 (minute 27) was found to be 30%. Those single colony isolates with the ana2 phenotype were stored on L.B. slopes. CC3005 was selected for further study.

Construction of CC3108

CC3108 series strains were constructed by the P1kc transduction of a HΔ13 lysate into CSH3B.

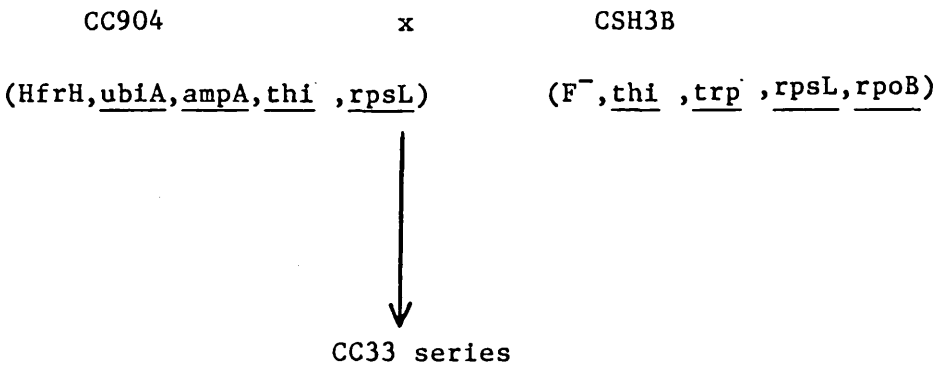


The aceE phenotype was cotransduced with aroP (Brown, 1970). The initial selection was on minimal M9 plates supplemented with thiamin, streptomycin, tryptophan, β-thienyl-alanine, 0.2% glucose and 0.2% acetate (Brown, 1970).

Single colony isolates of extransductants were tested for the aceE phenotype by their inability to grow aerobically on minimal M9 plates supplemented with thiamin, tryptophan and 0.2% glucose, but their ability to grown on minimal M9 plates supplemented with thiamin, tryptophan, 0.2% glucose and 0.02% acetate. The cotransduction frequency of aroP (minute 3) and aceE (minute 3), was unusually low for such closely linked markers, at 30%. Those single colony isolates with the aceE phenotype were stored on L.B. slopes. CC3108 was selected for further study.

Construction of strain CC3310

CC33 series strains were produced by conjugation between CC904 and CSH3B.

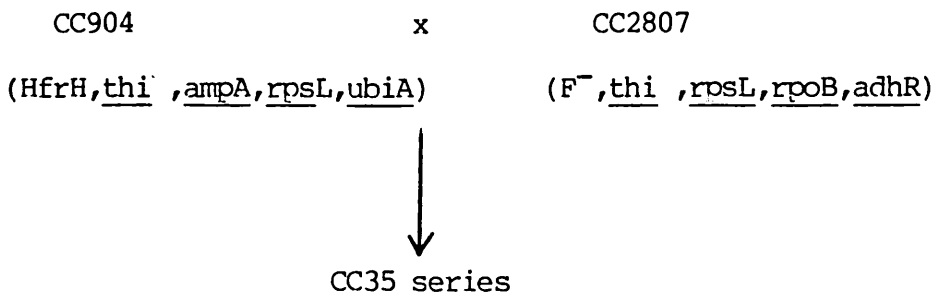


The ubiA phenotype was co-conjugated with ampicillin resistance (ampA). The inital selection was on L.B., ampicillin, rifampicin plates, selecting for ampicillin resistance with rifampicin resistance as counter-selection.

Single colony isolates of exconjugants were tested for the ubiA phenotype by their ability to grow on minimal M9 plates supplemented with thiamin, tryptophan and 0.2% glucose, but not thiamin, tryptophan and 0.2% succinate. The co-conjugation frequency of ubiA (minute 91) and ampA (minute 94) was 20%. Those single colony isolates with the ubiA phenotype were stored on L.B. slopes. CC3310 was chosen for further study.

Construction of CC3502

Construction of CC35 series strains was by conjugation of CC904 with CC2807.



The ubiA phenotype was co-conjugated with ampicillin resistance (ampA). Inital selection was on L.B. ampicillin rifampicin plates, selecting for ampicillin resistance, with rifampicin resistance as the counter selection.

It was deduced that in the ubiA, adhR construct, ubiA would prevent the growth of adhR on ethanol and therefore upon growth on T.I.M., white colonies would result. It was also deduced that the adhR phenotype would relieve the inhibition of growth of ubiA on succinate; however, ubiA would have no effect on the adhR phenotype of aerobic ethanol excretion. Table 11 shows all the possible phenotypes from an ubiA x adhR conjugation.

Single colony isolates of exconjugants underwent the growth tests indicated in table 11 to indicate which genotype they contained. The co-conjugation frequency of ampA with ubiA into an adhR background was found to be 10%.

Construction of CC3703

The construction of series CC37 strains was by conjugation of CC904 with CC2913.

Table 11 Expected phenotypes from aerobically grown single colony isolates of an *ubiA* x *adhR* conjugation

Genotype	Glucose	Succinate	T.I.M.	Ethanol Production
w/t	+ve	+ve	-ve	-ve
ubiA	+ve	-ve	-ve	+ve
adhR	+ve	+ve	+ve	+ve
ubiA+*adhR*	+ve	+ve	-ve	+ve

CC904 x CC2913

(HfrH, *thi* , *ubiA*, *ampA* *rpsL*) (F^-, *thi* , *rpsL*, *rpoB*, *anal*)

↓

CC37 series

The *ubiA* phenotype was co-conjugated with ampicillin resistance (*ampA*). The initial selection was on L.B. ampicillin, rifampicin plates; selecting for ampicillin resistance, with rifampicin resistance as the counter selection.

The *anal* phenotype of single colony isolates of the exconjugants was tested by their ability to grow on minimal M9 plates supplemented with thiamin and 0.2% glucose aerobically, but not anaerobically. The *ubiA* phenotype was tested by the ability to grow on minimal M9 plates supplemented with thiamin and 0.2% glucose but not on plates supplemented with thiamin and 0.2% succinate. Only 10% of the exconjugants contained both *ubiA* and *anal* phenotypes.

Construction of CC3805

CC38 series strains were constructed by conjugation of CC904 with CC3005.

CC904 x CC3005

(HfrH, *thi* , *ubiA*, *rpsL*, *ampA*) (F^-, *thi* , *ana2*, *rpsL*, *rpoB*)

↓

CC38 series

ubiA was co-conjugated with ampicillin resistance into the CC3005 background. Initial selection of exconjugants was on L.B. ampicillin, rifampicin plates; selecting for ampicillin resistance, with rifampicin resistance as the counterselction.

The ana2 phenotype of single colony isolates of the exconjugants were tested by their ability to grown on minimal M9 plates supplemented with thiamin and 0.2% glucose aerobically, but not anaerobically. The ubiA phenotype was tested by the ability to grow on minimal M9 plates supplemented with thiamin and 0.2% glucose, but not on plates supplemented with thiamin and 0.2% succinate. Only 10% of the exconjugants contained both ubiA and anal phenotypes.

Attempt to conjugate ndh into CSH3B

CC1410B, a spontaneous revertant to Trp^+ of CC1410, was conjugated with CSH3B in an attempt to co-conjugate the ndh^- character (at minute 22) with trp^+ (at minute 27.5).

CC1410B x CSH3B

(HfrH, ampA, ndh, his, ilv, rpsL) (F^-, thi, trp, rpsL, rpoB)

↓

CC14 series

Initial selection was on minimal M9 plates supplemented with thiamin and 0.2% glucose selecting for trp^+ with the absence of histidine, isoleucine and valine as counterselection

Single colony isolates of the exconjugants were then tested for *ndh* phenotype by their ability to grow on minimal M9 plates supplemented with thiamin and 0.2% glucose, but not on plates supplemented with thiamin and 0.2% mannitol. Although the conjugations were repeated many times, no exconjugants containing the *ndh* phenotype were identified.

Young and Wallace (1976) noticed a linkage in the conjugation of *his*$^+$ (at minute 44) with *ndh* phenotype. Also the spontaneous revertant to *ndh*$^+$ IY13 shows an unusually small colony morphology. These two observations, linked with the fact that *ndh* phenotype had never been expressed outside the IY12 genome, suggests that IY12 contains a second mutation which along with *ndh* is responsible for the reported phenotype.

Fermentation balances of Constructed Strains

The strains constructed in the CSH3B background by conjugation and transduction were grown aerobically overnight in L.B. and subcultured into a small volume of minimal M9 medium (containing 0.2% glucose and 0.02% acetate as carbon sources with thiamin, tryptophan and streptomycin as supplements) and grown to mid-log phase aerobically and anaerobically. The cultures were then spun down to produce spent culture supernatants which were assayed for metabolic intermediates by a combination of gas chromatography and enzyme assay.

The results (in Tables 12 and 13) showed the carbon flux from glucose to end products and indicated the pathways operating under those conditions.

Table 12 Fermentation balances of Aerobically grown mutant strains constructed in the CSH3B background

Strain	Formate (mM)	Acetate (mM)	Pyruvate (mM)	Lactate (mM)	Succinate (mM)	Ethanol (mM)	Glucose (mM)
CSH3B (wk)	N.D.[a]	3.9	N.D.	N.D.	N.D.	N.D.	N.D.
CC2807 (adhR)	N.D.	2.7	N.D.	N.D.	N.D.	0.4	N.D.
CC2913 (ana1)	N.D.	3.6	N.D.	N.D.	N.D.	N.D.	N.D.
CC3005 (ana2)	N.D.	4.2	N.D.	N.D.	N.D.	N.D.	N.D.
CC3108 (acaE)	N.D.	3.9	4.8	0.6	N.D.	N.D.	7.6
CC3310 (ubiA)	N.D.	3.9	N.D.	N.D.	N.D.	0.3	N.D.
CC3502 (ubiA adhR)	N.D.	3.8	N.D.	N.D.	N.D.	0.2	N.D.
CC3703 (ubiA ana1)	N.D.	2.6	N.D.	N.D.	N.D.	N.D.	N.D.
CC3805 (ubiA ana2)	N.D.	1.0	N.D.	N.D.	N.D.	N.D.	N.D.

(a) N.D. = Not Detected

Table 13 Fermentation balances of Anaerobically grown mutant strains constructed in the CSH3B background

Strain	Formate (mM)	Acetate (mM)	Pyruvate (mM)	Lactate (mM)	Succinate (mM)	Ethanol (mM)	Glucose (mg/ml)
CSH3B (wlt)	22.4	4.3	N.D[a]	N.D.	0.9	7.4	N.D.
CC2807 (adhR)	20.7	3.2	N.D.	N.D.	1.2	7.3	N.D.
CC2913 (ana1)			NO GROWTH ANAEROBICALLY				
CC3005 (ana2)							
CC3108 (aceE)	19.9	9.4	N.D.	0.7	1.4	4.6	N.D.
CC3310 (ubiA)	20.5	9.7	N.D.	N.D.	1.7	12.3	N.D
CC3502 (ubiA adhR)	42.0	5.1	N.D.	N.D.	1.9	9.2	N.D.
CC3703 (ubiA + ana1)			NO GROWTH ANAEROBICALLY				
CC3805 (ubiA + ana2)							

(a) N.D. = Not Detected

One of the most striking observations from these results was the absence of lactate in the anaerobic fermentation balances of all strains except CC3108. As it was reported in 'Microbial Metabolism' (Doelle, 1975) that the anaerobic fermentation balance of E.coli contained a high proprotion of carbon as lactate, the accumulation of lactate in CSH3B was examined.

Also noted from fermentation balances shown in Table 8 was the production of ethanol aerobically by ubiA strains but not ndh$^-$ strains. This phenomenon was also examined further.

Lactate production in E.coli K_{12}

The fermentation balance of anerobically growing E.coli reported in 'Microbial Metabolism' (Doelle, 1975) which showed lactate levels as 40% of the retained carbon, was traced back by a literature search to a paper by A.C.Blackwood (Blackwood et al, 1956) which was based upon work done in 1948. The medium upon which the E.coli strain was grown was a rich medium containing 5% glucose (Neish and Ledingham, 1949). It was therefore decided to grow CSH3B on a similar rich medium and compare lactate production and lactate dehydrogenase activities to CSH3B grown on a minimal medium.

An overnight culture of CSH3B grown aerobically on minimal M9 medium containing 0.2% glucose and supplemented with thiamin and tryptophan was subcultured into 200ml of either L.B. medium containing 3% glucose or minimal M9 medium containing 0.4% glucose and supplemented with thiamin

and tryptophan. The cultures were incubated overnight in anaerobic jars at 37°C and then the cells harvested by centrifugation at 6000g for 5 minutes. The spent bacterial culture supernatants were then assayed for metabolic intermediates whilst crude bacterial cell extracts (for lactate dehydrogenase determinations) were prepared from the pellets.

The results shown in tables 14 and 15 indicated that although specific activities of lactate dehydrogenase were similar for both cultures, lactate was only accumulated in the rich culture medium. The phenomenon of absence of lactate in cultures growing anaerobically with low levels of glucose has also been observed in Klebsiella aerogenes (Teixera de Mattos , 1983). From these results it was deduced that CSH3B was not a mutant strain and that it could be used to examine the aerobic to anaerobic switch in E.coli K_{12}.

Ethanol production in ubiA strains

Two mutations in the oxidative electron transport chain resulted in two different aerobic fermentation balances. The ndh$^-$ lesion of IY12 resulted in high levels of lactate accumulation only, whereas the ubiA lesion of AN385 and CC3310 resulted in ethanol accumulation in both strains and also the accumulation of low levels of other metabolic intermediates in AN385 (Table 8). This phenomenon could have been solely due to the strains in which the lesions existed, or could have indicated a region of the electron transport chain that governs the induction of anaerobic enzyme systems. To

Table 14 Partial fermentation balances for Anaerobically grown CSH3B on rich and minimal media

Culture Medium	Formate (mM)	Acetate (mM)	Pyruvate (mM)	Lactate (mM)	Succinate (mM)
Rich 3% glucose	8.1	7.8	N.D[a]	13.7	2.7
Minimal 0.4% glucose	8.4	2.1	N.D.	N.D.	0.4

(a) ND = Not Detected

Table 15 Lacatate Dehydrogenase activities in Anaerobically grown CSH3B on Rich and Minimal Media

Culture Medium	Activity Units/ml	Protein/ml Culture (mg)	Specific Activity Units/mg protein
Rich 3% glucose	22.7	0.192	1182
Minimal 0.4% glucose	13.6	0.104	1307

differentiate between these two possibilities the aerobic fermentation balances of ndh$^-$ and ubiA strains in an isogenic background were determined.

Construction of CC4009

As the transfer of ndh$^-$ out of the IY12 background proved so difficult, ubiA was transfered into the ndh$^-$ revertant, IY13, by conjugation between CC904 and IY13B (a spontaneous mutation to rifampicin resistance of IY13).

CC904 (HfrH, rpsL, thi, ubiA, ampA) x IY13B (F^-, his, ilv, trp, rpsL, Pl^r rpoB)

↓

CC40 series

The ubiA phenotype was co-conjugated into IY13 with ampicillin resistance (ampA). Initial selction was on L.B. ampicillin, rifampicin plates, selecting for ampicillin resistance, with rifampicin resistance as counterselection.

Single colony isolates of exconjugants were tested for the ubiA phenotype by their ability to grow on minimal M9 plates supplemented with thiamin, histidine, isoleucine, valine, tryptophan and with either 0.2% glucose or 0.2% succinate as carbon source. Exconjugants with the Glu^+, $Succ^-$ phenotype were stored on L.B. slopes. Co-conjugation frequency of ubiA (minute 91) with ampA (minute 94) was 20%. CC4009 was chosen for further study.

Fermentation balance of CC4009

An overnight culture of CC4009 grown on L.B. was subcultured in 5ml of minimal M9 medium supplemented with histidine, isoleucine, valine, tryptophan and with 0.2% glucose as carbon source. The culture was incubated at 37°C with vigorous aeration and harvested in mid-log phase to produce a spent culture supernatant. The supernatant was then assayed by gas chromatography and enzyme assay to determine the aerobic fermentation balance.

The results, shown in Table 16, along with the aerobic fermentation balances of an ndh$^-$ strain (IY12), ubiA strains (AN385, CC3310) and the 'wild-type' strain indicated that the ubiA lesion will produce ethanol aerobically.

Chemostat culture results

Three strains in the CSH3B background were chosen for study in continous culture, CC3310, CC2807 and CSH3B itself. CSH3B was chosen to examine the wild-type switch from aerobic to anaerobic metabolism. CC3310, the ubiA harbouring strain, was chosen to examine the effects of the lesion on NAD^+ and NADH levels and the effects of these altered levels on the switch. CC2807 was chosen to examine the effect of its lesion (adhR) on NAD^+ and NADH levels and the effects of these altered levels on the switch and also to examine the pleiotropic effects of the adhR lesion (Clark and Cronan, 1980).

Table 16 Fermentation balances of Aerobically grown wild-type, ndh⁻ and ubiA strains of E.coli K_{12}

Strain	Formate (mM)	Acetate (mM)	Pyruvate (mM)	Lactate (mM)	Succinate (mM)	Ethanol (mM)
CSH3B	N.D.	3.9	N.D.	N.D.	N.D.	N.D.
IY13	0.1	3.8	N.D.	N.D.	N.D.	N.D.
IY12	N.D.	2.3	N.D.	6.6	N.D.	N.D.
CC4009	N.D.	4.0	N.D.	N.D.	N.D.	0.33
CC3310	N.D.	3.9	N.D.	N.D.	N.D.	0.30
AN385	1.2	9.6	0.2	2.5	0.1	0.33

Continuous culture of CSH3B

The continuous culture was set up as described in 'Materials and Methods' in two chemostats with different air: N_2 ratios. Steady state was judged to be established after 48 hours; that is 4.8 volumes of medium passed through the chemostat, and was monitored by observing stable levels of CO_2 in the effluent gases (by I.R.Spectroscopy) and a constant $O.D._{550nm}$ of the culture.

Samples were then taken via the rapid sampling port and the overflow line to ascertain the physiological state of the cells at those aeration levels. Samples were also taken to assess the integrity of the chemostat and the sterility of the medium in the aspirator.

After the samples had been taken aeration was altered by changing the air and N_2 flows into the chemostats. Aeration was always altered in the aerobic to anaerobic direction (ie. the ratio of N_2: air in the influent gas always increased).

NAD^+ and NADH levels

Samples for cultures supplied with 100% air and 100% N_2 were assayed from pool sizes of NAD^+ and NADH. The other cultures were simply assayed for the ratio between NAD^+ and NADH levels, which gave an indication of internal redox potential (Wimpenny and Firth, 1972).

For the measurement of pool sizes the rate of change of optical density at 570nm of standards was related to the rate of change observed in samples (see 'Materials and Methods') and relating this to dry weight by measuring the $O.D._{550nm}$ of the culture and comparing with a dry weight vs. $O.D._{550nm}$ standard plot.

For the measurement of NAD^+/NADH ratios the rate of changes in optical density at 570nm of samples were corrected with blank readings and then directly related to each other.

Pool sizes of NAD^+ and NADH given in table 17 showed that anaerobically the total NAD^+ and NADH pool size was only 73% of the aerobic pool size, and that the ratio of NAD^+ to NADH had dropped from an aerobic level of 7.45 to an anaerobic level of 2.70.

NAD^+/NADH ratios for all CSH3B cultures are shown in figure 10. These results showed that NAD^+/NADH ratios fell into three categories:-

1. Fully aerobic - ratio 7.45
2. Oxygen limited - ratio ca.5.6
3. Anaerobic - ratio 2.7

Biomass levels

Biomass levels were determined by taking the optical densities of the cultures at 550nm and relating them to dry weights from an $O.D._{550nm}$ vs. dry wt. standard plot; and are shown in table 19, and in graphic form in figure 11.

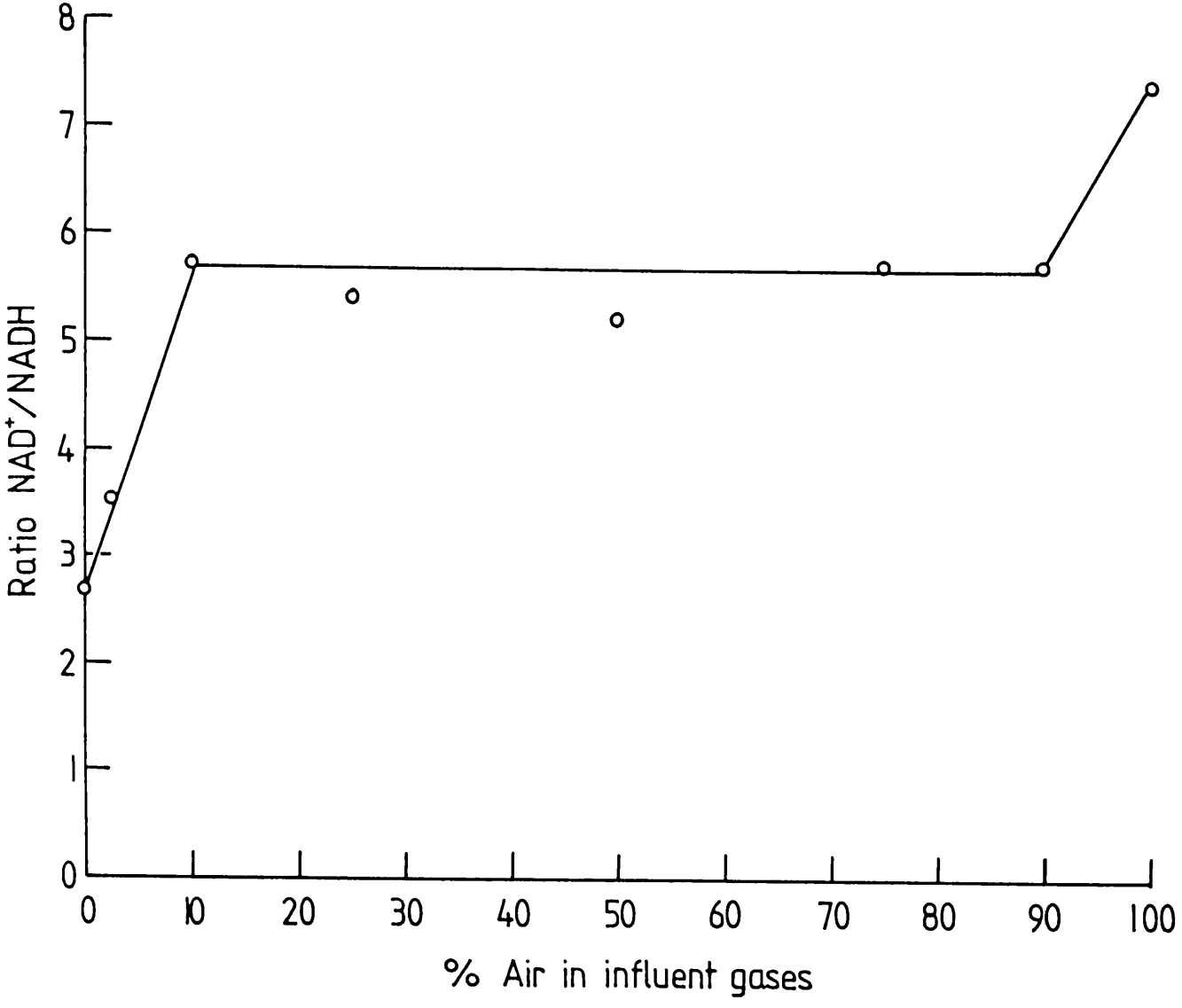
From figure 11 it can be seen that the culture experienced oxygen stress at air supply rates as high as 90% (as biomass had decreased at that air supply level). Also seen from figure 11 is the classical 'Pasteur Effect' a decrease in biomass under anaerobiosis. Concommittant with this effect was another 'typical' phenomenon, the increase in the glucose flux through the cell anaerobically. As the same level of glucose was utilised both aerobically, (100% air in influent gas) and anaerobically (100% N_2 in influent gas), but biomass was only

Table 17 Pool Sizes of Nicotinamide dinucleotide in cultures of CSH3B grown in a Chemostat

Culture Condition	NAD^+ Pool (μmol/g dry wt)	NADH Pool (μmol/g dry wt)	Total Pool (μmol/g dry wt)	Ratio NAD^+/NADH
100% Air	1.79	0.24	2.03	7.45
100% N_2	1.10	0.39	1.49	2.7

Redox balance vs. Aeration for CSH3B

Figure 10



1/3 of the aerobic level, the glucose flux anaerobically must have increased by three times. This figure agreed with that quoted by Gottschalk (G.Gottschalk, 1979). Concomitant with this effect was the increase in substrate required to provide energy for biomass production. Results shown in Table 18 indicated the percentage of glucose metabolised into biomass and the percentage of glucose required to fix that amount of glucose as biomass.

As Y_{ATP} remains constant (Pirt, 1975) and biomass levels are an indication of ATP production (Bauchop and Elsden, 1960), the fallen levels of biomass was an indication of the lowered efficiency of ATP production from glucose.

In calculating the carbon balance, the method of Herbert (Herbert, 1975) was used in which 50% of the cell dry weight was assumed to have been supplied from glucose.

Metabolite levels

Metabolite levels were determined by infra-red spectroscopy of the effluent gas, (CO_2 levels) and a combination of gas chromatorography (formate, acetate, pyruvate, lactate and succinate) and enzyme assays (ethanol and glucose) of spent culture medium. The results were standardised to mmol/hr in order to relate CO_2 levels to other metabolite levels. The results are shown along with a carbon balance (ie. $\frac{\text{mmol carbon/hr out}}{\text{mmol carbon/hr in}} \times 100$) in Table 19

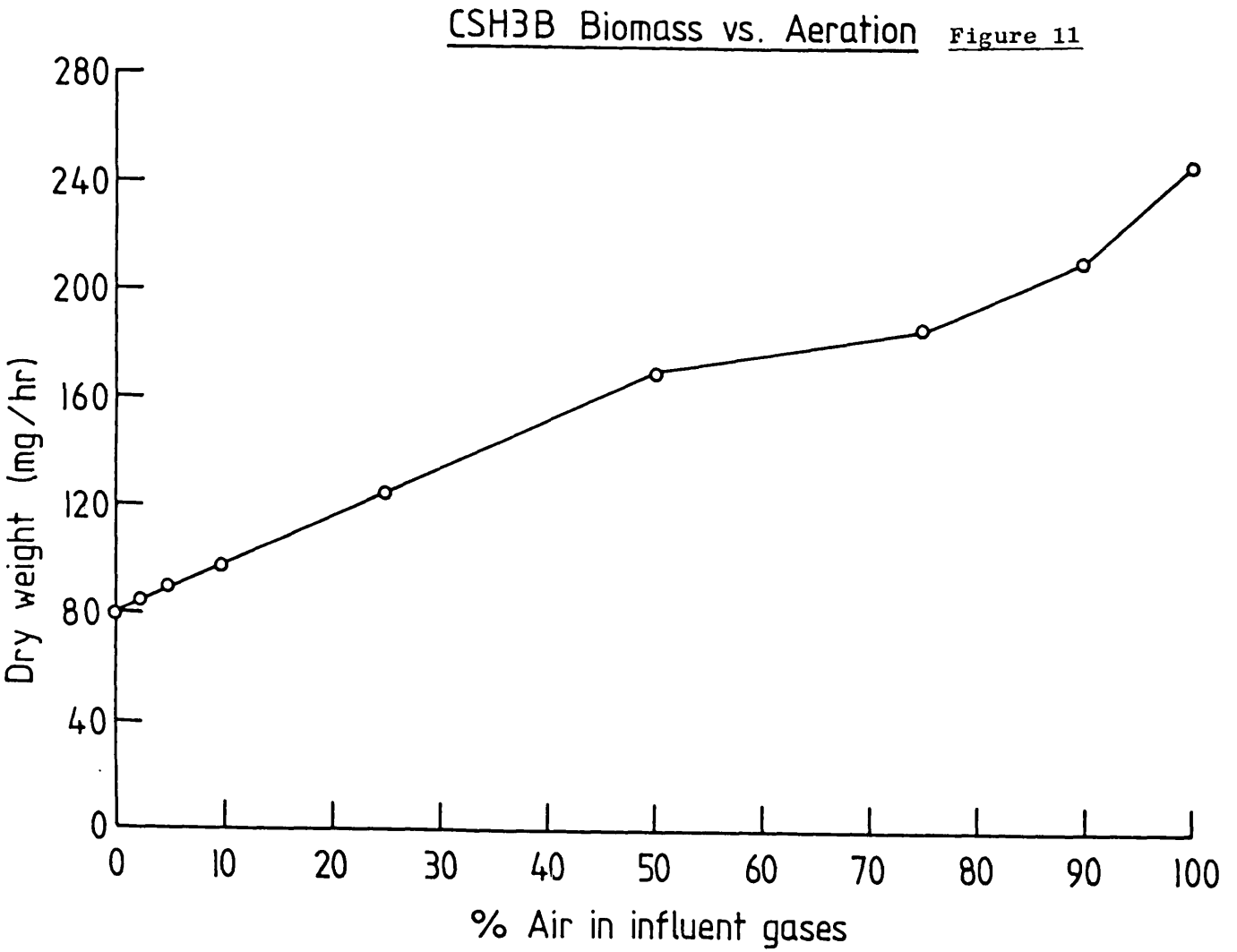
Anaerobically, ethanol and succinate levels must equal acetate levels to produce a balance of reducing equivalents

Table 18 Percentile glucose utilised as an energy source and carbon source in CSH3B continuous culture

Aeration	% Glucose as Cell Carbon	% Glucose as Engergy Source
100% Air	14.0	86.0
90% Air	12.0	88.0
75% Air	10.6	89.4
50% Air	9.7	90.3
25% Air	7.1	92.9
10% Air	5.6	94.4
5% Air	5.1	94.9
2.5% Air	4.8	95.2
100% N_2	4.6	95.4

Table 19 Product levels from Continuous Cultures of CSH3B

Aeration	CO_2 (mmol/hr)	Formate (mmol/hr)	Acetate (mmol/hr)	Ethanol (mmol/hr)	Succinate (mmol/hr)	Biomass (mg/hr)	Glucose (g/litre)	Carbon Balance
100% Air	13.70	0	0.03	0	0	247.5	0	80.1%
90% Air	10.90	0	2.93	0.52	0	215.5	0	87.3%
75% Air	7.66	0.20	4.90	0.57	0.05	186.3	0	85.6%
50% Air	4.42	1.68	8.00	0.58	0.53	170.0	0	104.4%
25% Air	1.92	3.03	5.95	1.45	1.10	125.0	0	94.8%
10% Air	2.36	3.10	5.23	3.52	0.75	98.8	0	97.0%
5% Air	3.24	1.80	4.40	3.68	1.35	90.0	0	97.5%
2.5% Air	2.95	1.40	3.50	3.55	1.25	85.0	0	95.6%
100% N_2	1.65	3.80	4.15	2.93	1.23	80.0	0	92.0%


CSH3B Biomass vs. Aeration Figure 11
280
240
200
160
120
80
40
0
Dry weight (mg/hr)
0
10
20
30
40
50
60
70
80
90
100
% Air in influent gases

(see fig 3). Also, under anaerobic conditions ethanol and acetate levels must equal CO_2, formate and succinate levels (see fig. 3). Therefore to assess the accuracy of the anaerobic fermentation balance these equations were calculated:-

a) Balance of reducing equivalents

Ethanol + succinate = Acetate

2.93/hr + 1.23mmol/hr = 4.15mmol/hr

4.16mmol/hr = 4.15 mmol/hr

$$\frac{4.16}{4.15} = 1.002$$

Expected value; $\frac{\text{Ethanol + succinate}}{\text{Acetate}} = 1.0$

Actual value is 0.2% off the expected value.

b) Ratio of Metabolite accumulation

Ethanol + acetate = CO_2 + Formate + succinate

2.93mmol/hr + 4.15mmol/hr = 1.65mmol/hr + 3.80mmol/hr + 1.23mmol/hr

7.08mmol/hr = 6.68mmol/hr

$$\frac{7.08}{6.68} = 1.059$$

$$\text{Expected value } \frac{\text{Ethanol + Acetate}}{CO_2 + \text{Formate + Succinate}} = 1.0$$

Actual value is 5.9% off the expected value.

H_2 levels

Hydrogen levels were assayed by gas chromatography as described in 'Materials and Methods'. Peak heights of a standard H_2 in N_2 mixture were compared with peak heights obtained from samples of effluent gases from the chemostat. Results are shown in Table 20, and with the other metabolite levels in Fig. 12.

Calculation of Respiration rate

By assuming that at each steady state a balance of reducing equivalents must be achieved, the oxygen utilised, for the reduction of excess NADH, was calculated.

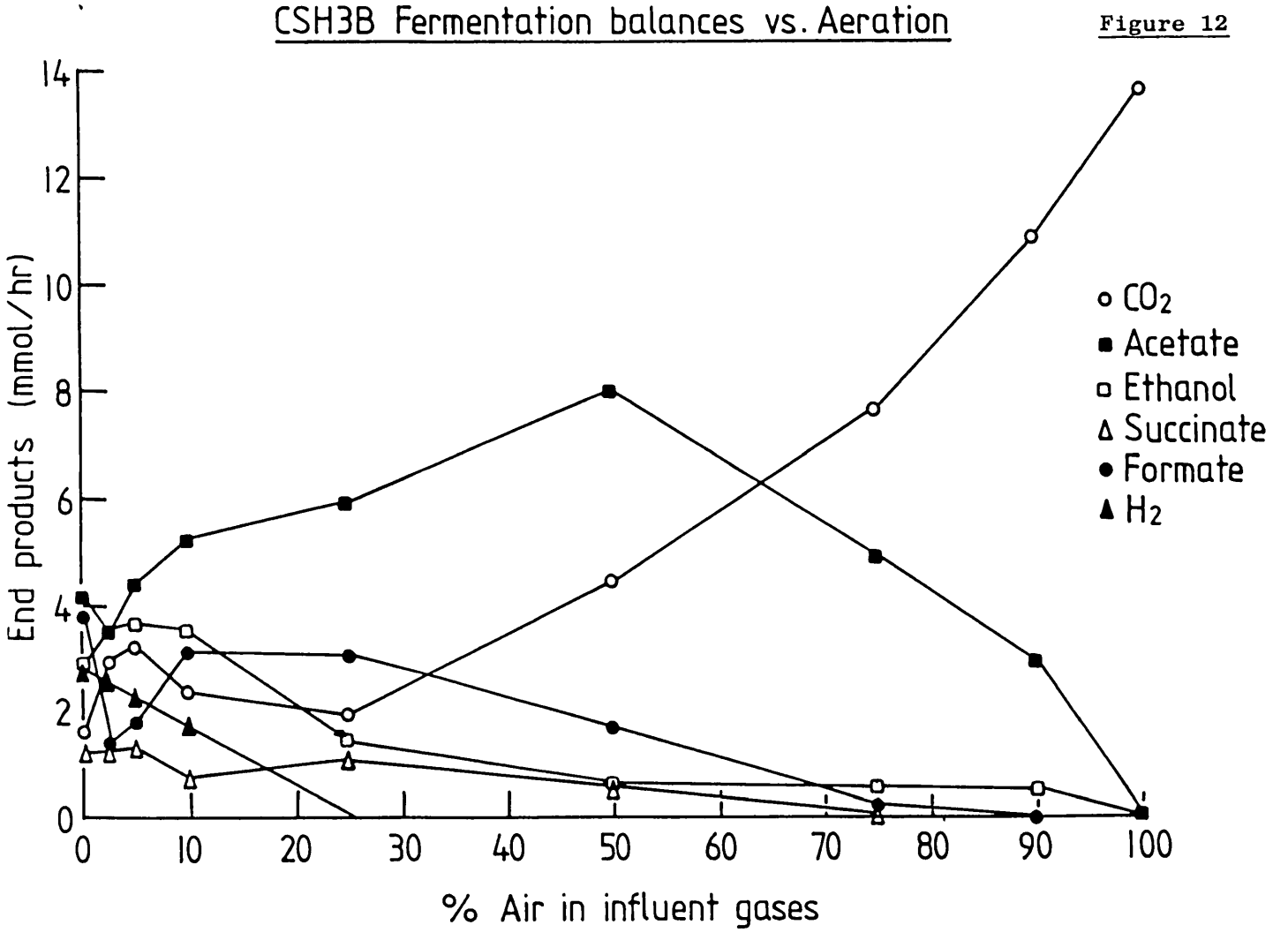
At 100% air the majority of the glucose was oxidised to CO_2, and by referring to the metabolic charts (figs. 1 and 3) the amount of NADH accumulated can be determined. Assuming that two reducing equivalents react with molecular oxygen to form $2H_2O$ (Downie and Cox, 1978) the oxygen utilised may be determined. When the cultures were anaerobic, similar calculations were performed, bearing in mind that different pathways and end products gave different NADH accumulations.

From the levels of oxygen utilised, the growth yield with respect to oxygen, Yo_2, was calculated from the equation:-

Table 20 Levels of H_2 in effluent gases from a CSH3B continuous culture

Aeration	H_2 levels (mmol/hr)
100% Air	0
90% Air	0
75% Air	0
50% Air	0
25% Air	0
10% Air	1.75
5% Air	2.30
2.5% Air	2.55
100% N_2	2.86

Figure 12


CSH3B Fermentation balances vs. Aeration
End products (mmol/hr)
% Air in influent gases
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
90
100
CO2
Acetate
Ethanol
Succinate
Formate
H2

$Yo_2 = -\frac{dx}{ds}$ where dx = steady state biomass formed

ds = Amount substrate used

(Herbert, 1958)

As:-

$qo_2 = \mu / Yo_2$ where qo_2 = respiration rate

μ = growth rate

Yo_2 = yield

(Birch and Pirt, 1969)

As (growth rate) at steady state, equalled D (dilution rate) and D was a known parameter, it was possible to calculate the respiratory quotient qo_2 once Yo_2 was calculated. The results of these calculations are shown in Table 21 and in figures 13, 14 and 15.

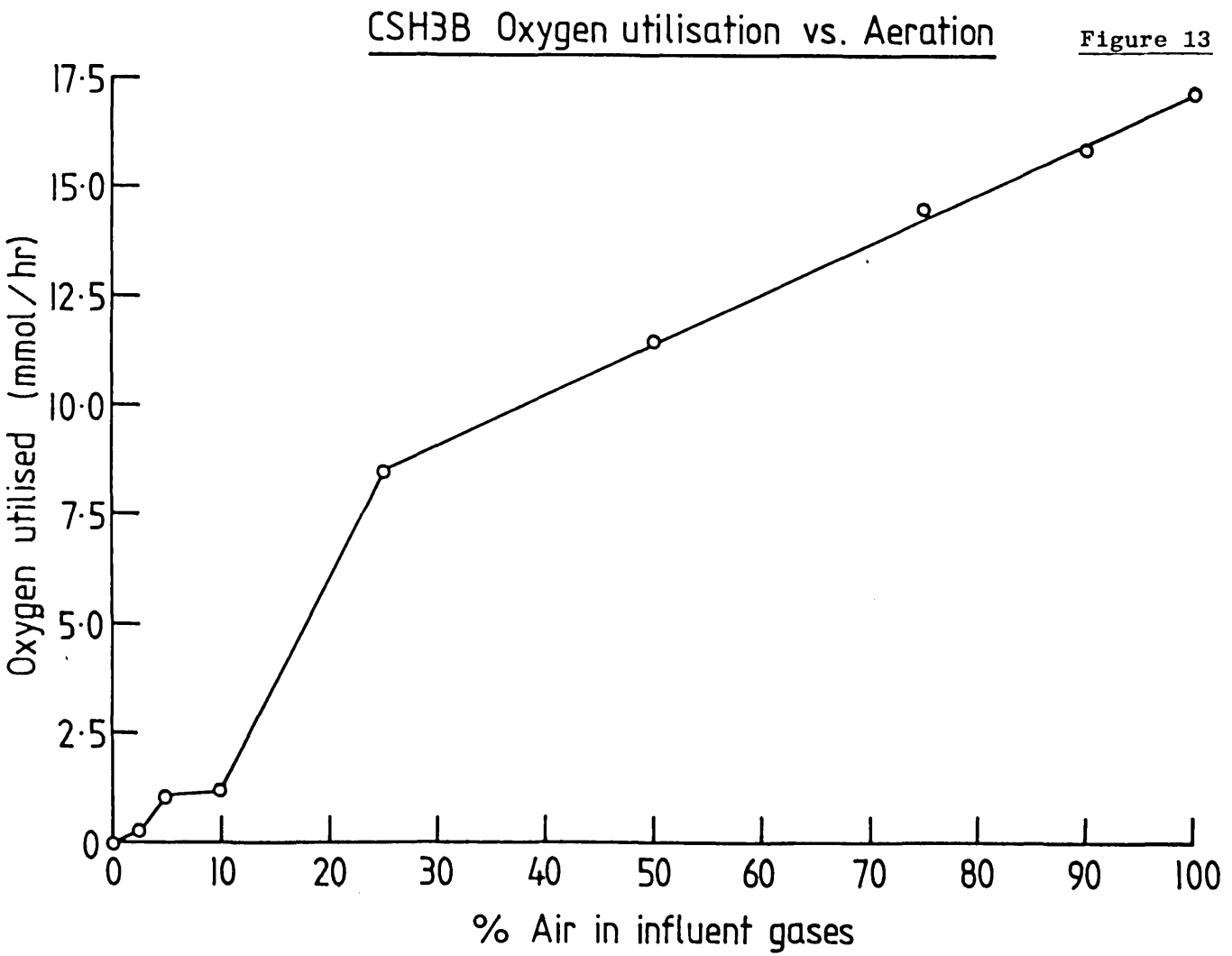
Enzyme assays of CSH3B

Crude cell-free extracts were prepared from samples collected from the overflow line and assayed for 2OGDH, ADH and NADHox by the methods described in 'Materials and Methods' within two days of sampling. Where storage of extracts was required they were placed in sealed containers at-20°C. The enzyme assays were all carried out in duplicate or triplicate and were reproducible to a level of $\pm$ 10% (Table 22 Figs. 16 and 17).

The specific activities of the extracts were determined by relating absolute activities, to the protein content of the extracts determined by the modified Lowry method described in 'Materials and Methods'.

Table 21 Calculated respiration rates for CSH3B grown in Continuous Culture

Aeration	NADH formed (mmol/hr)	O_2 utilised (mmol/hr)	Yo_2 (g/mol)	qo_2 (hr^{-1}/g/mol)
100% Air	34.3	17.2	14.4	7.0×10^{-3}
90% Air	31.2	15.6	13.7	7.2×10^{-3}
75% Air	28.9	14.5	12.9	7.8×10^{-3}
50% Air	23.0	11.5	14.8	6.8×10^{-3}
25% Air	16.7	8.4	14.9	6.8×10^{-3}
10% Air	2.4	1.2	82.3	12.2×10^{-4}
5% Air	2.1	1.1	81.8	12.2×10^{-4}
2.5% Air	0.6	0.3	283.3	3.6×10^{-4}
100% N_2	0	0		0


CSH3B Oxygen utilisation vs. Aeration
Figure 13
Oxygen utilised (mmol/hr)
17·5
15·0
12·5
10·0
7·5
5·0
2·5
0
0
10
20
30
40
50
60
70
80
90
100
% Air in influent gases

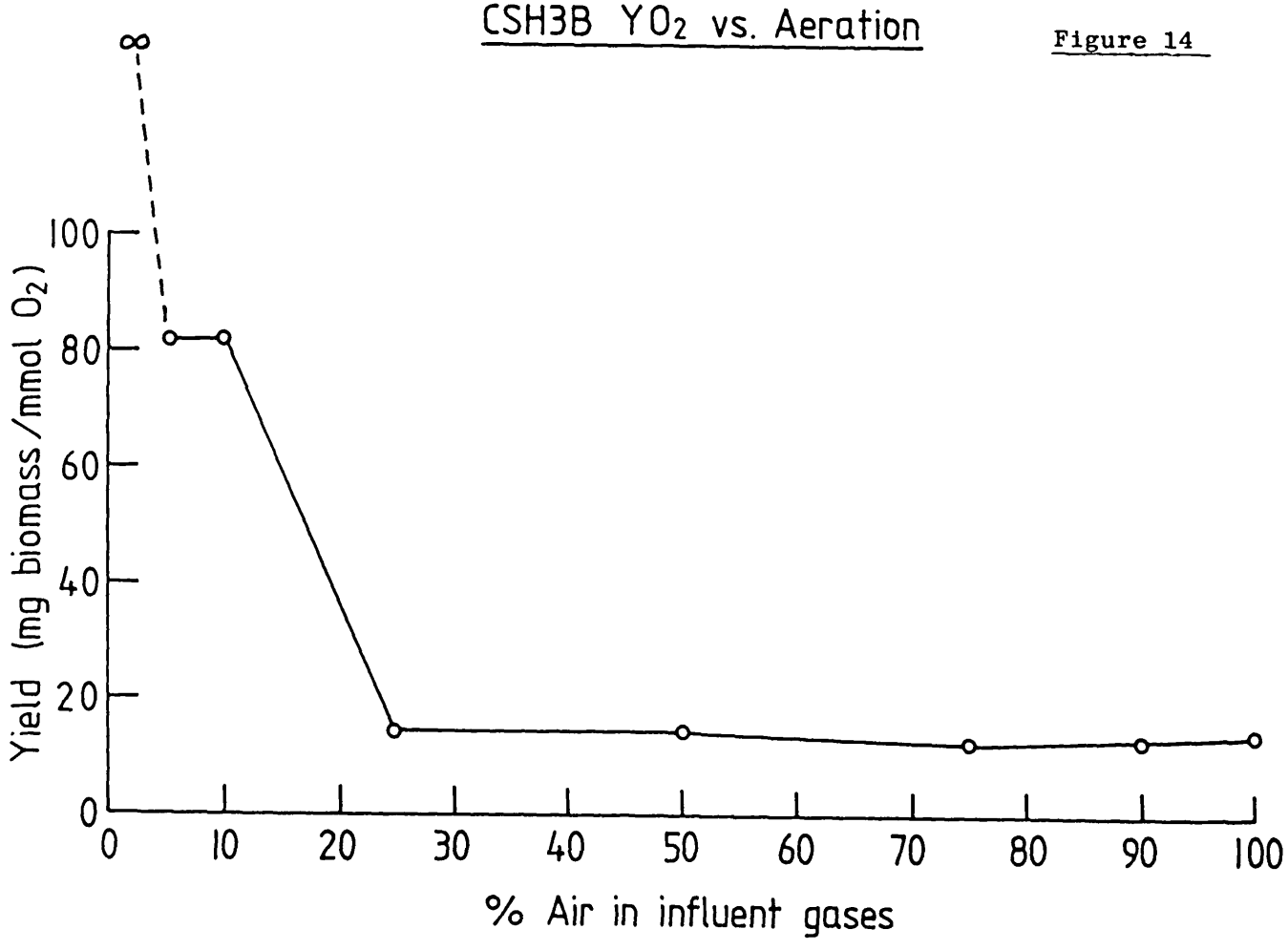

CSH3B Y_{O_2} vs. Aeration
Yield (mg biomass/mmol O_2)
∞
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
% Air in influent gases

Figure 14

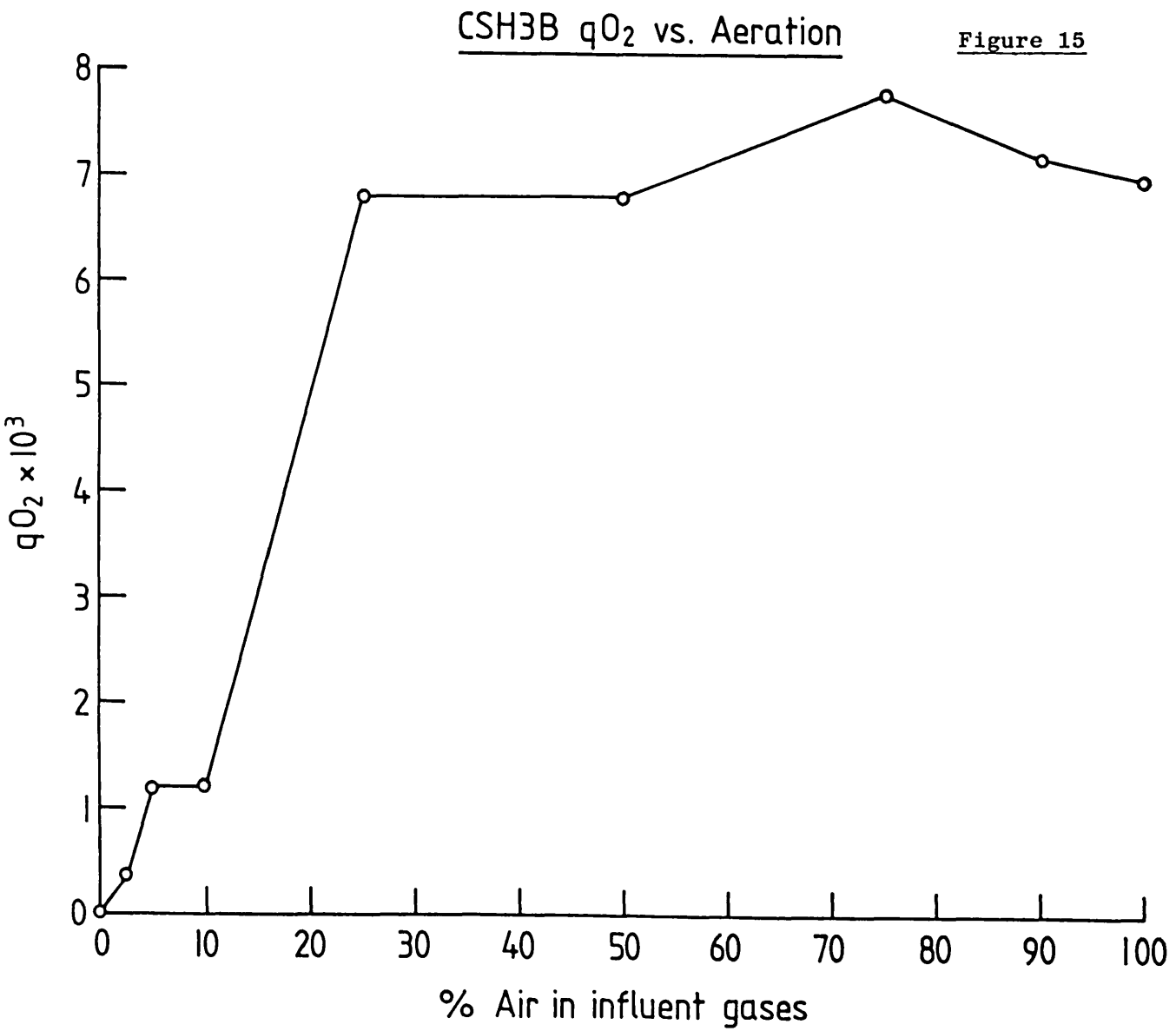

CSH3B qO_2 vs. Aeration
$qO_2 \times 10^3$
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
80
90
100
% Air in influent gases

Figure 15

Upon reducing air supply to the medium the steady state activities of 2OGDH reached a maximum at 50% air. From this point the activity decreased sharply to zero at 10% air in the influent gases and remained at zero to complete anaerobiosis.

The steady state activites of NADHox mirrored the levels of its substrate NADH (see Fig. 16). The lowest activity was found at 100% air in the influent gases. At 90% air the activity rose by xl.75 and stayed at this level until 5% air in the influent gases when it started to rise to a maximum activity, of about x4 the plateau activity, at 100% N_2.

The activities of ADH were mirrored by its product levels, (ie. ethanol) see Fig. 17. A low but steady activity was found in the extracts obtained from cultures grown at 100% air to 50% air. Then the activities started to rise to a maximum activity at 100% N_2. The rate of increase of activities was markedly greater after 10% air and may have indicated the accumulation of a second inducer of ADH activity.

Continuous Culture of CC3310

The continuous culture apparatus was set up as described in 'Materials and Methods' in two chemostats with different air:N_2 ratios in the influent gases. Steady state was judged to be established after 48 hours; that is 4.8 volumes of medium passed through the chemostat, and was monitored by observing stable levels of CO_2 in the effluent gases (by I.R. Spectroscopy) and constant OD_{550nm} of the cultures.

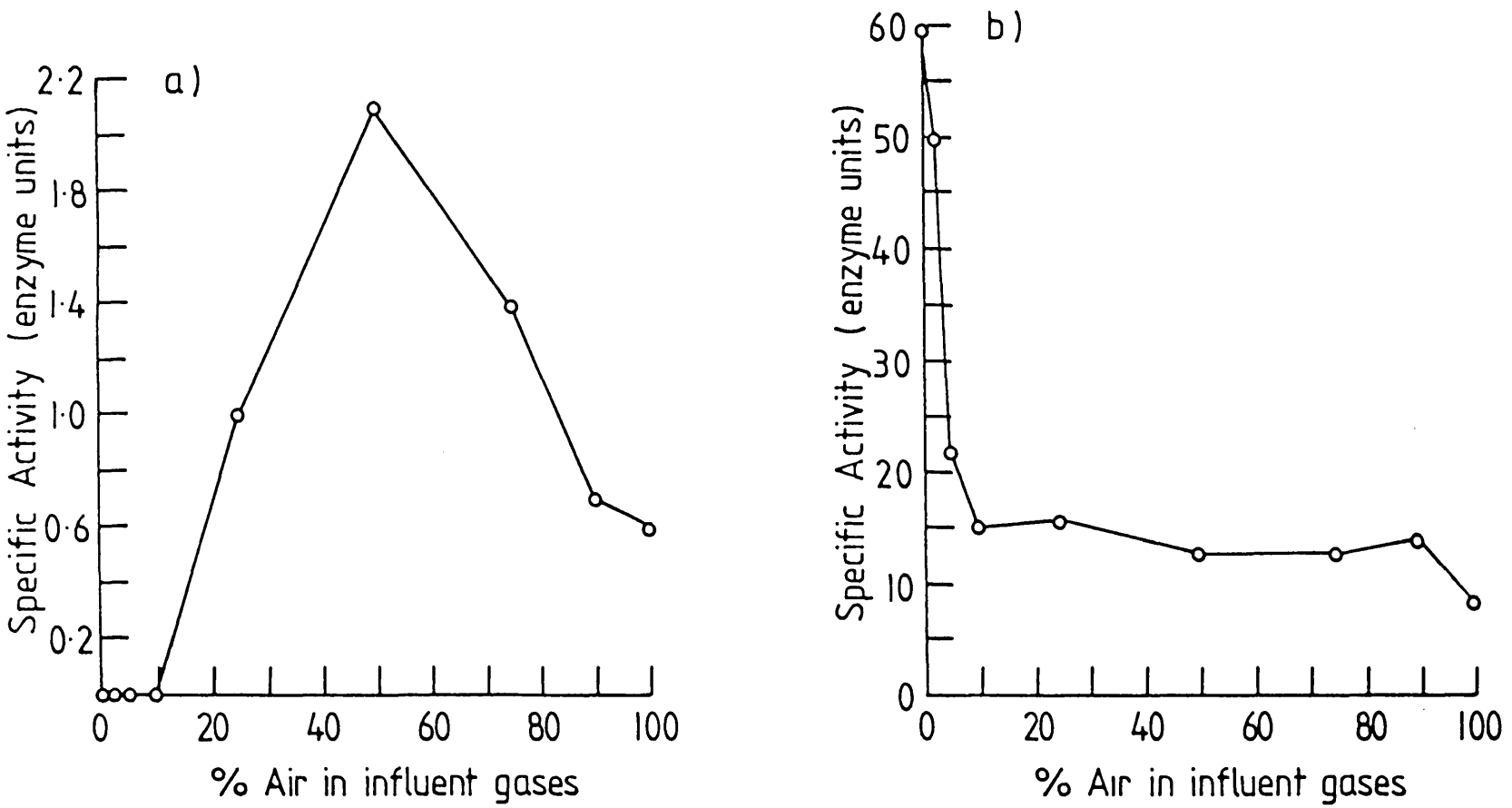
Samples were then taken via the rapid sampling port and the overflow lines to ascertain the physiological state of the cultures at those aeration levels. Samples were also taken to

Table 22 Specific Enzyme Activities for CSH3B grown in Continuous Culture

Aeration	2OGDH (Units/mg protein)	ADH (Units/mg protein)	NADHox (Units/mg protein)
100% Air	0.6	9.6×10^{-2}	8.6
90% Air	0.7	9.1×10^{-2}	14.3
75% Air	1.4	8.7×10^{-2}	12.9
50% Air	2.1	9.7×10^{-2}	12.9
25% Air	1.0	0.8	15.7
10% Air	0	0.9	15.2
5% Air	0	10.9	21.9
2.5% Air	0	56.3	50.0
100% N_2	0	62.4	59.5

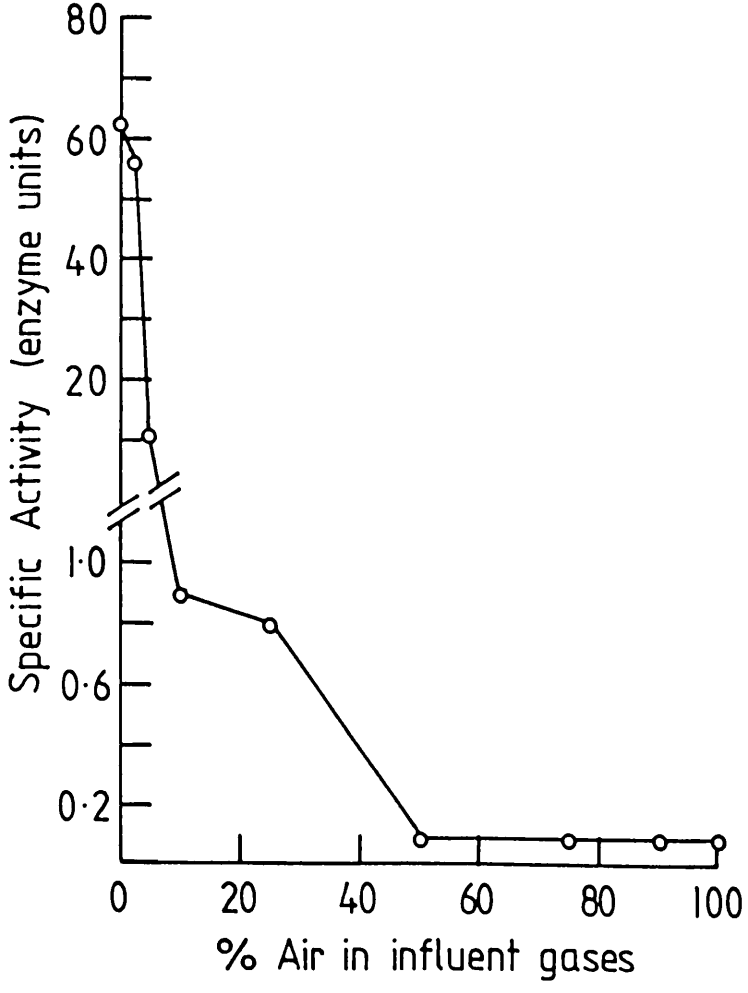
CSH3B Specific Activities of a) 2-OGDH + b) NADHOX with aeration

Figure 16



CSH3B Specific Activities of ADH with aeration

Figure 17



assess the integrity of the chemostat and the sterility of the medium in the aspirator. As the organism to be cultured had a point mutation of the wild-type genotype resulting in a deleterious phenotype (Young et al, 1971), the possiblity of a wild-type revertant 'taking-over' the chemostat was present. Therefore, the phenotype of the culture was tested twice a day to ensure results obtained were from a monoculture of CC3310 and not a mixture of CC3310 and revertants.

Aeration was altered by changing the air:N_2 flows into the chemostats. Aeration was always altered in the aerobic to anaerobic direction (ie. the ratio of N_2/air in the influent gas always increased).

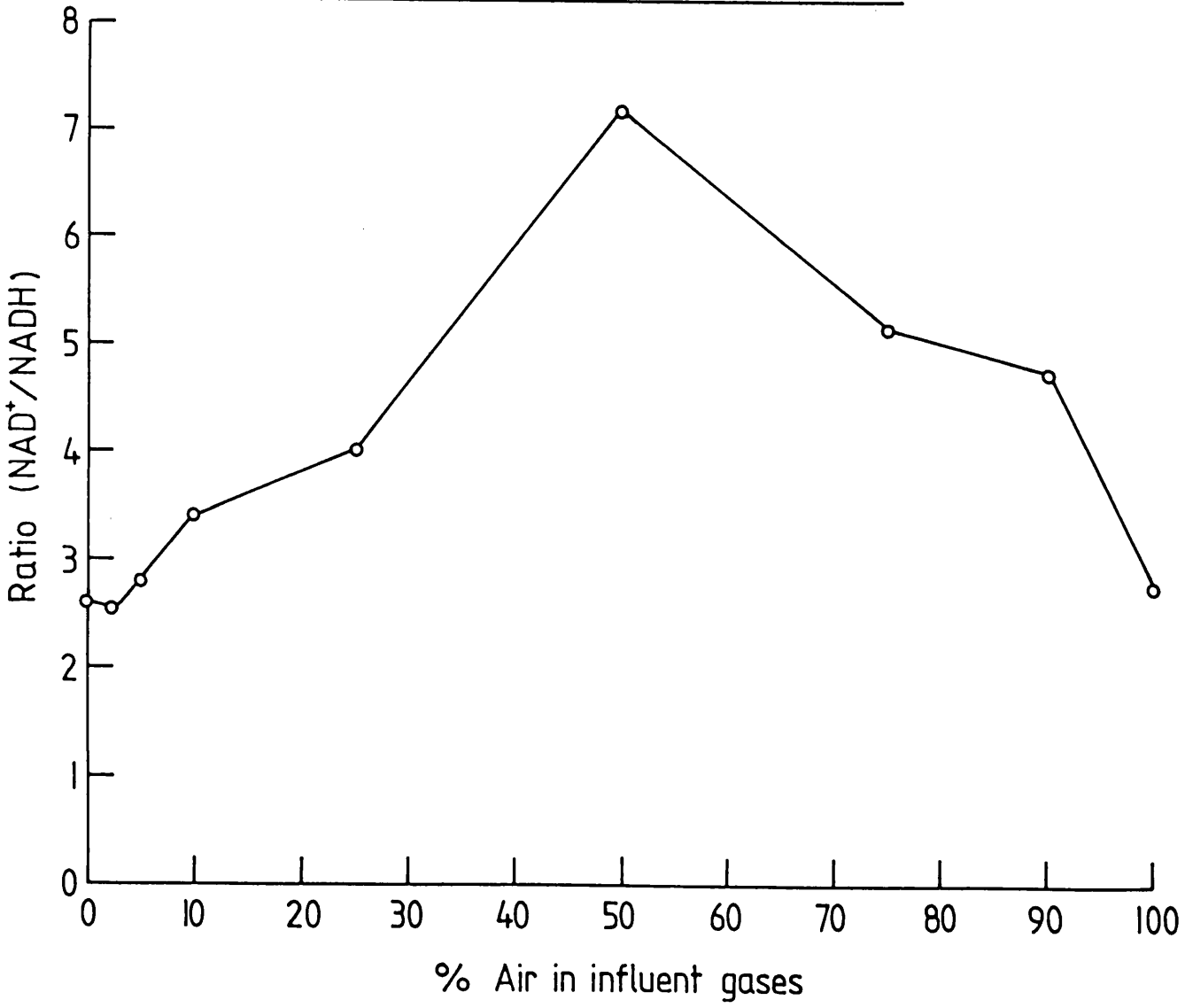
NAD^+ and NADH Ratios

Cultures extracted from the rapid sampling port were treated as described in 'Materials and Methods' to provide samples for the cycling assay of NAD^+ and NADH. Total pool sizes were not measured but the ratios of NAD^+/NADH were recorded. This ratio was determined by comparison between the rate of change of optical density at 570nm of samples treated to extract NAD^+ or NADH. All readings were taken in duplicate and were reproducible to a level of $\pm$ 10%.

The results (Fig. 18) showed a deviation from the pattern seen in the wild-type strain, CSH3B. As expected with a strain which contained a lesion in electron transport, redox balance at 100% air was lower (2.8) than in the wild-type strain. However, as the percentage of air in the influent gases decreased, the redox balance increased to a maximum at 50% air equal to the wild-type fully aerobic redox balance of 6.7.

Redox balance vs. Aeration for CC3310

Figure 18



From this maximum, the redox balance dropped to the fully anaerobic value of 2.6. Both wild-type and the ubiA strain were similar (2.7 and 2.6 respectively). This was to be expected as the ubiA phenotype anaerobically is indistinguishable from that of the wild-type. This gave further evidence of the reliability of the sampling, extraction and the assay method for nicotinamide dinucleotides.

Biomass levels

Biomass levels were determined by taking the optical densities of the cultures at 550nm and relating them to dry weights, from an OD_{550nm} vs. dry weight standard plot. The results are shown in Table 24 and in graphic form in Fig. 19

From Fig. 19 it can be seen that the cultures experienced oxygen stress at air supply rates as high as 90% (biomass had decreased at that level of aeration). Again, the classical 'Pasteur Effect' of a decrease in biomass under anaerobiosis can be seen along with the implied increase of glucose flux. The flux under anaerobiosis was x3.3 greater per gram dry weight of culture than under fully aerobic conditions. This figure agreed with that quoted by Gottschalk (Gottschalk, 1979). Concomitant with this effect was the increase in substrate required to provide energy for biomass production. Results, in Table 23, showed the percentage of glucose channeled into biomass and the percentage of glucose required to fix that amount of glucose as biomass.

As Y_{ATP} remains constant and biomass levels are an indication of ATP production (Bauchop and Elsden, 1960), the fallen level of biomass production anaerobically was an indication of the lowered efficiency of ATP production from glucose.

CC3310 Dry weight vs. Aeration

Figure 19

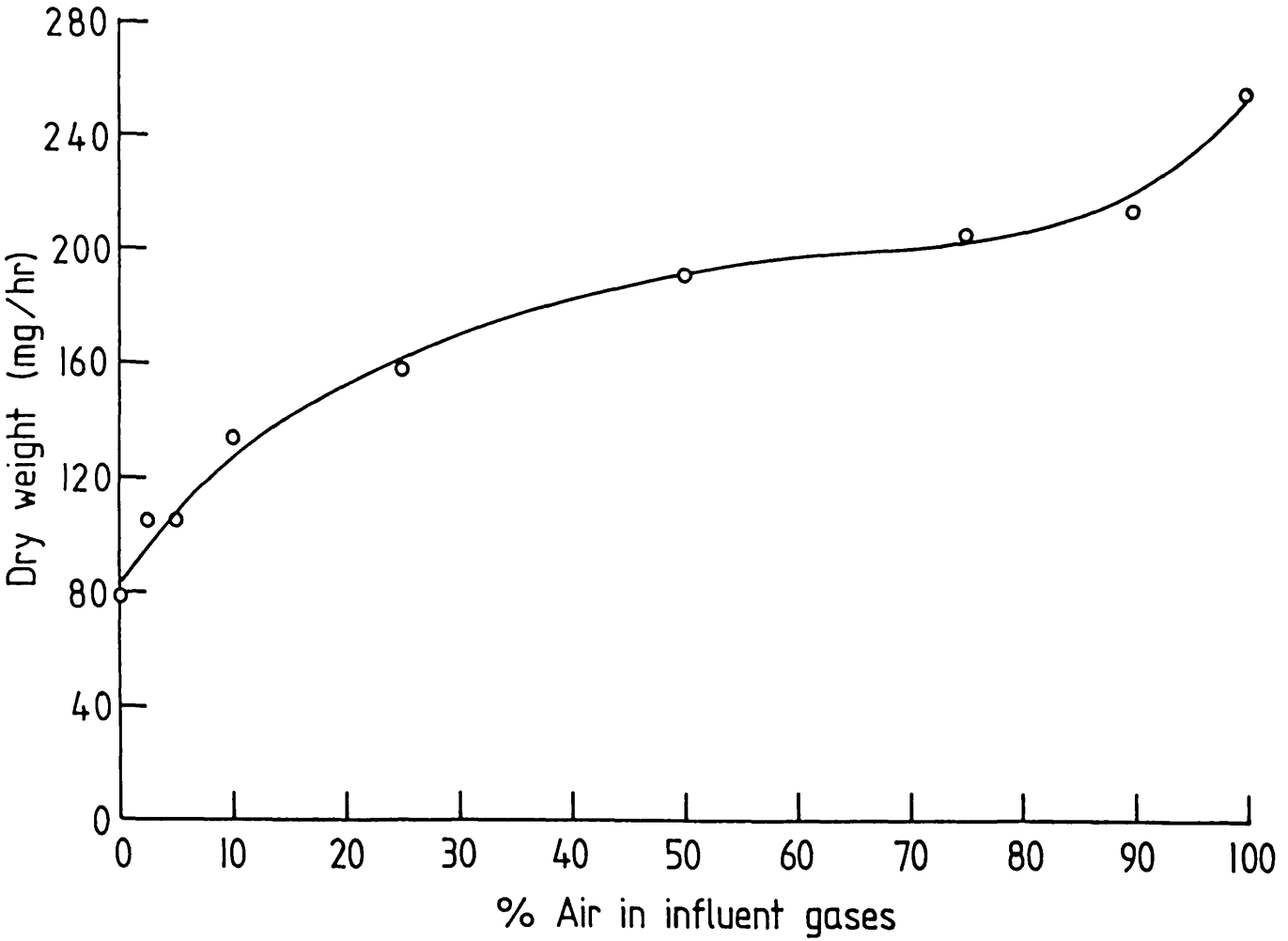


Table 23 Percentile glucose utilised as an energy source and as a carbon source in CC3310 continuous culture

Aeration	% Glucose for Biomass	% Glucose for Energy
100% Air	14.7	85.3
90% Air	12.3	87.7
75% Air	11.8	88.2
50% Air	10.9	89.1
25% Air	9.0	91.0
10% Air	7.7	92.3
5% Air	5.9	94.1
2.5% Air	5.9	94.1
100% N_2	4.5	95.5

In calculating the carbon balance, the method of Herbert (Herbert, 1975) was used in which 50% of the cell dry weight was assumed to have been supplied from glucose.

Metabolite levels

Metabolite levels were determined by a combination of infra-red spectroscopy of the effluent gas (for CO_2), and a combination of gas chromatography (for formate, acetate, pyruvate, lactate and succinate), and enzyme assays (for ethanol and glucose) of spent culture medium. The results were standardised to mmol/hr in order to compare CO_2 levels with other metabolite levels. The results are shown along with a carbon balance (ie. $\frac{\text{mmol carbon/hr out}}{\text{mmol carbon/hr in}} \times 100$).

in Table 24 and in graphic form in Fig. 20.

Under anaerobic conditions acetate and ethanol levels must equal CO_2, formate and succinate levels (see Fig 3). Also under anaerobic conditions, ethanol and succinate levels must equal acetate levels to produce a balance of reducing equivalents (see Fig. 3). Therefore, to assess the accuracy of the anaerobic fermentation balance these equations were calculated (see below):-

a) Ratio of Metabolite accumulation

Ethanol + Acetate = CO_2 + Formate + Succinate

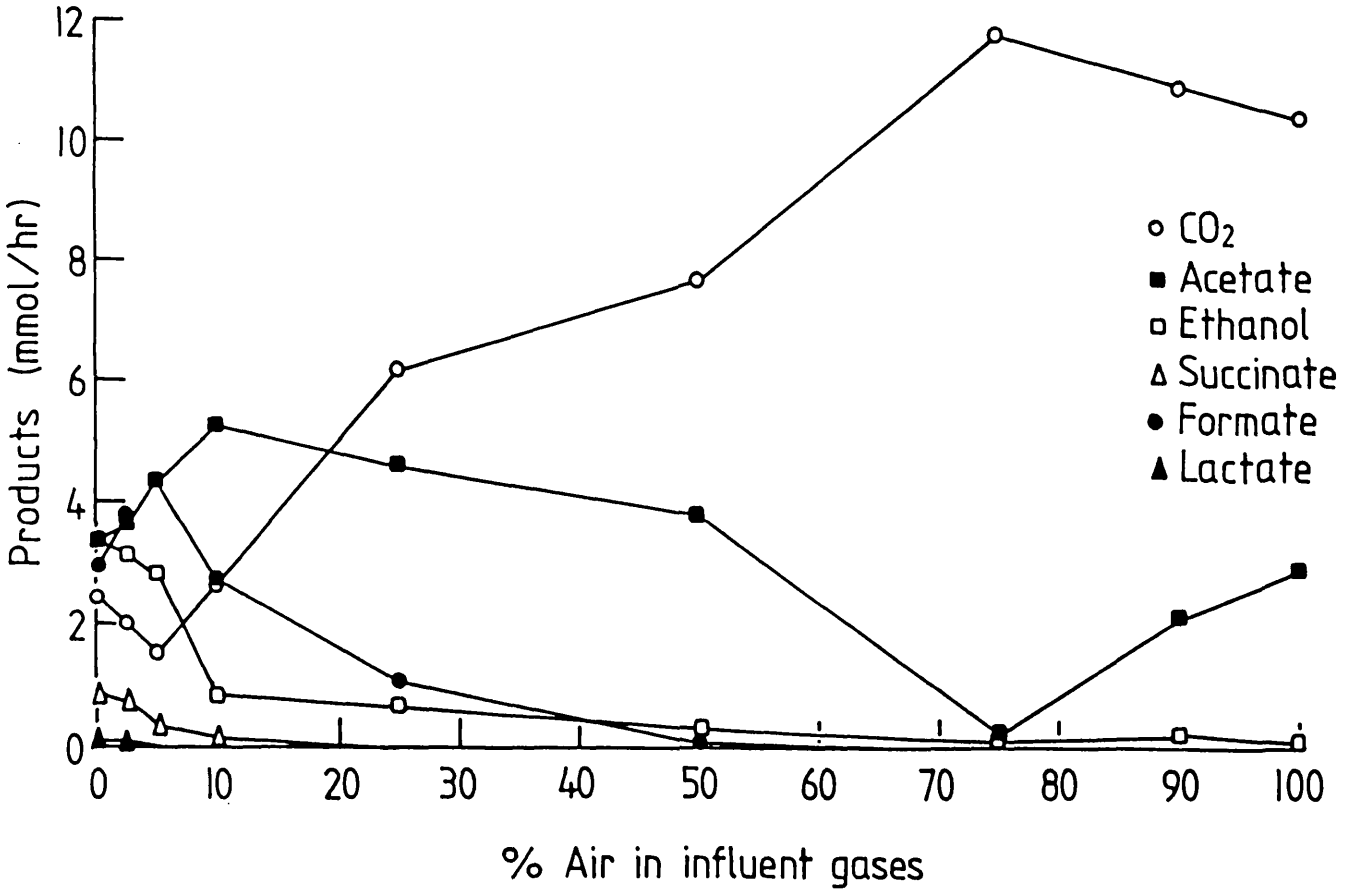
3.2 mmol/hr + 3.4 mmol/hr = 2.5 mmol/hr + 0.9 mmol/hr + 3.0 mmol/hr

Table 24 Product levels from continuous cultures of CC3310

Aeration	CO_2 (mmol/hr)	Formate (mmol/hr)	Acetate (mmol/hr)	Lactate (mmol/hr)	Ethanol (mmol/hr)	Succinate (mmol/hr)	Biomass (mg/hr)	Carbon Balance
100% Air	10.3	0.4	2.9	N.D.	0.1	N.D.	256.3	88.5%
90% Air	10.9	N.D.(a)	2.1	N.D.	0.2	N.D.	215.0	82.6%
75% Air	11.8	N.D.	0.2	N.D.	0.1	N.D.	206.3	73.0%
50% Air	7.7	0.1	3.8	N.D.	0.3	N.D.	191.3	77.8%
25% Air	6.2	1.1	4.6	N.D.	0.7	N.D.	158.3	81.2%
10% Air	2.7	2.8	5.3	N.D.	0.9	0.2	135.0	79.8%
5% Air	1.6	4.4	4.4	N.D.	2.9	0.4	102.5	87.9%
2.5% Air	2.1	3.9	3.7	0.15	3.2	0.8	102.5	91.6%
100% N_2	2.5	3.0	3.4	0.13	3.2	0.9	78.8	87.6%

(a) N.D. = Not Detected
N.B. No glucose was detected in any spent culture supernatant.

CC3310 Fermentation balances vs. Aeration Figure 20



6.6 = 6.4

$\frac{6.6}{6.4}$ = 1.03

Expected value is; $\frac{\text{Ethanol + Acetate}}{CO_2 + \text{Formate + Succinate}}$ = 1.0

Actual value is 3.0% off the expected value

b) Balance of Reducing equivalents

Acetate = Ethanol + Succinate

3.4 mmol/hr = 3.2 mmol/hr + 0.9 mmol/hr

3.4 = 4.1

$\frac{4.1}{3.4}$ = 1.20

Expected value; $\frac{\text{Ethanol + Succinate}}{\text{Acetate}}$ = 1.0

Actual value is 20% off the expected value

The ratio of metabolite accumulation, being 3.0% off the expected value, fell within the bounds of experimental error. The balance of reducing equivalents however, was well outside the expected value and indicated an unusual NADH generating pathway (there being excess NAD^+ indicated by the balance).

Continuous culture of CC2807

The continuous culture apparatus was set up as described in 'Materials and Methods' in two chemostats with different air:N_2 ratios in the influent gases. Steady state was judged to be established after 48 hours; that is 4.8 culture volumes of medium passed through the chemostat, and was monitored by observing stable levels of CO_2 in the effluent gases (by I.R.Spectroscopy) and constant $O.D._{550nm}$ of the cultures.

Samples were taken via the rapid sampling port and the overflow lines to ascertain the physiological state of the cultures at those aeration levels. Samples were also taken to assess the integrity of the chemostat and the sterility of the medium in the aspirator.

After samples had been taken, aeration was altered by changing the air and N_2 flows into the chemostats. Aeration was always altered in the aerobic to anaerobic direction (ie. the ratio of N_2/air in the influent gas always increased).

NAD^+ and NADH Ratios

Cultures extracted from the rapid sampling port were treated as described in 'Materials and Methods' to provide samples for the cycling assay of NAD^+ and NADH. Total pool

sizes were not measured but the ratio of NAD^+/NADH (as an indication of internal redox potential; Wimpenny and Firth, 1972) was recorded. This ratio was determined by comparison between the rate of change of optical density at 570nm of samples treated to extract NAD^+ or NADH. All readings were taken in duplicate and were reproducible to a level of ±10%.

The results (Fig. 21) showed a deviation from the pattern seen in the wild-type strain (CSH3B) but showed similarity to that pattern of NAD^+/NADH ratios obtained from the continuous culture of CC3310 (the ubiA harbouring strain). From a lowered (compared to wild-type) ratio at 100% air (3.9), the ratio rose to a broad maximum (8.4) at 50% air, equal to the fully aerobic ratio of the wild-type. From this maximum the ratio dropped sharply to the anaerobic level of 2.2. The anaerobic ratios of NAD^+/NADH obtained from the continuous cultures of CSH3B, CC3310 and CC2807 were (within the bounds of experimental error) all the same. This was to be expected, as under full anaerobiosis the ubiA, adhR and wild-type phenotypes were indistinguishable.

Biomass levels

Biomass levels were determined by taking the optical densities of the cultures at 550nm and relating them to dry weights, from an OD_{550nm} vs. dry weight standard plot. The results are shown in Table 26 and in graphic form in Fig. 22.

From Fig. 22 it can be seen that the cultures experienced oxygen stress at air supply rates as rates as high as 90% (as biomass had decreased at that level of aeration). Biomass levels then remained constant (at ca. 287mg/hr) till 50% air in the influent gas supply, when the levels dropped to 66.3mg

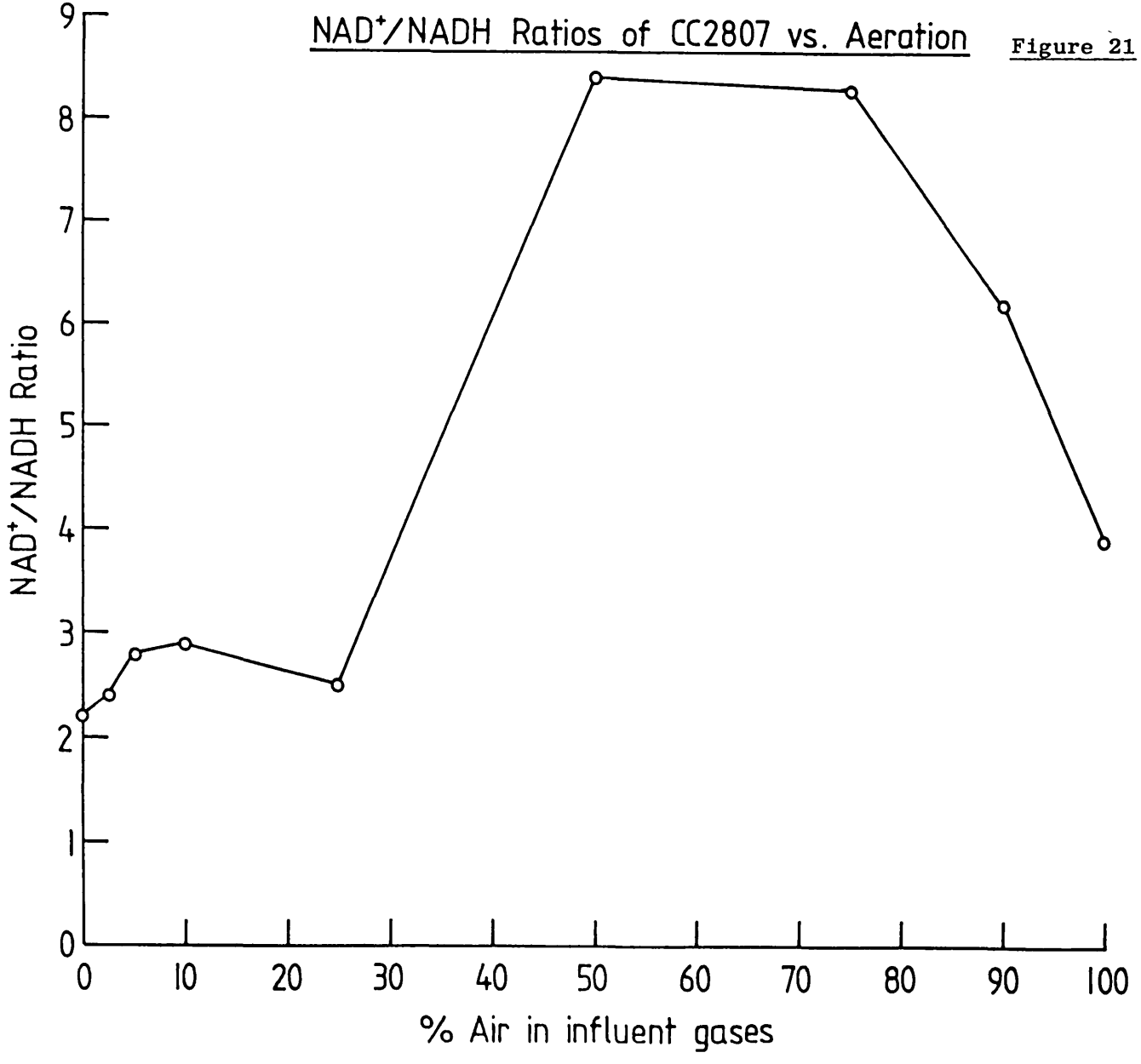
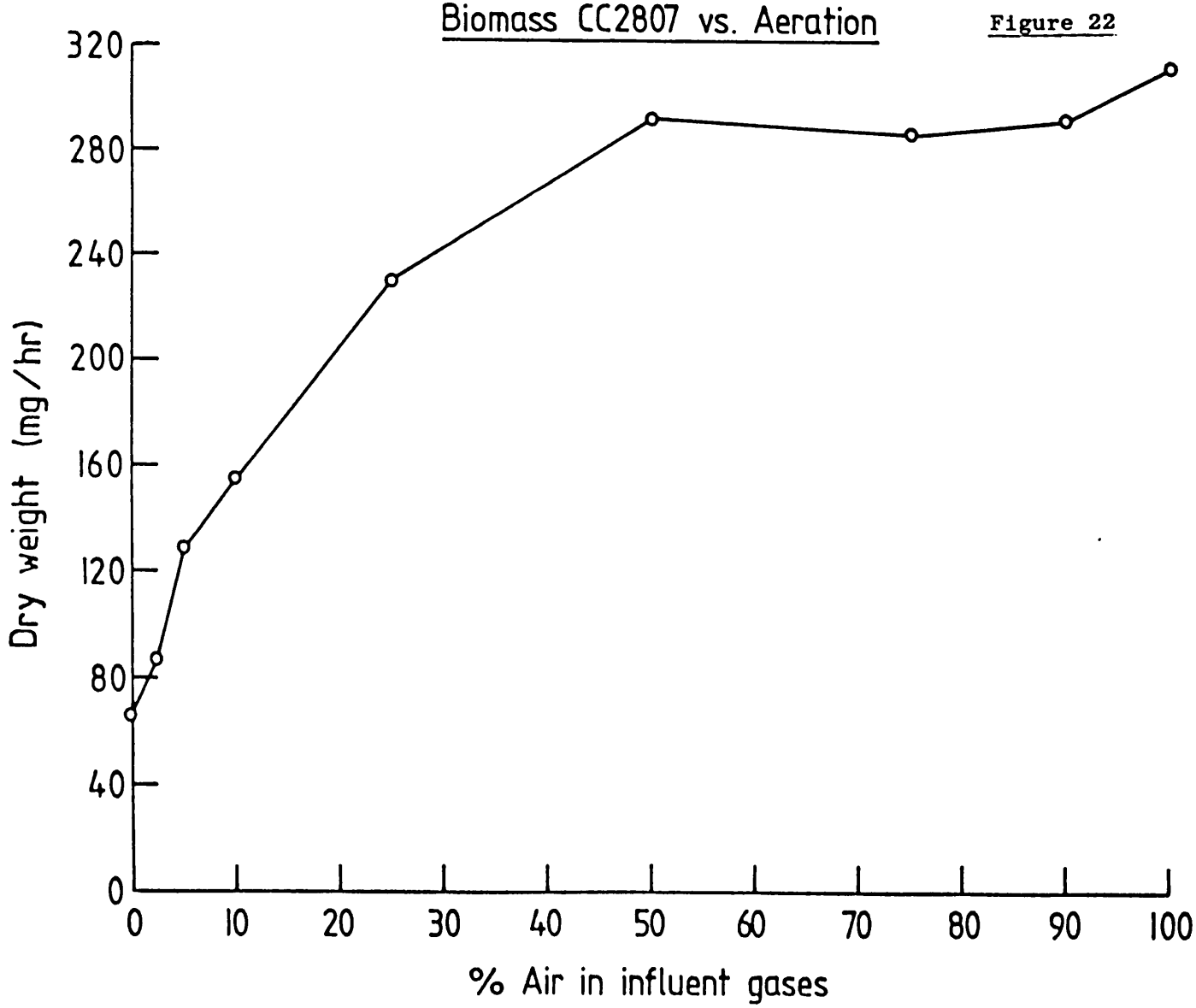

NAD+/NADH Ratios of CC2807 vs. Aeration
NAD+/NADH Ratio
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70
80
90
100
% Air in influent gases

Figure 21


Biomass CC2807 vs. Aeration
Figure 22
Dry weight (mg/hr)
320
280
240
200
160
120
80
40
0
0
10
20
30
40
50
60
70
80
90
100
% Air in influent gases

under fully anaerobic conditions. The classical 'Paster Effect' of a decrease in biomass under anaerobiosis was observed along with the implied increase of glucose flux. The flux under anaerobiosis was x4.7 greater per gram dry weight of culture than under fully aerobic conditions. This figure was a little higher than that quoted by Gottschalk (Gottschalk, 1979). Concomitant with this effect was the increase in substrate required to provide energy for biomass production. Results, in Table 25, showed the percentage of glucose channeled into biomass and the percentage of glucose required to fix that amount of glucose as biomass.

As Y_{ATP} remains constant and biomass levels are an indication of ATP production (Bauchop and Elsden, 1960). The fallen level of biomass anaerobically was an indication of the lowered efficiency of ATP production from glucose.

In calculating the carbon balance, the method of Herbert (Herbert, 1975) was used, in which 50% of the cell dry weight was assumed to have been supplied from glucose.

Metabolite levels

Metabolite levels were determined by infra-red spectroscopy of the effluent gas (for CO_2) and a combination of gas chromatography (for formate, acetate, pyruvate, lactate and succinate) and enzyme assay (for ethanol and glucose) of spent culture medium. The results were standardised to mmol/hr in order to compare CO_2 levels with other metabolite levels. The results were shown along with a carbon balance (ie. $\frac{\text{mmol carbon/hr out}}{\text{mmol carbon/hr in}} \times 100$) in Table 26 and in graphic form in Fig. 23.

Table 25 Percentile glucose utilised as an energy and as a carbon source in CC2807

Aeration	% Glucose for Biomass	% Glucose for Energy
100% Air	17.9	82.1
90% Air	16.1	83.9
75% Air	16.5	83.5
50% Air	16.8	83.2
25% Air	13.3	86.7
10% Air	8.9	91.1
5% Air	8.0	92.0
2.5% Air	5.0	95.0
100% N_2	3.8	96.2

Table 26 Product levels from continuous culture of CC2807

Aeration	CO_2 (mmol/hr)	Formate (mmol/hr)	Ethanol (mmol/hr)	Acetate (mmol/hr)	Succinate (mmol/hr)	Biomass (mg/hr)	Glucose (mmol/hr)	Carbon Balance
100% Air	12.7	N.D.[a]	N.D.	0.9	N.D.	312.5	N.D.	87.9%
90% Air	15.3	N.D.	N.D.	0.1	N.D.	281.3	N.D.	93.7%
75% Air	12.7	N.D.	N.D.	N.D.	N.D.	287.5	N.D.	80.3%
50% Air	13.6	0.6	0.1	N.D.	N.D.	293.8	N.D.	88.9%
25% Air	13.3	0.4	0.4	0.2	N.D.	231.8	N.D.	85.7%
10% Air	7.2	0.8	1.7	4.5	0.3	155.0	N.D.	89.8%
5% Air	5.3	0.8	2.3	4.3	0.4	138.8	N.D.	85.8%
2.5% Air	3.5	3.1	2.6	4.0	1.0	87.5	N.D.	93.5%
100% N_2	4.7	1.1	3.7	3.1	1.0	66.3	N.D.	87.4%

(a) N.D. = Not Detected

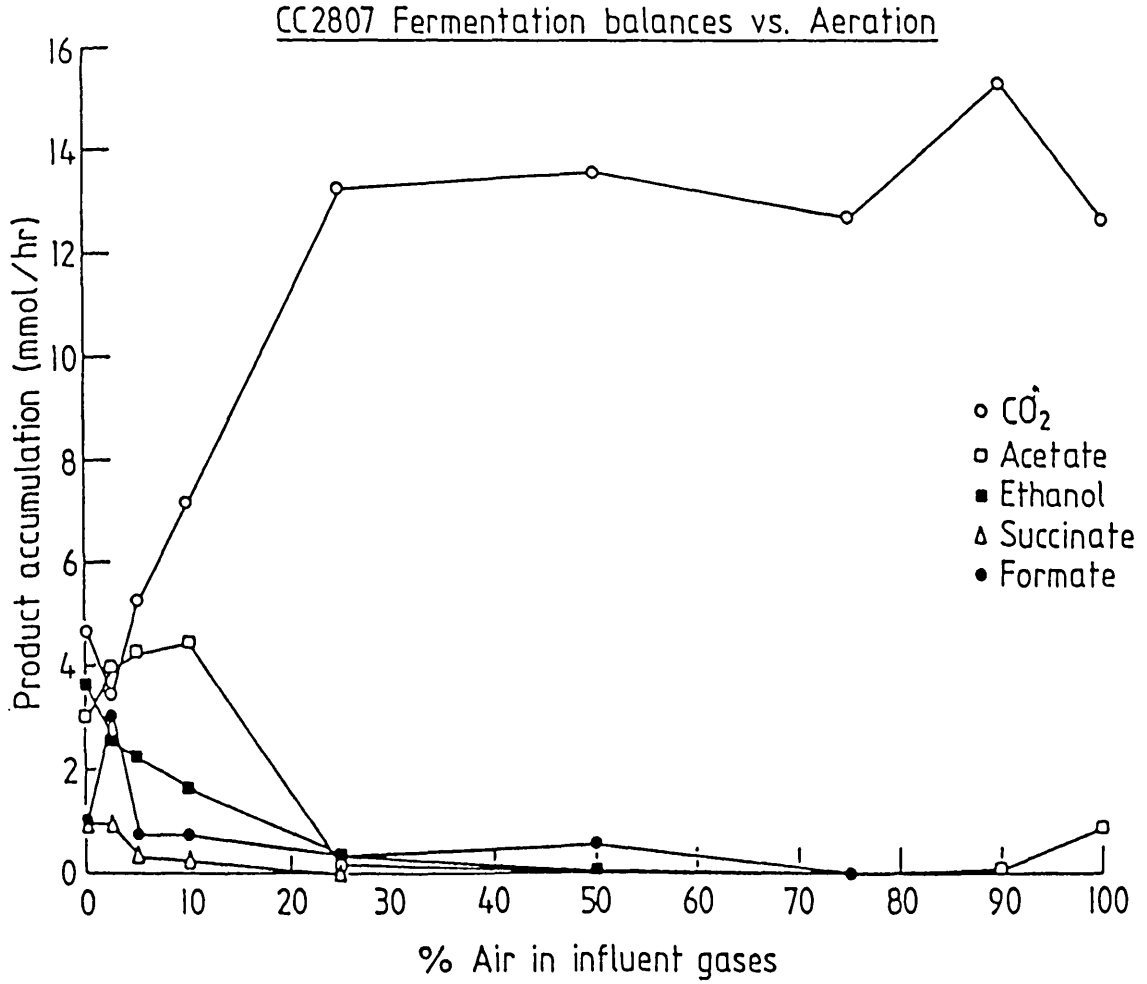

CC2807 Fermentation balances vs. Aeration
Product accumulation (mmol/hr)
% Air in influent gases
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70
80
90
100
CO2
Acetate
Ethanol
Succinate
Formate

Figure 23

Under anaerobic conditions, acetate and ethanol levels must equal CO_2, formate and succinate levels (see Fig. 3). Also under anaerobic conditions, ethanol and succinate levels must equal acetate levels to produce a balance of reducing equivalents (see Fig. 3). Therefore, to assess the accuracy of the anaerobic fermentation balance, these equations were calculated (see below):-

a) Ratio of Metabolite accumulation

Ethanol + Acetate = CO_2 + Formate + Succinate

3.7mmol/hr + 3.1 mmol/hr = 4.7mmol/hr + 1.1 mmol/hr + 1.0 mmol/hr

6.8 = 6.8

$$\frac{6.8}{6.8} = 1.0$$

Expected value, $\frac{\text{Ethanol + Acetate}}{CO_2 + \text{Formate + Succinate}} = 1.0$

Therefore the actual value of the ratio equals the expected value.

b) Balance of reducing equivalents

Succinate + Ethanol = Acetate

1.0 mmol/hr + 3.7 mmol/hr = 3.1 mmol/hr

4.7 = 3.1

$\frac{3.1}{4.7} = 0.66$

Expected value $\frac{\text{Acetate}}{\text{Ethanol + Succinate}} = 1.0$

Therefore the actual value is only 66% of the expected value. This value was well outside the bounds of experimental error and indicated an unusual NADH generating pathway (there being excess NAD^+ indicated by the balance).

Anaerobic metabolism of the facA strain, JRG1078

In the fermentation balances obtained from the wild-type continuous culture experiments, acetate was a major product upon lowering air supplies to the chemostats (Table 19).
In the batch culture fermentation balances of mutant strains, those mutants with lesions in their acetate producing pathways (ie. JRG1061 and JRG1086) were unable to grow anaerobically.

From Fig. 3 it was seen that a balance of reducing equivalents and the production of ATP was possible without acetate production, and therefore growth should have been possible for JRG1061 and JRG1086. To investigate this phenomenom strain JRG1078 (facA) was placed in a growth medium under anaerobic conditions and the spent medium assayed for end-products.

20ml of an overnight culture of JRG1078 grown aerobically in L.B. medium supplemented with 0.2% glucose was washed once in sterile, distilled water and resuspended in 10ml of sterile, distilled water. This suspension was used to inoculate 300ml of minimal medium supplemented with tryptophan, streptomycin and nalidixic acid with 0.2% glucose as carbon source. The medium was degassed by passing O_2 free N_2 throught it and then incubated at 37°C anaerobically after the withdrawal of a post inoculation sample. Anaerobiosis was maintained by passing N_2 through the head space. After 24 hours growth the incubation was halted and samples taken. The withdrawn samples were assayed for biomass (by measuring $O.D._{550}$ and relating to a dry weight vs. $O.D._{550nm}$ standard plot) and metabolite levels by a combination of gas chromatography and enzyme assays.

The results (Table 27) showed that during the incubation period biomass increased by x2.8. That is, there was less than 2 doublings. This minimal growth could be explained by growth factors accumulated during the aerobic growth of the organism. Although the organism did not grow,

Table 27 Fermentation balance of an anaerobic batch culture of JRG1078

Sample	Biomass (mg/litre)	Formate (mM)	Acetate (mM)	Ethanol (mM)	Pyruvate (mM)	Lactate (mM)	Succinate (mM)	Glucose (mM)
Post inoc.	41.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	11.0
24hr Culture	116	0.5	N.D.	1.7	N.D.	16.6	0.9	0.02

it did exhibit glucolysis, the majority of the glucose (75%) went to produce lactate, with smaller accumulations of ethanol, succinate and formate.

The ATP yield from this pathway was 2ATP/glucose, instead of a theoretical maximum of 3ATP/glucose if acetate was formed. This level of ATP generation should enable growth, albeit at a slower rate, so the catabolism of glucose without growth could not be explained in terms of lowered ATP production.

The favoured metabolism of anaerobically grown JRG1078 can be seen in Fig. 24.

Effects of cGMP, cAMP and GTP upon ADH and 2OGDH activities

From work performed in batch (Table 8) and continuous culture (Table 19), evidence of an effector of the anaerobic switch in the region of the electron transport chain shown in brackets, in Fig. 25 was inferred. The batch culture studies showed that an ubiA mutant but not a ndh mutant would result in ethanol accumulation aerobically. Therefore, a component of the chain in this region when reduced, or a metabolite level affected by that reduction may have been the anaerobic effector of ADH induction.

Work by Shaw and Guest (Shaw and Guest, 1982) indicated a pleiotropic mutation, fnr, which when expressed on a multi-copy plasmid aerobically, overcame the aerobic repression of fumarate and nitrate reductases. Further study

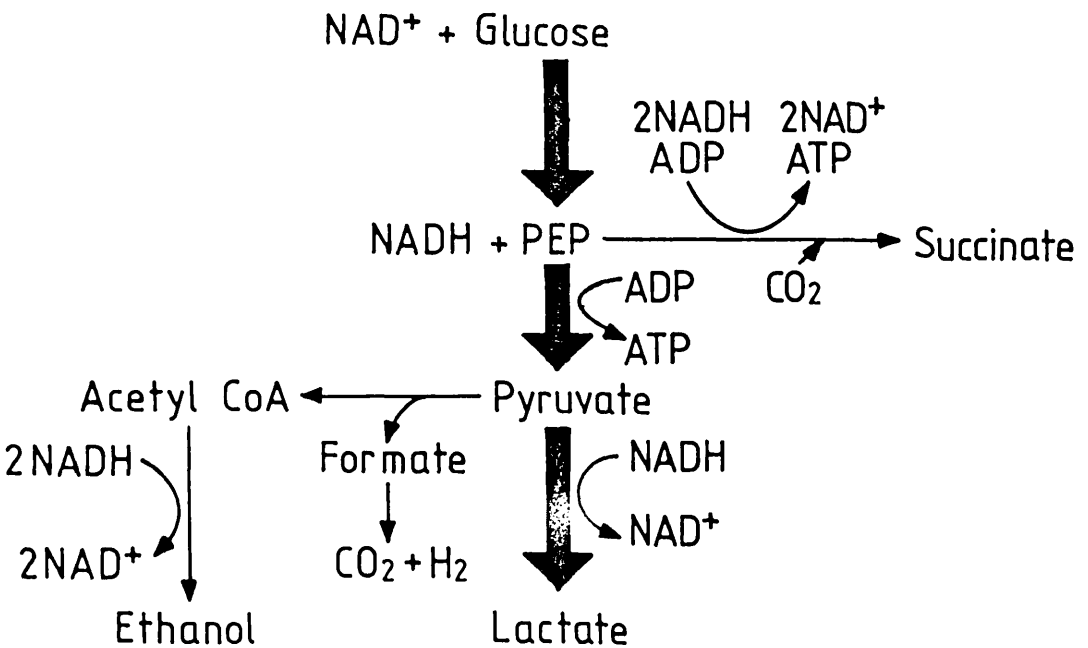


Diagram of Anaerobic glucolysis of JRG1078

Figure 24

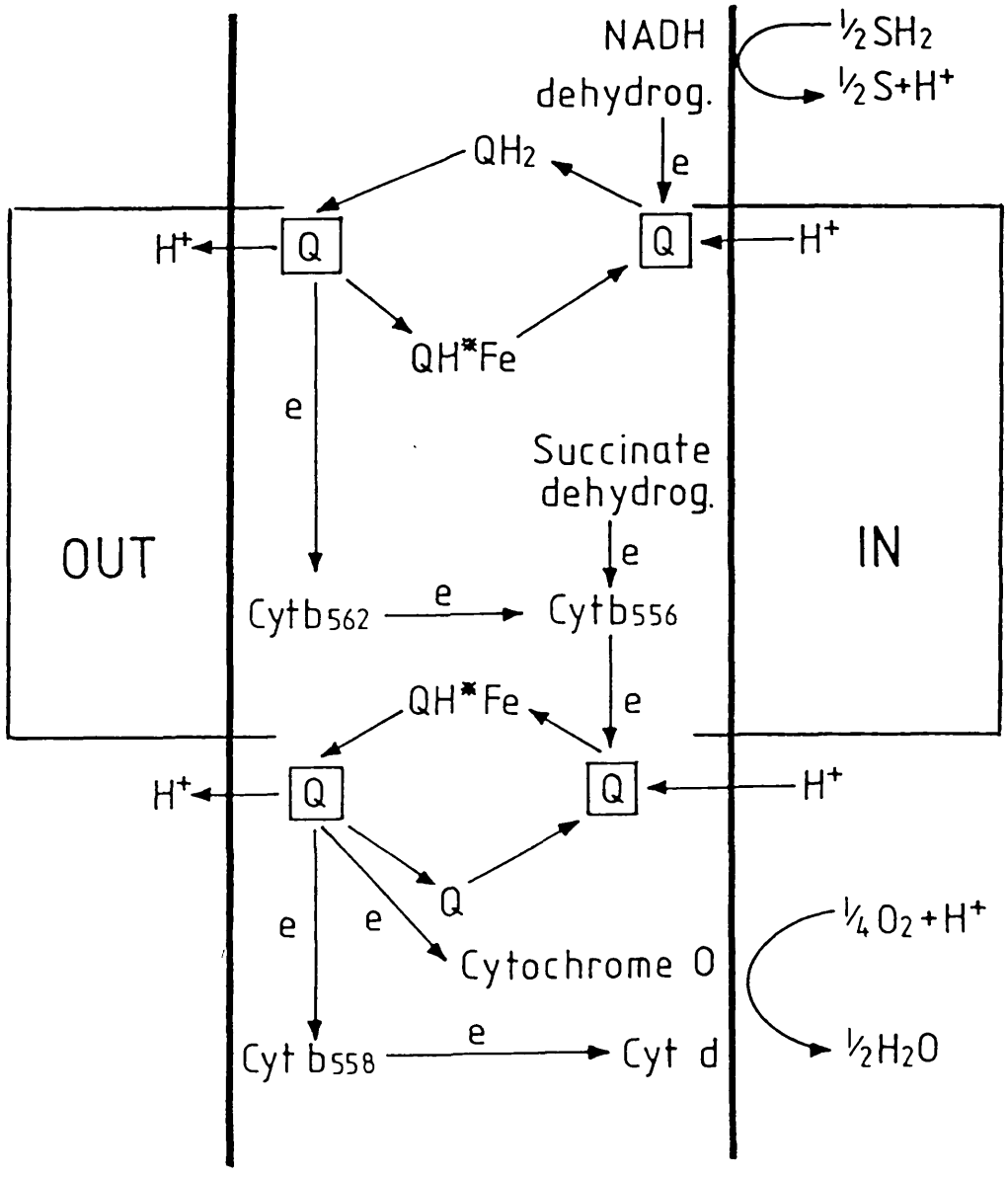


Figure 25

AEROBIC ELECTRON TRANSPORT CHAIN INDICATING REGION ASSOCIATED WITH THE SWITCH

for legend see page 11

(Shaw et al, 1983) showed that the primary protein sequence of the fnr gene product had extensive areas of homology with CRP (Catabolite repression protein). CRP is known to bind cAMP (Perlman and Pastan, 1971), but the purified gene product of fnr would not bind cAMP (pers.comm. Prof. J.Guest). Therefore it was decided to examine the effect of another cyclic nucleotide, cGMP (Pastan and Adhya, 1976).

Phosphorylated guanylate compounds have been associated with the control of protein induction/repression for many years. The magic spot compound, guanosine tetraphosphate, was found to be responsible for the 'stringent response' (Kaplan, Atherly and Barrett, 1973) in E.coli (Reiness et al, 1975). Also increased levels of guanosine tetraphosphate were found to alter the metabolism of E.coli (Debanor, 1982). In strains of bacilli, cGMP was found to act instead of cAMP in controlling catabolite repression (Ide, 1977).

The major GTP generating step in E.coli is between succinyl CoA and succinate in the tricarboxylic acid cycle. The following step is catalysed by succinate dehydrogenase, which was linked into that portion of the electron transport chain indicated in Fig. 25 by FADH. From the continuous culture experiments, succinate was accumulated in the wild-type strain from 90% air in the influent gas. Therefore, it was assumed as succinate was accumulated, GTP production slowed down, and if GTP production was lowered, the production of cGMP from GTP by guanylate cyclase (Macchia et al, 1975) may also have been lowered. Therefore, the switch to anaerobiosis may have been effected by low cGMP levels.

To test this hypothesis an experiment analogous to that which determined cAMP as the effector of catabolite repression (Perlman and Pastan, 1968) was undertaken. Levels of ADH were investigated as being indicative of anaerobic enzyme induction and levels of 2OGDH were measured as being indicative of aerobic enzyme induction.

500ml of an aerobic, carbon limited culture of CSH3B from a chemostat set up as described in 'Materials and Methods' was spun down at 6000g for 10 minutes at 4°C and then resuspended in 50ml of cold 100mM Tris, 10mMEDTA buffer pH 8.0. The culture was left on ice for 5 minutes before being resuspended in 10ml of cold 10mM buffer pH 8.0. A loopfull of this suspension was then withdrawn and plated out onto L.B. medium containing 10μg/ml actinomycin D along with an untreated sample of CSH3B.

The treatment of the cells with EDTA was to make them permeable to nucleotides (Leive, 1965). To ensure that the treatment was successful the treated cells were grown on 10 μg/ml actinomycin D. E.coli K_{12} is normally resistant to this antibiotic (Hurwitz et al, 1962) but treatment with EDTA allows the antibiotic to enter the cell and inhibit RNA synthesis (Leive, 1965). Therefore, cells treated with EDTA that were sensitive to 10μg/ml actinomycin D, also allowed nucleotides to enter the cell. The remaining suspension was then divided into 2.0ml aliquots and inoculated into 5 flasks containing minimal M9 medium supplemented with thiamin, tryptophan, 0.2% glucose and 1μC/ml [^{14}C]-leucine. Three of

the flasks also contained either 0.1mM cGMP, 0.1mM cAMP or 0.1mM GTP. Four of the flasks were sparged with O_2 free N_2 before being sealed with rubber bungs and incubated at 37°C. The fifth flask containing no nucleotide was incubated at 37°C with vigorous shaking. After 3 hours of incubation, 3ml aliquots were withdrawn from each of the flasks and spun down at 6000g for 10 minutes. The pellets were resuspended twice in 3ml 0.1M potassium phosphate buffer pH 7.4, before being placed in scintillation vials and the number of counts from incorporated [^{14}C]-leucine recorded.

The remaining cultures were spun down and resuspended in 0.5ml of 0.1M potassium phosphate buffer pH 7.4. The suspensions were then sonicated on ice to produce a crude cell extract as described in 'Materials and Methods'. The cell extract was then assayed for 2OGDH, ADH and protein.

The results (Tables 28, 29 and 30) showed that under these circumstances neither cAMP, cGMP nor GTP had any effect upon the induction/repression of ADH or 2OGDH. Neither did the nucleotides have any appreciable effect upon protein biosynthesis (as indicated by the amount of [^{14}C]-leucine incorporation). Only the flask containing cAMP differed from the other flasks, with 14% less than the average of the remaining flasks' [^{14}C]-leucine incorporation.

Examination of EDTA treated and untreated cells after overnight incubation on L.B. plates and L.B. plates supplemented with 10μg/ml actinomycin D, indicated that the EDTA treatment was successful as only the untreated cells grew

Table 28 Effect of nucleotides on specific activity of ADH and 2OGDH

Nucleotide	Growth Condition	Specific Activity ADH(U/mg Protein)	Specific Activity 2OGDH(U/mg Protein)
None	Anaerobic	47.3	0.1
cGMP	Anaerobic	45.4	0.2
cAMP	Anaerobic	44.8	0.1
GTP	Anaerobic	42.9	0.1
None	Aerobic	1.7	1.1

Table 29 Incorporation of [^{14}C]-Leucine

Nucleotide	Growth Condition	Av.CPM/ml -Background	%^{14}C Incoporation
None	Anaerobic	667,685	41.3
cGMP	Anaerobic	679,895	42.0
cAMP	Anaerobic	579,580	35.8
GTP	Anaerobic	675,285	41.7
None	Aerobic	662,565	41.0

N.B. Scintilliation counter efficiency was found to be 82% by the method described in 'Materials and Methods'.

Table 30 ADH and 2OGDH Specific activities/% ^{14}C Incorporated

Nucleotide	Growth Condition	Sp.Act.ADH/%^{14}C Incorporated	Sp.Act.2OGDH/%^{14}C Incorporated
None	Anaerobic	1.16	2.42×10^{-3}
cGMP	Anaerobic	1.08	4.76×10^{-3}
cAMP	Anaerobic	1.25	2.79×10^{-3}
GTP	Anaerobic	1.03	2.40×10^{-3}
None	Aerobic	0.04	26.83×10^{-3}

N.B. Sp.Act. = Specific Activity

on the actinomycin D supplemented plates, whereas both treated and untreated cells grew on the L.B. plates containing no antibiotic supplement.

CHAPTER 4

DISCUSSION

Strain Construction

Much effort was put into the accumulation of genetic lesions in an isogenic background. The reason for this was to eliminate the possibility of factors other than the known genetic lesion affecting fermentation balances. This approach was vindicated upon the discovery of three, separate unreported lesions. They were the P1 resistant character of IY12 and IY13, and the second lesion responsible for their small colony morphology, and the lesion in CSH59B preventing the aerobic growth of an ampicillin resistant mutant on minimal medium containing ampicillin with succinate as carbon source. These results highlighted the problems of using E.coli K_{12} strains obtained by N.T.G. mutagenesis and characterised for genetic experiments only. For the investigation of physiological phenomena, the ideal subject would be a wild-type E.coli which was specifically mutated by insertion inactivation (Taylor, 1963).

The proceedures followed in this work (ie. transduction and conjugation) were used to minimise the possibility of incorporating unknown lesions along with the desired character into the host strain. Throughout this work the assumption was made that no unknown genetic marker had been transferred into the recipient strain and therefore, that apart from the desired lesion all constructed strains have an isogenic background. However, the validity of this assumption has been questioned.

Batch Culture Results

From the batch culture experiments on the assembled E.coli K_{12} mutant strains available in the laboratory (see Tables 8 and 9), the fermentation balances of both aerobically and anaerobically growing cells were obtained. Five strains 6161B, LCB900, LCB901, JRG1061 and JRG1086 were unable to grow anaerobically. In strain 6161B the level of pyruvate formate lyase activity was greatly reduced (Varenne et al, 1975) and therefore, although ATP could be accumulated and NADH reoxidised by glucolysis to lactate, no acetyl CoA could be formed for fatty acid biosynthesis, etc.

Strains LCB900 and LCB901 have lowered levels of alcohol and acetaldehyde dehydrogenases (Pascal, et al, 1981). These strains cannot make acetyl CoA from glucose, for if they did NADH would be accumulated (see Fig. 3). Strains JRG1061 and JRG1086 have lowered activities of acetate kinase and phosphotransacetylase respectively (Guest, 1979). These strains are able to form acetyl CoA, ATP and obtain a redox balance during glucolysis and therefore should have been capable of growth. Results obtained from Bacillus Stearothermophilus indicated that a cessation of growth, but not glucolysis, occurred if the acetate producing pathway was blocked (Shama unpub.results). This phenomenon was examined in E.coli K_{12} by using a mutant with lowered activities of both phosphotransacetylase and acetate kinase (Guest, 1979) JRG1078. The anaerobic fermentation balance for this strain showed no growth but the catabolism of glucose mainly to

lactate (86%) (see Table 27). The absence of growth in acetate non-forming mutants can be attributed to the inability of these strains to recycle accumulated acetyl CoA to CoA-SH and acetate (see Fig. 3). Therefore as acetyl CoA builds up the free CoA-SH pool becomes depleted and reactions which depend upon this co-enzyme (eg. fatty acid degradation Metzler, 1977), cease resulting in the inability of the organism to grow.

The aerobic accumulation of acetate (see Fig. 1) may also be ascribed to the recycling of acetyl CoA to free co-enzyme by phosphotransacetylase and acetate kinase (Pascal, et al, 1981). During aerobic growth on glucose, if the flux is so great as to result in a rate of production of acetyl CoA greater than the rate of its usage by citrate synthase (see Fig. 1), acetyl CoA levels would rise with a concomittant decrease in free co-enzyme A. To prevent this acetyl CoA is processed by phosphotransacetylase and acetate kinase to acetate, free co-enzyme A and one ATP. The recycling of acetyl CoA to free co-enzyme A and acetate allows the rate of glucose uptake to be maintained by the organism which results in a higher growth rate and the accumulation of ATP by substrate level phosphorylation, and the reoxidation of NADH linked to oxidative phosphorylation at a greater rate than would occur without this pathway. This process is beneficial to the organism as although the glucose is not used as efficiently by the organism, it does acquire extra ATP, and thus is capable of sustaining a higher growth rate (Anderson and Von Meyenburg, 1980).

The strain lacking pyruvate dehydrogenase activity, HΔ13 (Langley and Guest, 1977), required an acetate supplement for aerobic growth in minimal glucose medium, and to be able to relate all the batch culture fermentation balances, this supplement was present in all minimal growth media for batch culture. HΔ13 was found to grow normally anaerobically, and to accumulate metabolites in roughly the same proportions as the 'wild-type' strains, CSH3B and IY13 (see Table 9). This was due to the pyruvate to acetyl CoA step being mediated by pyruvate formate lyase and not pyruvate dehydrogenase during anaerobiosis. Aerobically however, HΔ13 unlike other strains accumulated large amounts of pyruvate and lactate, and there was no acetate accumulation (see Table 8). This was due to the pyruvate dehydrogenase lesion preventing glucolysis from proceeding past pyruvate, this intermediate then accumulated, and as lactate dehydrogenase is present both aerobically and anerobically (Doelle et al, 1981), the aerobic formation of lactate was brought about by mass action ratios. Acetate was not accumulated, as the flux to acetyl CoA from the 0.02% (w/v) acetate supplement and from pyruvate via pyruvate oxidase was slower than its rate of use by citrate synthase.

Strain DC272 which contained the lesion responsible for over production of alcohol dehydrogenase, adhR (Clark and Cronan, 1980), had a fermentation balance similar to 'wild-type' strains anaerobically. Aerobically, strain DC272's only deviation from a 'wild-type' fermenation balance

was the accumulation of ethanol (see Table 8). As the strain, has an additional aerobic pathway for acetyl CoA cycling to free co-enzyme A, the aerobic accumulation of ethanol may be seen as the competition of the ethanol producing pathway for acetyl CoA and not as a response to redox pressure.

The two mutants with lesions in the electron transport chain, IY12 (with low levels of NADH dehydrogenase activity) and AN385 (containing a mutation in the ubiquinone biosynthetic pathway), both had anaerobic fermentation balances comparable with 'wild-type' balances. Aerobically however, the patterns of metabolite accumulation were different. IY12 accumulated acetate as did 'wild-type' strains, but also lactate. In this instance, unlike in HΔ13, lactate accumulation was not accompanied by pyruvate accumulation. As the ndh$^-$ lesion of IY12 would result in lowering the aerobic $NAD^+/NADH$ ratio, the accumulation of lactate was seen as the way the cell re-oxidised the NADH that the impaired electron transport chain could not.

In AN385 levels of all the metabolites assayed for could be found, including ethanol (see Table 8). This mutant had a block in the electron transport chain at a different position to the ndh$^-$ containing strain (see Fig. 2), but it would also result in lowering $NAD^+/NADH$ ratios aerobically. However, this strain did not simply accumulate lactate but a whole range of metabolites normally associated with anaerobic batch cultures. The differences in fermenation balances between

IY12 and AN385 could have been due to strain line variation or could have been due to the different mutations in the electron transport chain.

To ensure the differences in fermenation balances weren't simply due to strain line variation, the ubiA lesion was conjugated into the IY13 background (see page 100). The fermentation balance of this construct, CC4009, also showed the aerobic accumulation of acetate and ethanol (Table 16), but of none of the other metabolites which accumulated in the AN385 parental strain. From this it was inferred that the ubiA lesion in AN385 was more severe, probably due to a second unreported lesion, and that the accumulation of ethanol in ubiA, but not ndh mutants was an effect of the type of block in the electron transport chain.

Both the mutations, in the NADH dehydrogenase complex and ubiquinone biosynthesis would result in the lowering of NAD^+/NADH levels. In the case of IY12, this resulted in the excess reducing equivalents being re-oxidised by glucolysis to lactate. In AN385 the muation led to the aerobic accumulation of ethanol (an indication of alcohol dehydrogenase activity). From this it was deduced that the effector of ADH induction (at least) was sited between the NADH dehydrogenase complex and the quinone cycles of the electron transport chain (ie. the area between brackets in Fig. 25).

The fermenation balances obtained from strains constructed in the CSH3B background showed similar patterns of product accumulation as the mutation-carrying parental strains (Tables 12 and 13), except for those strains containing the aceF and ubiA lesions (CC3108 and CC3310 respectively).

Strain CC3108 was seen to have accumulated acetate as well as pyruvate and lactate aerobically. This could be interpreted as a decreased severity of the block in pyruvate dehydrogenase activity, or the CSH3B background exhibiting a higher activity of pyruvate oxidase, than the HΔ13 strain.

Strain CC3310, accumulated only ethanol and acetate, whereas the ubiA parental strain, AN385, was found to accumulate succinate, lactate, pyruvate and formate in addition to ethanol and acetate. This phenomenon was also seen when the ubiA lesion was conjugated into the IY13 background, and gave a further indication of the presence in the AN385 background of an unreported lesion that accentuated the ubiA phenotype in that strain.

The constructed strain, CC3502 which contained the ubiA and adhR lesions was expected to accumulate large amounts of ethanol aerobically due to the redox pressure (caused by the ubiquinone block in the electron transport chain) and the large amounts of alcohol dehydrogenase produced aerobically by the adhR genotype (Clark and Cronan, 1980). The actual accumulated level of ethanol however was found to be 0.2mM compared to 0.3mM for the strain carrying the ubiA lesion (CC3310) and 0.4mM for the strain carrying the adhR lesion (CC2807). The reason for the lower than expected accumulation of ethanol in CC3502 is not clear, but may be due to the ubiA lesion interfering with a secondary effect of the pleiotropic adhR mutation.

The other strains constructed to carry two lesions, CC3703 (ubiA and anal lesions) and CC3805 (ubiA and ana2

lesions), acted in an expected manner. That is, both strains were unable to grow anaerobically (due to the ana lesions) and the ubiA phenotype, of aerobic ethanol accumulation, was hypostatic due to the ana lesions' effects on alcohol and acetaldehyde dehydrogenase. Therefore, aerobically only acetate was accumulated by CC3703 and CC3805.

The absence of accumulated lactate in anaerobic fermentation balances of CSH3B in both batch and continuous cultures led to an examination of the causes of lactate production (see page 96). From the results obtained (Tables 14 and 15) it can be seen that similar lactate dehydrogenase activities were found in batch cultures of CSH3B whether lactate was accumulated or not. Lactate was only accumulated by the culture growing in rich medium containing 3% glucose. This evidence coupled to the work done with Klebsiella aerogenes (Teixera de Mattos and Tempest, 1983), indicated that lactate accumultion anaerobically in E.coli K_{12} performed a role analogous to that of aerobic acetate accumulation (see page 157). That is, of removing an inhibition on glycolysis caused by a high flux of glucose through that pathway. In aerobiosis, the block was caused by a depletion of the free co-enzyme A pool, which resulted in the recycling of acetyl CoA to free co-enzyme A and acetate (Pascal et al, 1981). During anaerobiosis, the blockage in glucoloysis could be caused by a depletion of the NAD^+ pool due to the inability of

the ethanol and succinate forming pathways (Fig 3) to re-oxidise NADH at a sufficient rate. To overcome this block, pyruvate would be diverted to lactate with the concomitant re-oxidation of NADH, thus allowing the rate of glucolysis to be maintained. As in the case of aerobic acetate accumulation, this pathway would be beneficial to E.coli as it allows a higher rate of glucose utilisation, with the concomitant higher rate of ATP generation (even though the efficiency of ATP generation would drop from 3 ATP per glucose to 2 ATP per glucose) that would allow a higher growth rate (Andersen and Von Meyenburg, 1980).

Therefore, the production of lactate anaerobically, should not be looked upon solely as the response of E.coli for the re-oxidation of NADH, but also as a response to high rates of carbon flux through glycolysis. This hypothesis is supported by the evidence that a 'pulse' of lactate is produced at the onset of skeletal muscle activity in mammals, due not to anaerobiosis, but to the massive increases in carbon flux through glycolysis triggered by muscle contraction (Newsholme and Start, 1973).

CONTINUOUS CULTURE RESULTS

CSH3B Culture

From the fermentation of a 'wild-type' strain of E.coli K_{12} in continuous culture, it was hoped that the normal

switch from aerobic to anaerobic metabolism could be determined. The information from the steady states between aerobiosis and anaerobiosis, whilst not complete, gave a greater understanding of the order in which anaerobic systems became active during this transition.

In the early stages of the transition to anaerobiosis, the greatest changes in fermentation balances occured in the CO_2 and acetate levels (Fig. 12). As discussed on page 157, acetate accumulation in the presence of air is a method of generating free co-enzyme A from acetyl CoA that the tricarboxylic acid cycle cannot utilise. Therefore the accumulation of acetate was seen as an indication of a loss of activity in the tricarboxylic acid cycle. This loss of activity was mirrored by a fall in CO_2 levels (ca 2/3 of all aerobically produced CO_2 coming from the tricarboxylic acid cycle [see Fig. 1]).

The level of acetyl CoA was not measured in these studies (the cycling assay of Bergmeyer, 1974, is too insensitive to measure the intracellular levels), but there are two indications that it rises to an elevated and possibly constant level during the phase from 90% to 50% influent air. First, there is a stable increase in ethanol levels although alcohol dehydrogenase activity remains constant. Second, there is a dramatic rise in acetate levels; an event that must mirror induction of acetate kinase and phosphotransacetylase.

An increase in acetyl CoA levels in fully anaerobic cells related to inactivation of the tricarboxylic acid cycle has long been documented (Hansen and Henning, 1966). The above results suggest that the rise occurs as soon as oxygen becomes limited.

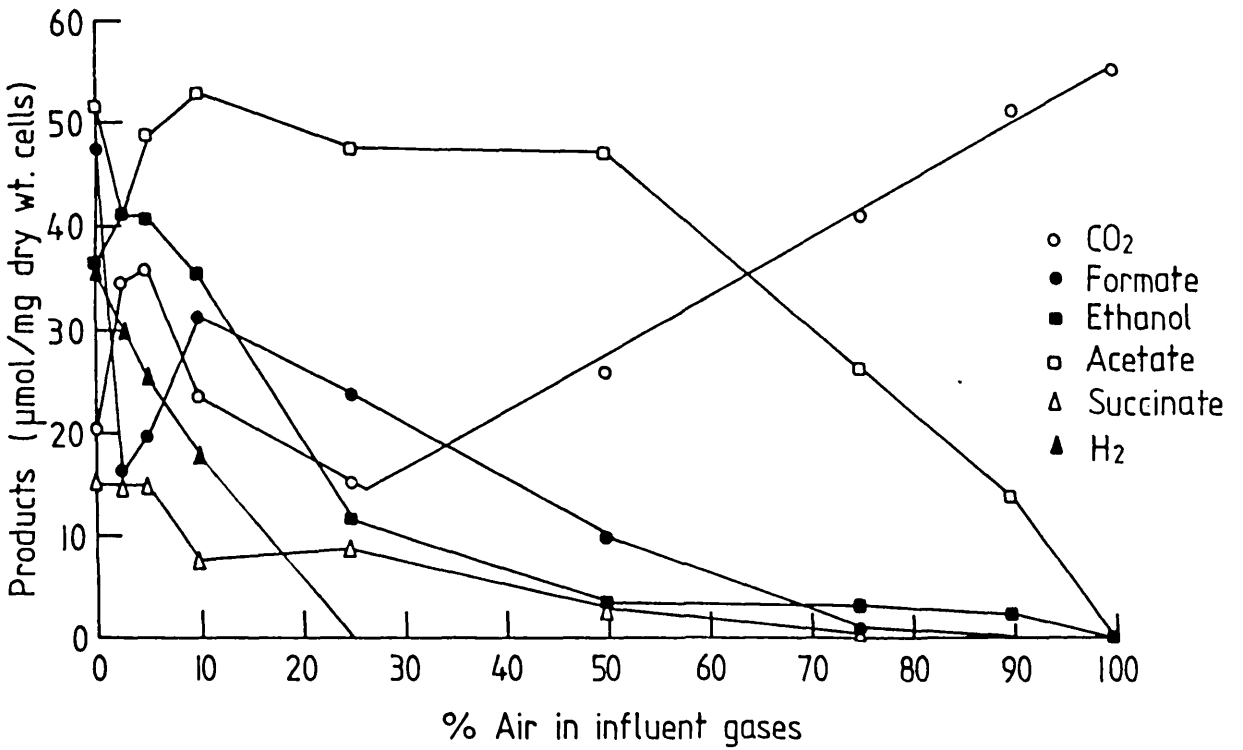
At 75% air in the influent gases formate and succinate were found in the spent culture supernatant (Fig. 12). The accumulation of formate indicated a flux of carbon through the pyruvate formate lyase pathway instead of through pyruvate dehydrogenase. The accumulation of succinate, indicated a block in the tricarboxylic acid cycle at the succinate dehydrogenase step, which resulted in a further decrease in the activity of the tricarboxylic acid cycle.

Between 50% and 25% air in the influent gases a major switch in metabolism occurred. Alcohol dehydrogenase activity started to increase (Fig. 17) which resulted in the accumulation of higher levels of ethanol (contiguous with this was the fall in acetate levels). Also 2-oxoglutarate dehydrogenase activity which had been increasing, peaked andstarted a decline to zero activity at 10% air in the influent gases (Fig. 16).

Between 25% and 10% air in the influent gases, a second switch was observed. CO_2 levels which were tending to zero at 100% N_2 in the influent gases (this was best seen in Fig. 26) levelled off and the accumulation of formate also levelled off (Fig. 26). Concomitant with this was the accumulation of hydrogen in the effluent gases. From this evidence the induction of a formate hydrogen lyase activity was inferred.

CSH3B Product formation per unit dry wt. cells vs. Aeration

Figure 26



As the amount of hydrogen detected did not account for all the carbon dioxide generated from formate (see Table 31), the re-oxidation of some reducing equivalents from the formate dehydrogenase moiety of the formate hydrogen lyase comlex must have been mediated by oxygen or fumarate (Oltmann and Stouthamer, 1975).

At 10% air in the influent gases, as there can be no tricarboxylic acid cycle (2 oxoglutarate dehydrogenase activity being zero, Fig. 16), succinate accumulation must have been solely due to a fumarate reductase activity, and the branched form of the tricarboxylic acid cycle must have been present (Payton and Haddock, in press).

The increase in carbon dioxide levels and the decrease in formate levels from their final anaerobic values (Fig. 12) are believed to have been caused by an increase in formate dehydrogenase activity linked to NADH re-oxidation by oxygen. When oxygen became limiting, reducing equivalents from formate dehydrogenase were re-oxidised by the formation of hydrogen and succinate (Oltmann and Stouthamer, 1975).

The linear increase in hydrogen accumulation from 25% to 0% air in the influent gases may have been due to a linear increase in hydrogenase induction, or due to the inhibitory effect of oxygen on hydrogenase activity (Back et al, 1946; Krab et al, 1982).

By calculating the amount of oxygen required for the oxidation of excess reducing equivalents, the amount of oxygen utilised by the culture was determined (the amount of oxygen utilised for other purposes being very small (Hayaishi and Nozaki, 1969). From this the Yo_2 and then the respiration

rate, qo_2, were calculated (page 112). The aeration procedures were arranged in such a manner as to make the amount of oxygen dissolved in the culture directly proportional to the amount of air in the influent gases (page 23). Therefore, the linear oxygen utilisation response produced between 25% and 100% air in the influent gases (Fig. 13) which, if extrapolated, would intersect the abscissa and would not pass through the origin, indicated that during this phase in the transition to anaerobiosis, the affinity for the electron transport chain for oxygen increased. This increased affinity was caused by an increase in cytochrome levels, as described by Moss (1952), and the transition between a terminal oxidase with a low affinity for oxygen, cytochrome o, to one with a higher affinity, cytochrome d (Haddock and Jones, 1977). The fall in oxygen utilisation after this point indicated the general collapse of the electron transport chain with oxygen supply (Thomas et al, 1972). The plateau observed in Fig. 13 at 5% air was caused by the channeling of reducing equivalents from the newly induced formate dehydrogenase activity to cytochromes of the electron transport chain (Linnane and Wrigley, 1977; Haddock and Jones, 1977).

Fig. 14 showed the yield of biomass in terms of oxygen utilised (Yo_2) to be constant during the period of transition in electron transport chain affinity for oxygen. As ATP generation and biomass yields are directly proportional

(Andersen and von Meyenburg, 1980). The constant Yo_2 indicated that ATP yields from the electron transport chain terminating in cytochrome d were the same as from the electron transport chain terminating in cytochrome o. This evidence was not consistent with the theory that the cytochrome d chain had only one energy conserving site (Brice et al, 1974), whereas the cytochrome o chain had two energy conserving sites (Lawford and Haddock, 1973), but supported the work by Rice and Hempfling (Rice and Hempfling, 1978) which indicated that both pathways were as efficient at ATP generation.

Fig. 15 which showed the response of qO_2 to aeration, mirrored all those phenomena present in Fig. 13 and Fig. 14. That is, a constant respiration rate to 25% air in the influent gases, followed by a fall to the plateau caused by formate dehydrogenase induction, and then a gradual decrease to zero with zero oxygen supply.

At the outset of this work, it was assumed that internal redox potentials would be the trigger for the induction of anaerobic enzyme systems (Ashcroft, 1975; Wimpenny, 1969; McPhedran et al, 1961). However, the internal redox potentials as indicated by the NAD^+/NADH ratios (Wimpenny and Firth, 1972) remained constant over a wide range of the transition to anaerobiosis (Figs. 10 and 12). The induction of pyruvate formate lyase (at 75% air in the influent gases), alcohol dehydrogenase (at 50% air in the influent gases, fumarate reductase and formate hydrogen lyase (at 25% air in the influent gases) activities and the induction of new cytochromes, all occured during the phase of intermediate

NAD^+/NADH ratios. In addition to this the tricarboxylic acid cycle became gradually less efficient, even though the NAD^+/NADH ratios remained constant. Therefore, the scope for the control of induction by internal redox potential (as measured by the NAD^+/NADH ratio) must be somewhat limited. The only parameter which NAD^+/NADH ratios directly affected was the NADH oxidase activity (Fig. 16), which gave an inversley proprotional response to the ratio (ie. proportionally more NADH led to a higher NADHox activity). The assay of NADHoxidase activity by the method of Young and Wallace, should be more accurately named as the DCPIP reductase activity of the NADH dehydrogenase complex; that is the NADH dehydrogenase itself, together with the other respiratory carriers involved in electron transport between NADH and ubiquinone (Young and Wallace, 1976).

The assumption that the NAD^+/NADH ratio would control the tricarboxylic acid cycle during oxygen limitation (Metzler, 1977) is clearly false, as tricarboxylic acid cycle activity decreased whilst the ratio remained constant. Instead, the control of tricarboxylic acid cycle was held to be at the succinate deydrogenase step. Control of the cycle at this step was deduced from the fermentation balances (Fig. 12), which showed the accumulation of succinate whilst the cycle was still operative. From this it was deduced that a block in succcinate dehydrogenase was the flux generating step in the tricarboxylic acid cycle under those conditions. The reducing

equivalents produced by the flavoprotein linked succinate dehydrogenase are re-oxidised by that portion of the electron transport chain already linked to alcohol dehydrogenase induction (see page 160) and therefore, this region of the electron transport chain's role in controlling the switch to anaerobiosis seems to be very important.

Although the lipoamide dehydrogenase component of the pyruvate dehydrogenase system shows in vitro inhibition by NADH (Hansen and Henning, 1966), this cannot be invoked to explain the carbon flux through the pyruvate formate lyase pathway (indicated by an accumulation of formate; Fig 3), as NAD^+/NADH ratios stay very much the same over this period. Therefore, the flux through the pyruvate formate lyase pathway must be due to a competition between this pathway and the pyruvate dehydrogenase pathway. The increased flux through the pyruvate formate lyase pathway, must therefore have been due to an increase in the active form of pyruvate formate lyase as oxygen supply diminished (Knappe, et al, 1974).

The decrease in NAD^+/NADH ratios after 10% air in the influent gases occured when the maximum ethanol concentration had been achieved and therefore indicated that ethanol accumulation was the major method of NAD^+ regeneration at that point. Therefore, it was deduced from these results that the NAD^+/NADH ratios played very little part in the transition to anaerobiosis, and that this ratio was itself the subject of an homoiostatic controlling mechanism over a wide range of the transition. This control was exerted by the accumulation of acetate, formate and ethanol, which spared the NAD^+ pool and

allowed the maintenance of a stable NAD^+/NADH ratio as the efficiency of NADH re-oxidation by the electron transport chain became impaired by low levels of oxygen.

Although the NAD^+/NADH ratios are not the cause of many enzyme inductions, the presence of lowered internal redox potentials anaerobically would have had a synergistic effect on the activities of enzymes such as pyruvate formate lyase and formate hydrogen lyase that require reduced factors for their activation (Knappe and Schmitt, 1976; Krab et al, 1982).

CC2807 and CC3310 Cultures

The cultures of CC2807 and CC3310, one impaired in NADH re-oxidation (CC3310, the ubiquinone deficient strain) and the other with elevated levels of NADH re-oxidising enzymes (CC2807, the strain which constitutively expressed the enzymes for ethanol accumulation), were performed in an attempt to peturbate the 'normal' pattern of internal redox potential (as described by the NAD^+/NADH ratio) with aeration (see Fig. 10) and equate changes in fermentation balances with the changed internal redox potentials. However, as was seen in the continuous culture of CSH3B, NAD^+/NADH ratios had very little effect upon the induction/repression of enzyme systems and this was apparent in the effects which the adhR and ubiA lesions had upon internal redox potentials (Figs. 18 and 21) and the subsequent fermentation balances (Figs. 20 and 23). The limited effects of internal redox potential (as described by the NAD^+/NADH ratios) upon fermentation

balances was best seen in the results obtained from the continuous culture of CC3310. At 100% and 5% air in the influent gases, the internal redox potentials were the same (at a NAD^+/NADH ratio of 2.8), however the fermentation balances differed greatly (Figs. 18 and 20).

The fermentation balances for both strains were found to be different from that obtained for the 'wild-type' strain, CSH3B. This was attributed to effects other than those caused by the impairment of the NAD^+/NADH homoiostatic control mechanisms. In the culture of CC3310 an aerobic accumulation of acetate gradually decreased to a minimum at 75% air in the influent gases, before a pattern of metabolite accumultion similar to that observed in CSH3B occured. The fermentation balance at 100% N_2 in the influent gases failed to give a redox balance by the method of calculation described on page 111. That method however, failed to take into account any succinate accumulation driven by formate. If there was succinate accumulation driven by formate, the redox balance would be described by:-

$$\text{Acetate} + \text{succinate} + CO_2 - H_2 = \text{Ethanol} + \text{Succinate}$$

or

$$\text{Acetate} + CO_2 = \text{Ethanol} + H_2$$

For the CSH3B anaerobic redox balance the new method of calculation would have no effect, as the amount of hydrogen

produced by the culture equalled the amount of succinate and carbon dioxide produced (see Tables 19 and 20). However, the anaerobic redox balance of CC3310 calculated on page 132 showed an excess amount of NADH re-oxidation. It was therefore hypothesised that the shortfall in reducing equivalents was made up by a level of formate driven succinate accumulation that would allow a redox balance to be achieved. In this case 77% of all succinate would have had to be accumulated by the reduction of fumarate by formate, resulting in a hydrogen level of 2.7 mmol/hr being produced by the culture if a redox balance was to have been achieved. As no equipment for the determintion of hydrogen levels was available at the time of the CC3310 culture, this hypothesis remains unproven. The hypothesis was strengthened however, by the knowledge that fumarate reduction could be driven by formate (Rosenberg et al 1975), that in ubiA strains levels of menaquinone were up to three times higher than in the wild-type strain (Cox et al, 1970) and that fumarate reductase had a menaquinone requirement (Guest, 1979). Therefore, an increase in menaquinone levels caused by the ubiA lesion could conceivably have resulted in the linkage of reducing equivalents from formate dehydrogenase to fumarate reductase, rather than to hydrogenase.

There was also no redox balance in the fully anaerobic culture of CC2807, if calculated by the method on page 142 . Therefore, it was again hypothesised that formate driven reduction of fumarate had taken place. To obtain a redox

balance, 60% of all succinate accumulated would have had to be produced by the reduction of fumarate by formate, resulting in a hydrogen level of 4.1 mmol/hr being produced by the culture. Again, no hydrogen detecting equipment was available when CC2807 was cultured and therefore the hyothesis was again unproven, but the linkage of formate dehydrogenase to fumarate reductase was held to be a result of the pleiotropic nature of the adhR lesion in CC2807 (Clark and Cronan, 1980).

The fluctuations of NAD^+/NADH ratios in continuous cultures of CC2807 and CC3310 over the region of the anaerobic switch are puzzling. In both cases the fully aerobic ratio is lower than for the wild-type. This was expected for the ubiA strain CC3310 as a consequence of the block in the electron transport chain, but there is no obvious reason why constitutive expression of alcohol and acetaldehyde dehydrogenase, the phenotype of CC2807, should yield a low aerobic NAD^+/NADH ratio. This observation signals an unexpected influence of the adhR lesion on the fully aerobic capacity of the electron transport chain to reoxidise NADH.

Moreover the $NADH^+$/NADH ratios rise in both mutant strains to a maximum at 50% air in the influent gases, which signals an increase in NADH oxidation by the electron transport chain, since there is no corresponding jump in secretion of reduced metabolites. This is not an obvious consequence of either the ubiA or the adhR mutation, but once again underlines the profound switch in metabolism that appears to occur at 50% air in the influent gases.

Both the mutant strains return to wild-type ratios of $NAD^+/NADH$ in fully anaerobic cultures. This was expected in both cases, but shows that the pleiotropic effects discussed above are exercised only in the presence of different oxygen concentrations.

Investigation into the effects of nucleotides on the batch culture of CSH3B

The aim of this experiment was to see if certain nucleotides could prevent the induction/repression of two enzymes in CSH3B, one characteristic of anaerobic growth (alcohol dehydrogenase), the other characteeistic of aerobic growth (2-oxoglutarate dehydrogenase), when placed in an anaerobic environment. That is, to peturbate the normal pattern of enzyme biosynthesis by the addition of a specific nucleotide and therefore ascribe to that nucleotide a physiological effect.

In the experiment devised (page 149) no significant differences could be found between alcohol dehydrogenase and 2-oxoglutarate dehydrogenase in those cultures used as controls (ie. those cells incubated anaerobically with no nucleotides added) and the test cultures (Table 28). The only significant difference was in the total level of protein biosynthesis of the culture containing cAMP. The cells in this culture accumulated only 86% of the $[^{14}C]$-leucine that the other cultures accumulated (Table 29). This may have been an effect of cAMP or due to inaccuracies in the exoerimental procedure, but even if it was an effect of cAMP, it was a gross effect on all protein biosynthesis as the pattern of enzyme biosynthesis (as measured by the activites of alcohol

dehydrogenase and 2-oxoglutarate dehydrogenase) was the same as those patterns found in the other anaerobic cultures.

Therefore, the results from this experiment suggested that the nucleotides tested (cGMP, cAMP and GTP) had no effect upon the induction or repression of aerobic and anaerobic enzymes. However, the experiment contained some elements that might have invalidated the results, of these the cold shock given to the cells during centrifugation was deemed most serious, as cold shock can have far ranging effects upon the physiology of micro-organisms (Postgate and Hunter, 1963). Also, the cold shock may have delayed the adaption of the cells to their anaerobic environments, therefore as the results were obtained from point readings taken three hours after the start of the experiment, the enzyme activities may not have reached their true anaerobic values.

The assay of alcohol dehydrogenase and 2-oxoglutarate dehydrogenase required a large biomass of the cells as the assay systems for these enzyme were not sensive, and this put another constraint upon the experiment. It was felt that this experiment should be repeated using Mu(lac) constructs inserted behind the promotors of proteins induced or repressed by anaerobiosis (Gowrishankar and Pittard, 1982), for this would allow the continuous analysis of the activity of those promotors as they responded to anaerobiosis, by using the sensitive β-galactosidase assay (Miller, 1972).

SUMMARY

The work described in this thesis was carried out in an attempt to describe the aerobic to anaerobic switch in wild-type E.coli K_{12} and ascribe an effector to the various phenomena that occurred during the tranisition to anaerobiosis As far as the availability of analysis systems and tolerances of equipment would allow a descripton of the switch has been obtained. The first adaption to anaerobiosis in CSH3B was at 90% air in the influent gases, with the induction of phosphotransacetylase and acetate kinase (page 164), the induction of 2-oxoglutarate dehydrogenase (Fig. 16) and the induction of NADHoxidase (Fig. 16) activities. The only enzyme activity induced that had a known effector was NADHoxidase (more correctly known as the NADH dehydrogenase complex activity, see page 170) which responded in an inverse manner to the NAD^+/NADH ratio.

The second event in the transition to anaerobiosis occured at 75% air in the influent gases, where formate accumulated due to the increase in pyruvate formate lyase activity, caused either by an increase in the activating enzyme, or by the lowered levels of inhibitory oxygen (page 165). Also at this point succinate began to be accumulated caused by an inhibition of the succinate dehydrogenase activity (page 165). The accumulation of succinate at this point indicated a decreased activity of the

tricarboxylic acid cycle during oxygen stress, caused not by NAD^+/NADH ratios but by the inhibition of succinate dehydrogenase activity, presumably due to the inability of the electron transport chain to re-oxidise the reducing equivalents produced by this enzyme.

At 50% air in the influent gases ethanol accumulation increased due to an induction of alcohol dehydrogenase (Figs. 12 and 17) and the increases in acetate accumulation levelled off (Fig. 26). Also at this point, 2-oxoglutarate dehydrogenase activity started to decline after reaching a maximum (Fig. 16), and the NAD^+/NADH ratios of the mutant strains CC2807 and CC3310 also reached maxima (Figs. 18 and 21).

After 25% air in the influent gases the electron transport chain ceased to increase it's affinity for oxygen (which had occured continuously from 100% air in the influent gases; page 168); 2-oxoglutarate activity fell to zero (Fig. 16) and the activation of formate hydrogen lyase and fumarate reductase activities occured (page 165). The activation of the formate hydrogenlyase activity allowed some re-oxidation of reducing equivalents from the formate dehydrogenase moiety of formate hydrogen lyase by oxygen (page 167).

After 10% air in the influent gases the NADH dehydrogenase complex was further induced (Fig. 16) by a decrease in the NAD^+/NADH ratio (Fig. 10).

After 25% air in the influent gases, the pattern of metabolite accumulation changed in a manner that gave a redox

balance in the absence of oxygen (Fig. 12), but rather than an induction and repression event, it was probably due to the inhibition and activation of enzyme systems by the nicotinamide adenine dinucleotides.

The identity of the effector (or effectors) of the switch however, remains unknown. A region of the electron transport chain (see Fig. 25) has been implicated in the switch by batch culture experiments (page 160), but whether the effector lies within the electron transport chain as is claimed in the work of DeGroot and Stouthamer (1970), or is some other element indirectly affected by the ubiquinone block has not been determined. This work has however, indicated that internal redox potentials (as described by NAD^+/NADH ratios) played only a limited role in the adaptation to anaerobiosis, and that this parameter was the subject of homoiostasis mechanisms over a large extent of the transition to anaerobiosis (page 169). The possibility that levels of succinate may trigger the induction/repression of anaerobic enzyme systems also appears unlikely due to two factors. Firstly continuous culture of CSH3B shows the succinate level dips at 10% air in the influent gases (Fig. 12) without any corresponding change in physiology. Secondly a literature search, could find no reports of increased levels of anaerobic enzymes in organisms grown aerobically on succinate, and as succinate is a common carbon source, such reports might have been expected.

These results along with the growing amount of evidence of an hierachy of control proteins (adhR, Clark and Cronan, 1980); fnr, Shaw and Guest, 1982) indicated that the mechanism of control of the switch is not the simple change in internal redox levels as suggested by Showe and Demoss (1968) and

Wimpenny (1969), but by a method analogous to catabolite repression, whereby, small molecules (effectors of the switch) modulate the affinity of 'pasteur control proteins' (eg. fnr, adhR gene products) for their attachment sites in promotor regions of 'pasteur proteins' (ie. those proteins induced or repressed by anaerobiosis), thereby increasing or decreasing the rate of transcription of those proteins (Kuritzkes, 1984; Pascal, 1982).

The hypothesis that control of the aerobic to anaerobic switch is mediated solely by the modification of a specific nucleotide in tRNA (Buck and Ames, 1984) is unlikely as the modified nucleotide, cis-2-methylthioriobosylzeatin mentioned in Buck and Ames's work on Salmonella typhimurium was found to be absent in E.coli K_{12} (Alexander and Young, 1978) and yet the control of the aerobic to anaerobic transition still occurs. Therefore, although some attenuation control of transcription as described by Buck and Ames may occur in other facultative anaerobes, the absence of the modified nucleotide and the presence of the 'pasteur control proteins', adhR and fnr gene products, in E.coli K_{12} make this form of control unlikely in E.coli K_{12}. However, there is the possibility that modification of tRNA of the type suggested by Buck and Ames (1984) may be triggered by the redox state of an unknown component in that region of the electron transport chain indicted in Fig. 8, which in turn might control the induction and repression of 'pasteur control proteins'.

In addition to a description of the switch and evidence of an effector associated with the sucinnate dehydrogenase component of the electron transport chain, this work has presented evidence of the role of lactate and acetate accumulation during the transition to anaerobiosis. Lactate accumulation was found to be unnecessary for the anaerobic growth of E.coli K_{12} (Fig. 12), and when it does occur is due to a high glucolytic flux rather than redox pressure (see page 162). The accumulation of acetate anaerobically was found to be essential for growth but not glucolyis (see page 156). This is due to the requirement of free co-enzyme A, which is regenerated from acetyl CoA by the action of the acetate producing enzymes, phosphotransacetylase and acetate kinase.

To identify the effector (or effectors) of the switch, further work should be carried out into the redox states during the transition to anaerobiosis of the components of the electron transport chain in the region of succinate dehydrogenase, and also to assay the steady state levels of cAMP, cGMP CoA-SH and acetyl CoA. By comparison of these parameters with enzyme induction and repression the effector (or effectors) of the switch could be identified. Recent work with strains lacking adenylate cyclase activity (Unden and Guest, 1984) has suggested that the induction of fumarate reductase, required the presence of cAMP, and therefore this cyclic nucleotide should be put under the strict scrutiny to see if any linkage between cAMP levels and enzyme induction can be seen. However, the very high levels of cAMP required

to alleviate the reduction of fumarated reductase activity, and the fact that the cAMP binding domain of the CRP is not the same as the fnr gene product although other regions are highly conserved suggested that the ligand of the fnr gene product is a cAMP-like compound (eg. cGMP or acetyl CoA/CoA-SH) rather than cAMP itself.

Further work should also be carried out to identify more 'pasteur control proteins', followed by sequencing and binding studies. From this work, common sequences or secondary structures in promotor regions responsible for the binding of control proteins may be identified.

References

Alexander, K. and I.G.Young (1978), Biochem. 17, 4750-4755

Amarasingham, C.R. and B.D.Davies (1965), J.Biol.Chem. 240, 3664

Andersen, K.B. and K. von Meyenburg, (1980), J.Bacteriol. 144, 114-123

Ashcroft, J.R. and B.A.Haddock (1975), Biochem. J. 148, 349-352

Back, K.J.C. (1946), Australian J.Sci.Res. 9,25-33

Bachmann, B.J. and K.Brooks-Low, (1980), Microbiol.Ref. 44, 1-56

Bauchop, T. and S.R.Elsden, (1960), J.Gen.Microbiol. 23, 457

Beckwith, J.R., A.B. Pardee, R Austrian and F.Jacob (1962), J.Mol Biol. 5,618-634

Bergmeyer, H.U. (1974) in "Methods of Enzymatic Analysis": (H.U.Bergmeyer, ed.) 1,480 Verlag Chemie, Academic Press, London

Bergmeyer, H.U. and H Mollering (1966), Biochem. Z. 344 167

Bergmeyer, H.U., K.Gawehn and M.Grassl (1974) in "Methods of Enzymatic Analysis" (H.U.Bergmeyer, ed.) 1,425-426 Verlag Chemie, Academic Press, London

Bernofsky, C. and M Swan (1973), Anal.Biochem. 53,452

Bernt, E. and I. Gutmann (1974) in "Methods of Enzymatic Analysis" (H.U. Bergmeyer, ed) 3, 1499-1502, Verlag Chemie, Academic Press, London

Birch, J.R. and S.J Pirt (1969), J.Cell Sci. 15, 135

Blackwood, A.C., A.C. Neish, and G.A.Ledingham (1956), J. Bacteriol. 72, 497-499

Bloxham, D.P. and H.A. Lardy (1973) in "The Enzymes" (P.D.Boyer, ed.) 8 239 Academic Press, New York

Bochner, B.R. and M.A.Savageau (1977), Appl. Environmen. Microbiol. 33, 434-444

Brice, J.M., J.F.Law, D.J.Meyer and C.W.Jones, (1974), Biochem.Soc.Trans. 2, 523-529

Brown, K.D. (1970), J.Bacteriol. 104, 177-188

Brown T.D.K., M.C.Jones-Mortimer, and H.L.Kornberg (1977), J.Gen.Microbiol. 102, 327-336

Buck, M. and B.N.Ames (1984), Cell 36, 523-531

Casse, F., M-C.Pascal, M Chippaux and J.Ratouchniak, (1970), Molec.Gen.Genet. 148, 337-340

Castor, L.N. and B.Chance (1959), J.Biol. Chem. 234, 1587-1592

Ceriotti L. and J.Frank (1969), Clin.Chim.Acta 24, 311-317

Clark, D. and J.E.Cronan (1980), J.Bacteriol. 141, 177-183

Clarke, P. and M.Payton (1983), Anal.Biochem. 130, 402-405

Cohen, G.N. and J.Monod (1957), Bacteriol.Rev. 21, 169-194

Cole, H.D., J.W.T.Wimpenny and D.E.Hughes (1967), Biochem.Biophys.Acta 143, 445-453

Cox, R., N.A.Newton, F.Gibson, A.M.Snowswell and J.A.Hamilton, (1970), Biochem.J. 117, 551-562

Cox, G.B., F.Gibson and J. Pittard, (1968), J.Bacteriol. 95, 1591-1598

Creaghan, I.T. and J.R. Guest (1978), J. Gen. Microbiol. 107, 1-13

Debanov, V.G. (1982), in "The Bacilli" (D.A.Dubnau, ed.) 1, 338 Academic Press, London

DeGroot, G.N. and A.H. Stouthamer (1970), Biochim. Buioiphys Acta 208, 414

Dills, S.S., A. Apperson, M.R. Schmidt and M.H. Saier (1980), Microbiol. Rev. 44, 385-418

Doelle, H.W. (1975), "Bacterial Metabolism" Academic Press, London

Doelle H.W. (1981), Adv. Biotech. 1, 249-254

Doelle, H.W. K.N. Ewings, N.W. Hollywood (1981), Adv. Biochem. Eng. 23, 1-35

Doelle, H.W. and S. McIvor (1980), FEMS Microbiol. Lettrs. 1, 337

Downie, J.A. (1970) Ph.D. Thesis, University of Dundee

Downie, A. and G.B. Cos (1978), J. Bacteriol. 133, 477

Drummond, I. and G. Shama (1982), Chromatog. 15, 180-182

Eisenberg, R.C. and W.S. Dobrogosz (1967), J. Bacteriol. 93, 941-949

Entner, N. and M. Doudoroff (1952), J. Biol. Chem. 196, 853-862

Finn, R.K. (1954), Bacteriol. Rev. 18, 254

Fletchner, V.R. and R.S. Hanson (1969), Biochim. Biophys. Acta 184, 252-262

Gawehn, K. and H.U. Bergmeyer (1974), in "Methods of Enzymatic Analysis" (H.U. Bergmeyer, ed.) 3, 1492-1495. Verlag Chemie, Academic Press, London

Gottschalk, G. (1979) "Bacterial Metabolism", Springer-Verlag, New York

Gowrishankar, J. and J. Pittard (1982), J. Bacteriol. 150, 1122-1129

Guest, J.R. (1979), J. Gen. Microbiol. 115, 259-271

Guest J. R. and I. T. Creaghan (1973), J. Gen. Microbiol. 75, 197-210

Hackett, N.R. and P.D. Bragg (1983), J. Bacteriol. 154, 708-718

Haddock, B.A. and C.W. Jones (1977), Bacteriol. Rev. 41, 47-99

Haddock, B.A. and H.U. Schairer (1973), Eur. J. Biochem. 35, 34-45

Hanson, R.S. (1976), in "Spores" (P. Gerhardt, ed.) 6, 318-326

Hansen, R.G. and U. Henning (1966), Biochim. Biophys. Acta 122, 355-358

Harrison, D.E.F., D.G. Maclennan and S.J. Pirt (1974), Fermentation Advances, 114-117

Harrison, D.E.F. and S.J. Pirt (1967), J. Gen. Microbiol. 46, 193-211

Hayashi, O. and M. Nozaki (1969), Science 164, 389-396

Heber, U.W. and K.A. Santarius (1965), Biochim. Biophys. Acta 109, 390

Herbert, D. (1958), "Recent Progress in Microbiology", (Symposium held at the VII International Congress for Microbiology)

Herbert, D. (1975), "6th International Symposium on Continuous Culture" 1

Herbert, D., R. Elsworth and R.C. Telling (1956), J. Gen. Microbiol. 14, 601-622

Herbert, D. and H.L. Kornberg (1976), Biochem. J. 156, 477-485

Hespell, R.B. (1976), J. Bacteriol. 128, 677-680

Hirsch, C.A., M. Raminsky, B.D. Davis and E.C.C. Lin (1963), J. Biol. Chem. 238, 3770-3774

Hunter, I.S. and H.L. Kornberg (1979), Biochem J. 178, 77

Hurwitz, J., J.S. Furth, M. Malamy and M. Alexander (1962), Proc. Natl. Acad. Sci. USA. 48, 1222

Ide, M. (1971), Arch. Biochem. Biophys. 144, 262-268

Izui, K., T. Yoshinaga, M. Morikawa, and H. Katsuki (1970), Biochem. Biophys. Res. Commun. 40, 949

Jacobs, N. J. and J. M. Jacobs (1978), Biochim. Biophys. Acta 544, 540-546

Jones, C.W. (1977), Symp. Soc. Gen. Microbiol. 27, 23-59

Jones, C.W., J.M. Brice, A.J. Downs and J.W. Drozd (1975), Eur. J. Biochem. 52, 265-271

Kaback, H.R. (1968), J. Biol. Chem. 243, 3711-3724

Kaplan, S., A.G. Atherby and A. Barrett (1975), Proc. Natl. Acad. Sci. USA. 70, 689-692

Knappe, J., H.P. Blaschkowski, P. Grobner and T. Schmitt (1974), Eur. J. Biochem. 50, 253-263

Knappe, J., J. Schacht, W. Mockell, T. Hopner, H. Vetter and R. Edenharder (1969), Eur. J. Biochem. 11, 316-327

Knappe, J. and T. Schmitt (1976), Biochem. Biophys. Res. Commun. 71, 1110-1117

Knappe, J., F.A. Neugebauer, H.P. Blaschkowski and M. Ganzler (1984), Proc. Natl. Acad. Sci. USA. 81, 1332-1335

Koch, A.L. (1976), Perspect. BViol. Medicine 20, 44-63

Koike, M., L.J. Reed and W.F. Carroll (1963), J. Biol. Chem. 238, 30-39

von Korf (1969), in "Methods in Enzymology" 13, 519-523

Krab, K., L.F. Oltmann and A.H. Stouthamer (1982), Biochim. Biophys. Acta 679, 51-59

Krebs, H.A. (1972), Essays Biochem. 8, 1-34

Kroger, A. (1978), Biochim. Biophys. Acta 505, 129-145

Kundig, W., F.D. Kundig, B. Anderson and S. Roseman (1966), J. Biol. Chem. 241, 3243-3246

Kuritzkes, D.R., X.Y. Zhang and E.C.C. Lin (1984), J. Bacteriol. 157, 591-598

Langley, D. and J.R. Guest (1977), J. Gen. Microbiol. 99, 263-276

Lawford, H.G. and B.A. Haddock (1973), Biochem. J. 136, 217-220

Leive, L. (1965), Biochem. Biophys. Res. Commun. 18, 13-17

Lenox, E.S. (1955), Virology 1, 190

Lilius, E.A. (1978), Ph.D. Thesis, University of Turku

Linnane, A.W. and C.W. Wrigley (1965), Biochim. Biophys. Acta 77, 408-418

Lwoff, A. and J. Monod (1947), Ann. Inst. Past. 73, 323

Macchia, V.S., H. Varrone, D. Weissbach, D. Miller and I. Pastan (1975), J. Biol. Chem. 250, 6214-6217

Mather, M., R. Blake, J. Koland, H. Schrock, P. Russel, T. O'Brien, L.P. Hager, R.B. Gennis and M. O'Leary (1982), Biophys. J. 37, 87-88

McPhedran, P.B. Sommer and E.C.C. Lin (1961), J. Bacteriol. 81, 852-857

Metzler, D.E. (1977), "Biochemistry. The Chemical Reactions Of Living Cells". Academic Press, London

Miller, G.L. (1959), Anal. Chem. 31, 964

Miller, J.H. (1972), "Experiments in Molecular Genetics" Cold Spring Harbour Laboratory, New York

Mitchell, P. (1975), FEBS Lettrs. 59, 137

Model, P. and D. Rittenberg (1967), Biochem. 6, 69 - 78

Moss, F. (1952), Aust. J. Exp. Biol. Med. Sci. 30, 531

Moss, F. (1956), Aust. J. Exp. Biol. Med. Sci. 34, 395

Neish, A.C. and G.A. Ledingham (1949), Can. J. Research 26, 335-342

Newsholme, E.A. and C. Start (1973), "Regulation in Metabolism". Willey, New York.

Oltmann, L.F. and A.H. Stouthamer (1975), Arch. Microbiol. 105, 135-142.

Pascal, M-C., J. F. Burini, J. Ratouchnick and M. Chippaux (1982), Mol. Gen. Genet. 188, 103-106.

Pascal, M-C., F. Casse, M. Chippaux and M. Lepelletier (1975), Mol. Gen. Genet. 141, 173-179.

Pascal, M-C., M. Chippaux, A. Abou-Jaoude, H.P. Blaschkowski and J. Knappe (1981), J. Gen. Microbiol. 124, 35-42.

Pastan, I. and S. Adhya (1976), Bacteriol. Rev. 40, 527-551.

Pasteur, L. (1861), Compt. Rend. Acad. Sci. Paris 52, 344-347.

Perlman, R.L. and I. Pastan (1968), Biochem. Biophys. Res. Commun. 30, 656-664.

Perlman, R.L. and I. Pastan (1971), Curr. Top. Cell Regul. 3, 117-134.

Pirt, S.J. (1975), "Principles of Microbe and Cell Cultivation", Blackwell Scientific Publications, London.

Po-Keung Wong and E. Barrett (1983), FEMS Microbiol. Lettrs. 22, 143-148.

Postgate, J.R. and J.R. Hunter (1963), J. Appl. Bact. 26, 405-414.

Racker, E. (1955), "Methods in Enzymology" 1, 500-503.

Reichelt, J.L. and H.W. Doelle (1971), Antonie Van Leeuwenhoek J. Microbiol. Serol. 33, 213-229.

Reiness, G., Huey-Lang Yang, G. Zubaz and M. Cashel (1975), Proc. Natl. Acad. Sci. U.S.A. 72, 2881-2885.

Rice, C.W. and W.P. Hempfling (1978), J. Bacteriol. 134, 115-124.

Rockwell, G.E. and J.H. Highberger (1927), J. Inf. Dis. 40, 438.

Romano, A.H., S.J. Eberhard, S.L. Dingle and T.D. McDowell (1970), J. Bacteriol. 104, 808-813.

Rose, I.A., M. Grunberg-Manago, S.R. Korey and S. Ochoa (1954), J. Biol. Chem. 211, 737-756.

Rosenberg, H., G.B. Cox, J.D. Butlin and S.J. Gutowski (1975), Biochem. J. 146, 417-423.

Sanwal, B.D. (1976), J. Biol. Chem. 245, 1626.

Shaw, D.J. and J.R. Guest (1982), J. Gen. Microbiol. 128, 2221-2228

Smith, M.W. and F.C. Neidhardt (1983), J. Bacteriol. 154, 336-343

Smith, M.W. and F.C. Neidhardt (1983), J. Bacteriol. 154, 344-350.

Sokatch, J.R. (1969), "Bacterial Physiology and Metabolism" Academic Pres, London.

Southamer, A.H. (1975), in "The Bacteria" (L.N. Osnston and J.R. Sokatch, eds.), 6, 389-462. Academic Press, New York.

Swanstrom, M. and M.H. Adams (1951), Proc. Natl. Soc. Exptl. Biol. Med. 78, 372-375.

Tanaka, N.K. Koike, K. Otsuka, M. Hamada, K. Ogasahara and M. Koike (1974), J. Biol. Chem. 249, 191-198.

Tarmy, E.M. and N.O. Kaplan (1963), J. Biol. Chem. 243, 2587-2596.

Taylor, A.L. (1963), Proc. Natl. Acad. Sci. U.S.A. 50, 1043-1051.

Teixeira de Mattos, M.J. and D.W. Tempest (1983), Arch. Microbiol. 134, 80-85.

Tempest, D.W. and O.M. Neijssel (1978), Adv. Microbiol. Ecol. 2, 105-153.

Thomas, A.D., H.W. Doelle, A.W. Westwood and G.L. Cordon (1972), J. Bacteriol. 112, 1099-1105.

Undeng and J.R. Guest (1984), FEBS Lettrs. 170, 321-325.

Uyeda, K. (1979), Adv. Enzymol. 48, 193.

Varenne, S., F. Casse, M. Chippaux and M-C. Pascal (1975), Mol. Gen. Genet. 141, 181-184.

West, I.C. and P. Mitchell (1974), FEBS Lettrs. 40, 1-4.

Wimpenny, J.W.T. (1969), Biotechnol. Bioeng. 11, 623-629.

Wimpenny, J.W.T., and J.A. Cole (1967), Biochim. Biophys. Acta. 148, 233-242.

Wimpenny, J.W.T. and A Firth (1972), J. Bacteriol. 111, 24-32.

Wimpenny, J.W.T. and D.K. Necklen (1971), Biochim. Biophys. Acta. 253, 352-359.

Wood, A.P. and D.P. Kelly (1980), J. Gen. Microbiol. 120, 333-345.

Wood, W.A. (1961), in "The Bacteria" (I.C. Gunsalus and R.Y. Stanier, eds.) 2, 59 Academic Press, New York.

Yanofsky, C. (1981), Nature 289, 751-758.

Young, I.G., R.A. Leppik, J.A. Hamilton and F. Gibson (1972), J. Bacteriol. 110, 18-25.

Young, I.G. and B.J. Wallace (1976), Biochim. Biophys. Acta 449, 376-385.