THE METABOLISM OF LACTULOSE BY *CLOSTRIDIUM PERFRINGENS* IN BATCH AND CONTINUOUS CULTURE

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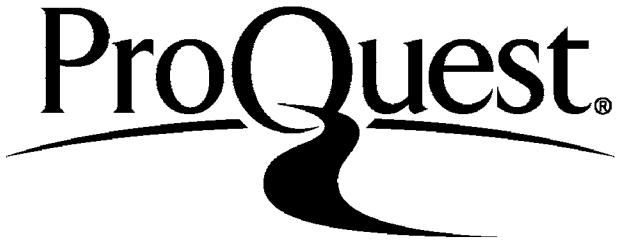

ABSTRACT

The aim of this project was to initiate studies on lactulose (4-O-β-D-galactopyranosyl D-fructofuranose) metabolism by human intestinal bacteria. The organism of choice was *Cl. perfringens*, due to its rapid metabolism of the sugar. Work was conducted with cells grown anaerobically, in both batch and continuous culture. Use of the latter system permitted closer approximation of the *in vivo* situation.

Initial work was concerned with the identification of the metabolic pathway(s) involved. Radiolabelled lactulose was not available, hence a variety of 'cold' procedures were employed; including fluorimetric determination of key metabolites, and assay of key enzymes.

Growth in medium supplemented with lactulose resulted in the induction of β-galactosidase, whereas phospho-β-galactosidase activity could not be detected. This suggested that lactulose was accumulated in the free form and that the first step in intracellular metabolism was hydrolysis to its constituent monosaccharides. This was confirmed by the detection of intracellular free galactose and fructose.

The galactosyl moiety was found to be metabolised by the Leloir pathway: the enzymes galactokinase, galactose 1-phosphate uridylyl transferase and UDPgalactose 4-epimerase were present in lactulose-grown cells, and both galactose 1-phosphate and glucose 1-phosphate were detected. The fructosyl moiety was most likely metabolised by an ATP-dependent fructokinase activity, present in lactulose-grown cells.

Measurement of lactulose utilisation in a buffered incubation system permitted the study of various transport system inhibitors. Utilisation was inhibited by both CCCP and DCCD, suggesting that transport requires a proton gradient; formed by the action of the membrane-bound ATPase activity. The presence of a proton gradient-dependent uptake system is consistent with the findings from the metabolic studies. A similar metabolic system was responsible for lactulose metabolism under conditions of carbon limitation in the chemostat.

Galactokinase was further studied. Basal levels of this enzyme were repressed by inclusion of glucose in the culture medium. Glucose also prevented induction of galactokinase by lactulose or galactose. This latter 'glucose effect' could not be abolished by the addition of cyclic AMP, and appeared to be mediated via inducer exclusion.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviations were the same as those recommended in [1], with the following additions:

ATPase	ATP phosphohydrolase (EC 3.6.1.3)
BC	batch culture
BM	basal medium
cAMP	adenosine 3',5'-monophosphate or cyclic AMP
CAP	catabolite gene activator protein
CC	continuous culture
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
D	dilution rate
DCCD	N,N'-dicyclohexylcarbodiimide
DHAP	dihydroxyacetone phosphate
dry wt	dry weight
EM	Embden-Meyerhof pathway
EI	enzyme I of the PTS
EII-A	enzyme II-A of the PTS
EII-B	enzyme II-B of the PTS
EIII	enzyme III of the PTS
FIII	factor III of the PTS
F	Faraday's constant
FDP	fructose 1,6-bisphosphate
GA3P	glyceraldehyde 3-phosphate
HPr	histidine-containing phosphocarrier protein of the PTS
K_d	equilibrium binding constant
K_m	substrate concentration at half-maximal reaction rate (Michaelis constant)
K_s	substrate concentration at half-maximal growth rate
K_t	substrate concentration at half-maximal reaction rate
m	maintenance coefficient
MGT	mean generation time
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
ΔpH	transmembrane chemical gradient of protons
pmf	protonmotive force
PSE	portal systemic encephalopathy
PTS	phosphoenolpyruvate: sugar phosphotransferase system

q_{LL}	specific rate of lactulose utilisation
R	the gas constant
RPr	putative regulatory protein of the PTS
SD	standard deviation
T	temperature
TDG	thio-β-D-digalactoside
TMG	thiomethyl β-D-galactoside
UDPG	uridine-5'-diphosphoglucose
UDPGal	uridine-5'-diphosphogalactose
V_{max}	maximal rate of reaction (or uptake)
Y	molar growth yield (Y_x = molar growth yield on sugar X)
Y_{max}	maintenance-free growth yield
μ	specific growth rate
μ_{max}	maximum specific growth rate
$\Delta\mu_{H+}$	electrochemical potential of hydrogen ions
$\Delta\Psi$	transmembrane electrical potential difference

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CHAPTER 1: INTRODUCTION

Lactulose (4-O-β-D-galactopyranosyl D-fructofuranose) is the synthetic ketoanalogue of lactose (4-O-β-D-galactopyranosyl D-glucopyranose) (Fig. 1). The synthesis of the disaccharide was first reported in 1929 by Hudson and Montgomery [2], who were investigating the relationship between sugar structure and optical rotatory properties. Subsequent studies by a variety of workers have identified important clinical uses for lactulose, including: the therapies of chronic portal-systemic encephalopathy and chronic constipation, the estimation of small bowel transit time, and the detection of sugar malabsorption [3].

1.1 Lactulose Metabolism in Man.

Lactulose cannot be hydrolysed by the human small intestinal lactases [4], and very little absorption occurs from the healthy small intestine [5]. Orally administered sugar therefore passes largely intact and unabsorbed to the colon, where it is rapidly fermented by the resident microbial flora [3].

A pH-sensitive, radiotelemetering device has been used to monitor changes in lumenal pH along the length of the gastrointestinal tract following oral lactulose (Table 1) [6]. Acidification is most marked in the proximal (right) colon, suggesting that this is the major site of lactulose breakdown. It should be noted that the dosage of lactulose (30-40 g) given in this study was much less than the normal therapeutic dose (20-30 g; 3-4 times daily), therefore, the observed changes in lumenal pH may be unrepresentative of the clinical situation. Indeed, therapeutic dose levels normally result in a significant reduction in stool pH; this falls from a mean value of 7.1 to a mean value of 5.3 [3].

The administration of oral lactulose produces a laxative effect [3]. Stool softening and increases in both the number and mean daily weight of stools are observed at low dose levels, whereas diarrhoea may result from high doses. This cathartic action of lactulose is a consequence of its metabolism to organic acid by the intestinal flora. Acid is a potent stimulator of intestinal peristalsis and the degradation products may also contribute an osmotic effect [3].

1.2 Lactulose and Portal-Systemic Encephalopathy (PSE).

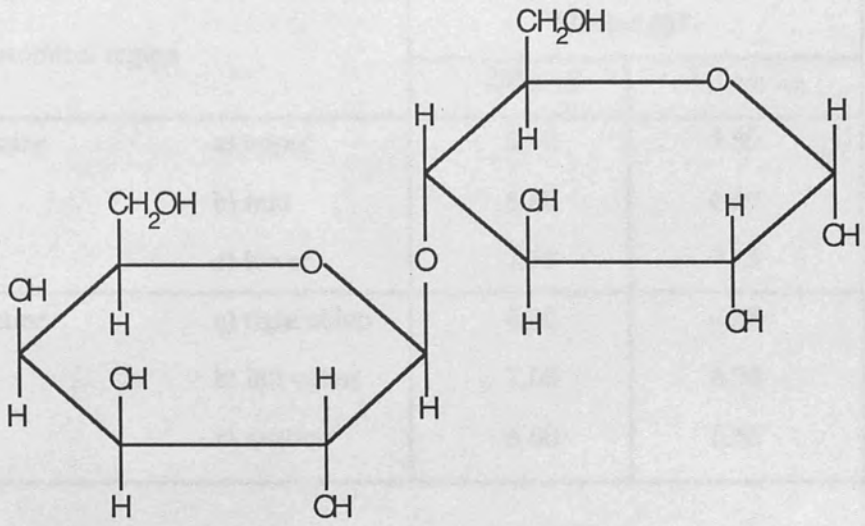
1.2.1 The pathogenesis of PSE.

PSE is a hepatic coma syndrome and is commonly found in cirrhotic patients; especially if portal-systemic shunts are also present [3]. Impaired liver function and/or shunting of portal blood around the liver results in high levels of toxic metabolites in the systemic circulation.

The pathogenesis of PSE has been extensively studied but remains poorly understood [3,7-10]. Cerebral intoxication may be due to a single etiological agent or, possibly,

Figure 1: Chemical structures of lactulose and lactose

lactose (4-O-β-D-galactopyranosyl-α-D-glucopyranose)



lactulose (4-O-β-D-galactopyranosyl-β-D-fructofuranose)

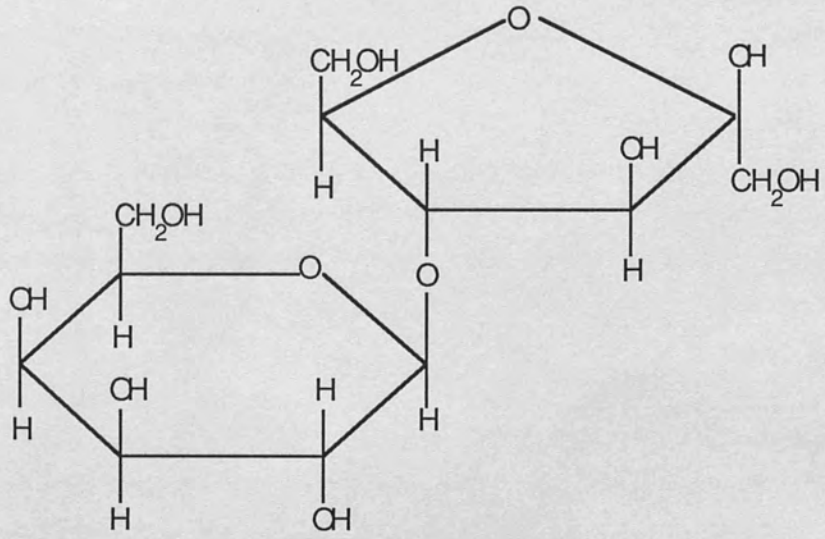


Table 1: Changes in lumenal pH along the length of the human gastrointestinal tract following administration of oral lactulose.

Anatomical region		Median pH	
		Control	+ Lactulose
Small intestine	a) upper	5.90	5.80
	b) mid	6.80	6.20
	c) lower	7.50	7.05
Large intestine	a) right colon	6.00	4.85
	b) left colon	7.00	6.70
	c) rectum	6.80	6.55

A dosage of 30-40 g lactulose was administered.
Data from [6].

several agents acting synergistically. Coma is frequently precipitated by heavy protein meals or gastrointestinal bleeding, therefore, the toxic agents are thought to be nitrogenous metabolites which arise in the gut. Although several different classes of compounds (ammonia, short chain fatty acids, mercaptans, aromatic amine analogues of central neurotransmitters) have been implicated, ammonia is thought to play a central role.

The gastrointestinal tract is considered to be a major site of ammonia production in the body, since the concentration of this compound in the hepatic portal vein is 5 to 10-fold higher than that in mixed venous blood [8]. Studies with germ-free and conventional animals have shown that a considerable fraction of this gut ammonia is produced by the action of the intestinal flora [11,12]. Intestinal bacteria can generate ammonia from a diverse range of substrates (urea, dietary protein, shed epithelial cells, gut secretions, dead bacterial cells) by mechanisms such as urea hydrolysis and deamination of amino acids, peptides and proteins [11,13,14].

The intestinal bacteria can also use ammonia as a precursor in amino acid and, consequently, protein biosynthesis. Indeed, a major portion (approx. 60%) of faecal nitrogen is contained within bacterial protoplasm [15]. Under normal conditions, bacterial generation of ammonia exceeds bacterial utilisation, and the majority of gut ammonia is absorbed by the colonic mucosa and subsequently transported to the liver [13].

1.2.2 The mode of action of lactulose in PSE therapy.

The therapy of PSE is normally directed towards decreasing the production and/or absorption of gut ammonia [3]. Thus, prior to the introduction of lactulose, conventional therapy normally required dietary protein restriction, purgation and the administration of broad-spectrum antibiotics [16]. Successful lactulose therapy is characterised by decreased blood ammonia levels [3] and increased faecal nitrogen excretion [17]. The mode of action of lactulose in bringing about these effects is not fully understood; a total of 4 different mechanisms have been proposed:

a) Catharsis.

Oral lactulose results in a decreased intestinal transit time, therefore, less time will be available for the production and absorption of ammonia [18]. Although this mechanism may play an important role in the therapy of PSE, it cannot be the main mode of action of lactulose since control cathartics, such as magnesium sulphate, do not produce clinical improvement [19,20].

b) Acid dialysis of ammonia.

Absorption of ammonia from the intestine to blood occurs by passive nonionic diffusion, and bicarbonate ion may assist absorption in a coupled process [11,13]. Ammonia has a high pK_a value and the process is markedly pH-dependent. Thus, acidification of the colonic contents may decrease absorption by increasing the proportion of ammonium ion. Furthermore, at sufficiently low lumenal pH values, ammonia

absorption (from intestine to blood) may even be reversed [3]. Although faecal nitrogen excretion is increased in response to lactulose [17], most studies have failed to demonstrate impressive increases in faecal ammonia excretion [3].

c) Changes in the composition of the intestinal flora.

Lactulose was first introduced into PSE therapy on the assumption that it would promote the growth of acidophilic, urease-negative bacteria (the lactobacilli) at the expense of proteolytic members of the flora [3]. Studies concerned with the effects of lactulose on the composition of the faecal flora have yielded inconsistent results, and it is difficult to draw any valid conclusions because of considerable criticism of experimental design [3]. Such studies indicate an increase in the lactobacilli, but clinical benefit can be obtained in patients whose stool is devoid of these organisms, due to previous neomycin therapy [21].

d) Alterations in bacterial nitrogen metabolism.

A large body of evidence is accumulating to support the view that unabsorbed dietary compounds have major effects on the metabolic activities of the intestinal flora, rather than its composition [14,22-24]. Studies with an *in vitro* faecal incubation system suggest that lactulose can influence bacterial nitrogen metabolism [25].

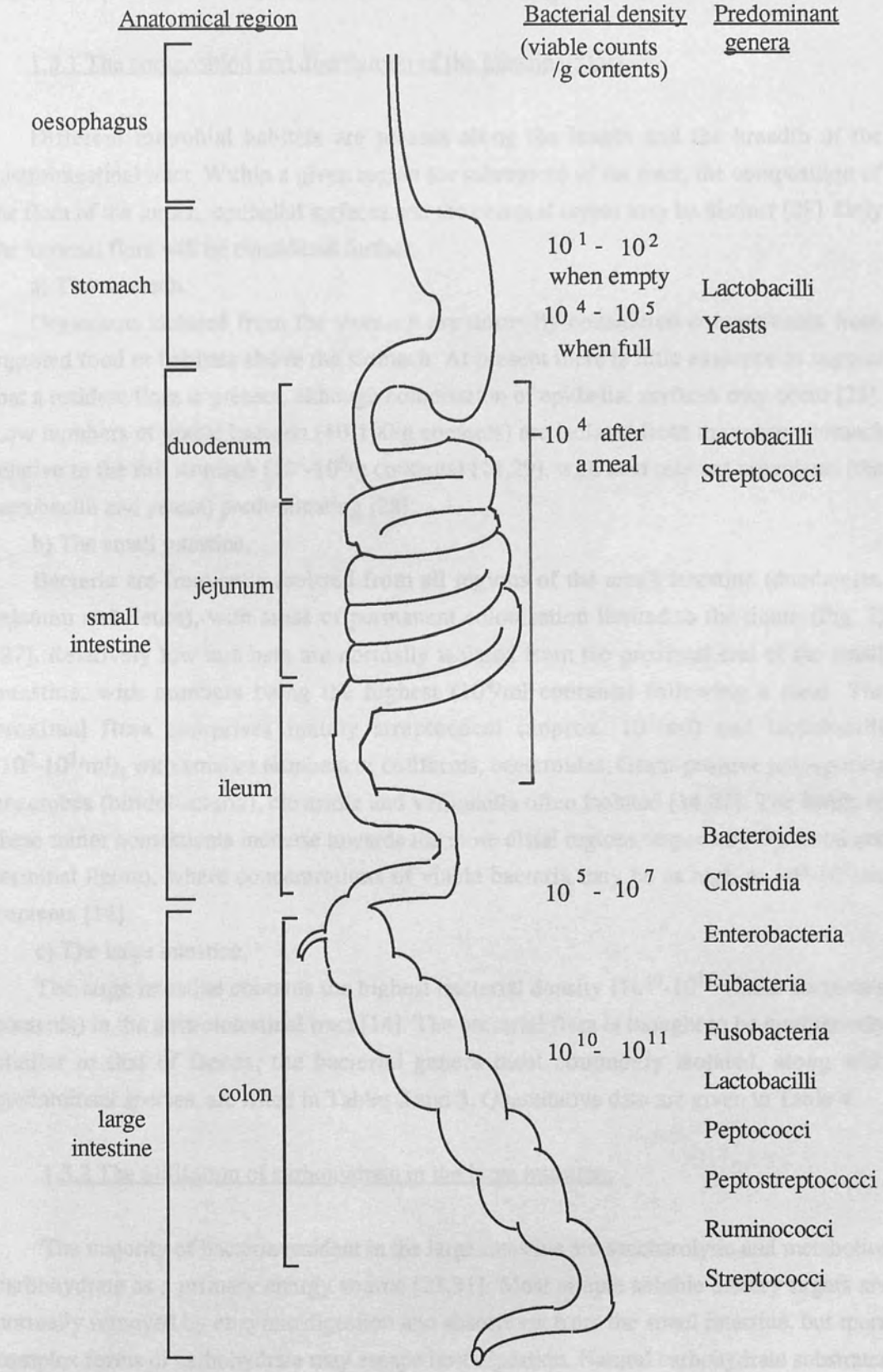
Under anaerobic conditions, the system generates ammonia by deamination reactions [26], and the effect of lactulose on this process has been investigated [25]. Both untitrated and titrated systems have been studied, in order to dissociate any pH effect from a substrate effect. Net generation of ammonia is converted to net utilisation in the presence of lactulose (30 mM), and the effect is more marked in untitrated cultures (pH falls from 7.1 to 5.1) relative to titrated cultures (pH falls from 7.1 to 5.5 and is maintained at this value). Net generation of ammonia resumes in both systems once lactulose has been metabolised, but this is significantly reduced in untitrated systems relative to titrated or control systems [25].

Vince and coworkers (1978) have suggested that the inability to detect increased faecal ammonia following lactulose administration may be due to a decreased bacterial generation combined with an increased assimilation into bacterial protein [25]. Presentation of a readily utilisable carbon and energy source to the intestinal flora may be expected to promote bacterial growth and, at the same time, spare energy production by proteolytic reactions. Superimposed on this substrate effect is a pH effect; acid appears to affect both nitrogen and carbon metabolism alike, since lactulose utilisation is also inhibited at low pH [25].

1.3 The Human Intestinal Flora.

The human gastrointestinal tract comprises 4 major structural regions: the oesophagus, the stomach, the small intestine and the large intestine (Fig. 2) [27,28]. The tract harbours a complex mixture of bacterial species, and the composition of this flora varies from region to region [14,27]. Over 400 different bacterial species have been isolated from the tract, the majority (>95%) of which are obligate anaerobes. Obligate aerobes (various *Bacillus* spp.)

Figure 2: Major anatomical structures of the human gastrointestinal tract and the distribution of predominant bacterial genera.



are often isolated, but these are considered to be enviromental contaminants [27]. At any given time, a specific region of the tract will contain both a resident flora and transient contaminants. The latter may be derived from sources such as food, drink, other regions of the tract or parts of the body, and the general surroundings [14,28].

1.3.1 The composition and distribution of the intestinal flora.

Different microbial habitats are present along the length and the breadth of the gastrointestinal tract. Within a given region (or subregion) of the tract, the composition of the flora of the lumen, epithelial surfaces and the mucosal crypts may be distinct [28]. Only the lumenal flora will be considered further:

a) The stomach.

Organisms isolated from the stomach are normally considered contaminants from ingested food or habitats above the stomach. At present there is little evidence to suggest that a resident flora is present, although colonisation of epithelial surfaces may occur [28]. Low numbers of viable bacteria (10-100/g contents) are isolated from the empty stomach relative to the full stomach (10^4-10^5/g contents) [14,29], with acid tolerant organisms (the lactobacilli and yeasts) predominating [28].

b) The small intestine.

Bacteria are frequently isolated from all regions of the small intestine (duodenum, jejunum and ileum), with areas of permanent colonisation limited to the ileum (Fig. 2) [27]. Relatively low numbers are normally isolated from the proximal end of the small intestine, with numbers being the highest (10^4/ml contents) following a meal. The proximal flora comprises mainly streptococci (approx. 10^3/ml) and lactobacilli (10^2-10^3/ml), with smaller numbers of coliforms, bacteroides, Gram-positive non-sporing anaerobes (bifidobacteria), clostridia and veillonella often isolated [14,22]. The levels of these minor constituents increase towards the more distal regions (especially the distal and terminal ileum), where concentrations of viable bacteria may be as high as 10^5-10^7/ml contents [14].

c) The large intestine.

The large intestine contains the highest bacterial density (10^{10}-10^{11} viable bacteria/g contents) in the gastrointestinal tract [14]. The bacterial flora is thought to be qualitatively similar to that of faeces; the bacterial genera most commonly isolated, along with predominant species, are listed in Tables 2 and 3. Quantitative data are given in Table 4.

1.3.2 The utilisation of carbohydrate in the large intestine.

The majority of bacteria resident in the large intestine are saccharolytic and metabolise carbohydrate as a primary energy source [23,31]. Most simple soluble dietary sugars are normally removed by enzymic digestion and absorption from the small intestine, but more complex forms of carbohydrate may escape host digestion. Natural carbohydrate substrates

Table 2: Genera and predominant species of obligately anaerobic bacteria which are frequently isolated from the human large intestine.

Genus	Gram stain/ morphology	Predominant species
Bacteroides	G-ve rods	*Bact. vulgatus*, *Bact. thetaiotamicron*, *Bact. ovatus*, *Bact. distasonis*, *Bact. uniformis*, *Bact. eggerthii*.
Fusobacterium		*F. prausnitzii*.
Desulfomonas		
Leptotrichia		
Butyrivibrio		
Succinimonas		
Vibrio		
Bifidobacterium	G+ve non-sporing rods	*Bif. adolescentis*, *Bif. longum*, *Bif. breve*.
Eubacterium		*Eu. aerofaciens*, *Eu. siraeum*, *Eu. rectale*, *Eu. eligens*, *Eu. biforme*.
Propionobacterium		
Lachnospira		
Clostridium	G+ve sporing rods	*Cl. perfringens*, *Cl. leptum*, *Cl. paraputrificum*.
Acidaminococcus	G+ve cocci	
Megasphera		
Peptococcus		
Sarcina		
Peptostreptococcus		*P. productus*.
Streptococcus		
Ruminococcus		*R. bromii*, *R. torques*, *R. albus*, *R. callidus*.
Veillonella	G-ve cocci	

G+ve - Gram positive
G-ve - Gram negative
Adapted from [14,22,27].

Table 3: Genera and predominant species of facultatively anaerobic bacteria which are frequently isolated from the human large intestine.

Genus	Gram stain/ morphology	Predominant species
Escherichia Enterobacter Klebsiella Proteus Citrobacter	G-ve rods	*E. coli.*
Lactobacillus	G+ve rods	*L. acidophilus.*
Neisseria	G-ve cocci	
Streptococcus	G+ve cocci	*Str. faecalis.*
Corynebacteria	G+ve irregular rods	

G+ve - Gram positive
G-ve - Gram negative
Adapted from [14,22,30].

Table 4: Numbers of selected bacterial groups in the large intestine.

Bacterial group	Viable count /g contents
Bacteroides	10^7-10^{10}
Gram-positive non-sporing anaerobes	10^5-10^{10}
Enterobacteria	10^6-10^7
Streptococci	10^5-10^7
Lactobacilli	10^4-10^6
Clostridia	10^4

Adapted from [14,27,30].

of the intestinal bacteria include the polysaccharides of plant cell walls (cellulose, hemicellulose, pectins), gums (galactomannans) and the oligosaccharide side chains of meat glycoproteins [23,31]. Endogenous compounds, such as the glycoproteins of mucin and sloughed epithelial cells, can also serve as substrates.

Most of the intestinal bacterial species which ferment complex polysaccharides *in vitro* are members of the bacteroides and bifidobacteria [23]. Several organisms (*Bacteroides uniformis, Bacteroides ovatus, Bacteroides thetaiotamicron*) produce a variety of glycosidases and polysaccharide-degrading enzymes, and can metabolise a wide range of complex sugars. These enzymes are both inducible and glucose-repressible, and changes in faecal levels occur in response to changes in diet (Chang, cited in [23]). The main end products of intestinal fermentation are volatile fatty acids (mainly acetic, propionic and butyric acids), gas (CH_4, CO_2, H_2) and energy for bacterial maintenance and growth [24,31]. Based on several assumptions, Wolin (1981) has derived the following equation for carbohydrate fermentation in the large intestine [31]:

$$34.5\ C_6H_{10}O_5 \text{ ----> } 48\ CH_3COOH + 11\ CH_3CH_2COOH + 5\ CH_3(CH_2)_2COOH + 23.75\ CH_4 + 34.25\ CO_2 + 9.5\ H_2O$$

Hydrogen is also produced in large amounts, with methane being a major product in only 33% of the population. The majority of gas is exhaled from the lungs, and most of the volatile fatty acids are absorbed by the blood and subsequently metabolised by the host [31].

Addition of fermentable polysaccharide (digestible dietary fibre) to the diet will also stimulate bacterial growth, with consequent changes in gut transit time (decreased) and stool output (increased) [24,32]. An important secondary effect is an increase in faecal nitrogen excretion; the majority of which is associated with bacterial protoplasm. Thus, the effects of degradable dietary polysaccharide are comparable to those of lactulose.

1.3.3 The metabolism of lactulose by intestinal bacteria.

The utilisation of lactulose by individual strains of intestinal bacteria has been studied in some detail by Sahota *et al.* (1982) [33]. These authors screened a total of 64 strains of bacteria for the ability to use lactulose, and their results are summarised in Table 5. The bifidobacteria, bacteroides, clostridia and lactobacilli all use large amounts of the sugar *in vitro*, therefore, these organisms may play an important role in its metabolism *in vivo*. In contrast, the enterococci and enterobacteria are relatively poor users of the sugar *in vitro*, and the eubacteria demonstrate negligible metabolic activity (Table 5). Essentially similar findings have been reported by other workers [34,35].

Lactulose fermentation products have also been studied by Sahota *et al.* (1982) [33]. The lactobacilli exhibit a homolactic fermentation, but most other organisms produce large

Table 5: Lactulose utilisation by intestinal bacteria.

Organism	Utilisation
Lactobacillus spp., Cl. perfringens, L. casei sub casei, Bif. bifidum.	>30%
Bact. oralis.	20-30%
Bact. vulgatus, Bact. distasonis, Cl. butyricum, Cl. cadaveris, Pr. vulgaris, Cl. difficile, Bact. uniformis, Bact. fragilis, L. brevis, L. acidophilus, Enterobacter aerogenes, Pr. mirabilis, Peptostrep. anaerobius.	10-20%
Klebsiella aerogenes, Streptococcus sp., Bact. ovatus, Bact. thetaiotamicron, Staph. aureus, Str. intermedius, Str. faecium, Str. viridans, E. coli.	5-10%
Bact. mel. asaccharolyticus, Eu. lentum, Eu. limosum, F. nucleatum, L. buchneri, N. catarrhalis, Cl. paraputrificum, P. acnes, V. parvula.	<5%

Cultures were incubated in the presence of lactulose (40 mM), for 48 hr, under anaerobic conditions. Sugar utilisation was determined by t.l.c..
Adapted from [33].

amounts of lactic and acetic acids. Butyric acid and succinic acid, respectively, are characteristic products of the clostridia and the bacteroides [33].

Studies with an *in vitro* faecal incubation system indicate that the fermentation product pattern is dependent on the pH of the system [33]. Major fermentation products in untitrated systems include acetic, lactic, butyric and propionic acids, whereas titration of the system to pH ≥5.5 results in a wider range of products (including acetic, butyric, propionic, caproic and succinic acids). These results imply that the contribution of particular bacterial groups to lactulose breakdown *in vivo* may change as a direct result of lactulose metabolism. Thus, the lactobacilli may only make a significant contribution when the colonic pH is reduced to a value ≤5.5, whereas the bacteroides may become less active as the pH is decreased. The presence of large amounts of butyric acid in both titrated and untitrated systems suggests that the clostridia make an important contribution regardless of changes in colonic pH.

Apart from the above work, lactulose metabolism by intestinal bacteria is poorly documented in the literature. Most studies have concentrated on the effects of lactulose on nitrogen metabolism, without any serious consideration for the metabolism of lactulose itself. In order to illustrate possible mechanisms of lactulose metabolism (sugar uptake and subsequent intermediary metabolism), the remainder of this Chapter is concerned with general aspects of carbohydrate metabolism in prokaryotes. Particular emphasis has been placed on lactose, the aldo-isomer of lactulose, due to the similarity in chemical structure. The clostridia are considered in detail, wherever possible, since *Cl. perfringens* was the organism studied in this Ph.D. project.

1.4 Mechanisms of Sugar Transport.

There are 4 distinct classes of carrier-mediated sugar transport systems in prokaryotes: facilitated diffusion, systems coupled to ion gradients, the phosphoenolpyruvate:sugar phosphotransferase system (PTS) and binding-protein systems [36-38]. The latter 3 systems are all concentrative mechanisms and are distinguished by their mode of energy coupling. Sugar binding-protein systems are found exclusively in Gram-negative bacteria, therefore, they will not be considered in further detail.

1.4.1 Facilitated diffusion.

Facilitated diffusion is the simplest mechanism of carrier-mediated transport [36]. Glycerol appears to be the only known carbohydrate which is transported exclusively by this mechanism in bacteria. The substrate cannot be accumulated against its concentration gradient since the system is not coupled to metabolic energy; thus, the carrier catalyses the equilibriation of substrate across the cell membrane. The continuation of the transport process is ensured by the rapid metabolism of glycerol via an ATP-dependent kinase (glycerol kinase; EC 2.7.1.30), which is subject to feedback inhibition [39].

1.4.2 Ion gradient-coupled systems.

The classical example of an ion gradient-coupled transport system is the lactose transport system of *E.coli*. This system has been extensively studied and, although many areas remain controversial, it is now without doubt that chemiosmotic phenomena play a central role in the mechanism of energy coupling [40-44].

The chemiosmotic hypothesis was first advanced by Mitchell in relation to oxidative phosphorylation and later applied to nutrient transport (reviewed in [45]). The hypothesis requires the existence of a topologically intact membrane which must be essentially impermeable to protons. It is postulated that energy is conserved at the level of the membrane in the form of an electrochemical gradient of hydrogen ions. This gradient is generated by the action of various membrane-bound, proton-translocating proteins (primary active transport systems) and can be used to drive several membrane-related, energy consuming processes (secondary active transport systems).

The transmembrane electrochemical gradient of hydrogen ions can be detected as a pH-difference (cytoplasmic alkaline) and an electrical potential difference (cytoplasmic negative), and can be represented as follows:

$$\Delta\mu_{H+} = F\Delta\Psi - 2.3RT\Delta pH \quad \text{..... (1)}$$

Where:

$\Delta\mu_{H+}$ is the electrochemical potential of hydrogen ions.

$\Delta\Psi$ is the electrical potential difference.

ΔpH is the chemical gradient of protons.

F is Faraday's constant.

R is the gas constant.

T is temperature (°Kelvin).

The electrochemical gradient of hydrogen ions will exert an inwardly-directed force on the protons, termed the protonmotive force (pmf). By analogy with the electromotive force of electrochemical cells, equation (1) can be arranged to give:

$$pmf = \Delta\mu_{H+} / F = \Delta\Psi - Z\,\Delta pH \quad \text{..... (2)}$$

Where:

Z is a conversion factor (2.3RT / F) and has a value of 59 mV/pH unit at 25 °C.

All other symbols are as in equation (1).

Chemotrophic bacteria possess two distinct primary active transport systems: the respiratory chain and the proton-translocating ATPase (ATP phosphohydrolase; EC 3.6.1.3) [36,40,46]. In aerobes and facultative anaerobes, the pmf is normally generated by the action of respiratory chains, with the proton-translocating ATPase functioning in the direction of ATP synthesis [40,47]. In strict fermentative bacteria which lack membrane-bound electron transport pathways, the pmf can only be generated by the proton-translocating ATPase acting in the direction of ATP hydrolysis. Examples of this latter group of bacteria include the clostridia, *Str. lactis* and certain strains of *Str. faecalis* [40].

The magnitude of the pmf and its components has been measured in a variety of organisms, and selected data are presented in Table 6. The relative contributions of the membrane potential and the pH gradient to the total pmf vary with external pH. However, at a given external pH, the values of the pmf in strict fermentative bacteria are lower than those of respiring organisms. In the case of the streptococci, it has been calculated that a value of 175-200 mV is required to drive ATP synthesis [48].

In the case of *Cl. pasteurianum*, no definitive data are available on the magnitude of the membrane potential, but a preliminary report indicates a value of -70 mV (interior negative) at an external pH of 6.5-6.6 [53]. This value is slightly less than that of *Cl. acetobutylicum* and rather less than that of other organisms (Table 6).

Generation of the pmf in strictly fermentative bacteria may be expected to consume a considerable fraction of the ATP generated by substrate level phosphorylation. This led Michels *et al.* (1979) to propose the existence of an additional proton pumping mechanism [54]. They suggest that carrier-mediated efflux of the end-products of fermentation in symport with protons may contribute to the total pmf. This contribution will only be significant when efflux is both continuous and electrogenic. Experimental support for this energy recycling model has come from studies on L-lactate efflux from *E. coli* [55] and *Str. cremoris* [56,57]. Any significant increase in growth yield is only observed above an external pH of 6.3 and when the external lactate concentration is low (<10 mM)[58].

Nutrient transport by secondary active transport systems is indirectly linked to metabolism via the proton circulation. The endergonic process of nutrient accumulation is coupled to the exergonic process of protons moving down their electrochemical gradient by substrate-specific, membrane-associated carrier proteins [45]. In the case of neutral substrates, such as lactose, the carrier molecules catalyse a symport reaction; both protons and substrate are transported in the same direction at the same time [45]. The direction of transport is determined by the direction of the pmf, and the maximum possible substrate concentration gradient is determined by the magnitude of the pmf.

The most extensively studied secondary active transport system is the lactose transport system of *E. coli*. A large body of evidence supports the view that this system functions as a galactoside:proton symporter under both non-energised (catalyses facilitated diffusion) and energised conditions; that obtained from whole cell studies is summarised below [40-42,49]:

Table 6: Values of the transmembrane electrical potential, pH gradient and total protonmotive force in selected bacteria.

Bacterium	pH_{out}	pH_{in}	ΔpH (mV)	ΔΨ (mV)	pmf (mV)	Ref.
Str. lactis	5.0	6.5	-85	-75	-160	[48]
(fermenting,	7.0	ND	ND	ND	-150	
resting cells)	8.0	7.5	+21	-135	-100	
Str. lactis	5.1	6.1	-60	-83	-143	[48]
(growing cells)	6.8	7.2	-25	-108	-133	
E. coli	5.9	7.8	-109	-130	-240	[49]
(respiring,	7.0	ND	ND	-160	ND	
resting cells)	8.7	8.8	-6	-160	-165	
E. coli	6.0	7.8	-106	-95	-200	[50]
(respiring,	7.0	7.6	-35	-135	-170	
resting cells)	8.0	7.8	+12	-150	-140	
Staph. aureus	6.5	7.8	-77	-134	-211	[41]
Cl. pasteurianum	5.1	5.9	-47	ND	ND	[51]
(growing cells)	7.1	7.5	-24	ND	ND	
Cl. acetobutylicum	4.5	6.0	-102	0	-102	[52]
(growing cells)	5.5	6.0	-43	-52	-95	
	6.5	6.7	-16	-90	-106	

pmf - total protonmotive force (interior alkaline and negative)

ΔΨ - electrical potential (interior negative)

ΔpH - pH gradient (interior alkaline)

ND - no data available.

a) Metabolising cells generate a pmf of the correct orientation and magnitude. Conditions which interfere with the production of this pmf also abolish active uptake of galactoside.

b) Artificial generation of either $\Delta\Psi$ (interior negative) or ΔpH (interior alkaline) in energy-depleted cells can drive the transient accumulation of galactoside.

c) A proton flux coupled to the movement of galactoside down its concentration gradient has been demonstrated in non-metabolising cells.

d) Mutants which are defective in galactoside accumulation are also defective in the coupling of the proton flux to galactoside uptake.

There is some confusion in the literature regarding the actual stoichiometry of the symport process. An early study by West and Mitchell (1973) reported a proton:lactose stoichiometry of 1 [59] and this value has been confirmed by independent groups [49,50]. However, more recent studies suggest that this value may be 2 [60,61], and the discrepancy remains unresolved [62].

The lactose transport system of *E. coli* recognises a large variety of galactosides, in addition to lactose [43]. There is an absolute requirement for a galactopyranosyl ring of D-configuration with a free 6-hydroxyl group, whereas the system is relatively non-specific with regard to the identity of the aglycone and both the type and position of the glycosidic link. Substrates therefore include α- and β-galactosides, with either O- or thio-glycosidic linkages. However, the identity of the linkage and the aglycone does influence substrate affinity [43].

Current evidence favours the existence of a single type of galactoside binding site, but substrates can be divided into two groups based on differences between the equilibrium binding constant (K_d) and the half-saturation constant for active transport (K_t) [63]. The ratio K_d/K_t is close to unity for certain substrates (e.g. thio-β-D-digalactoside (TDG) and melibiose), but greater than unity for others (e.g. lactose and *o*-nitrophenyl-β-D-galactoside (ONPG)). Appropriate values in the case of lactose are: K_d = 14 mM ($\Delta\mu_{H+}$ = 0), K_t = 0.085 mM (under conditions of energisation) and K_d/K_t = 160 [63]. Galactoside binding is a spontaneous, energy-independent process, and 1 mole of galactoside is bound per polypeptide chain of carrier protein [63,64]. Although binding has been shown to be a pH-dependent process [65], the carrier protein is considered to be largely protonated in the physiological pH range [63].

The carrier protein has been extensively purified and reconstituted into *E. coli* phospholipid vesicles [47,66-68]. Studies with such systems indicate that a single polypeptide species (*lac Y* gene product) is sufficient to catalyse all of the transport reactions of whole cells [44]. There is some controversy over whether the carrier functions as a monomer or a dimer [62]. Based on kinetic studies, Kaback's group favour a model whereby a monomeric form of the protein catalyses facilitated diffusion (low affinity pathway), and a dimeric species catalyses active transport (high affinity pathway) [44].

Imposition of the pmf is postulated to promote aggregation of monomers to dimers [44]. Other workers favour a functional monomeric species of carrier protein, since energisation does not produce affinity changes for certain galactosides which are still actively transported [62,69,70].

1.4.3 The phosphoenolpyruvate:sugar phosphotransferase system (PTS).

The bacterial PTS was first characterised by Kundig in 1964 [71]. This system has been extensively studied, especially in *E. coli*, *S. typhimurium* and *Staph. aureus* (reviewed in [36,72-76]). The PTS catalyses the concomitant uptake and phosphorylation of various hexoses and hexitols at the immediate expense of phosphoenolpyruvate (PEP). The majority of substrates are phosphorylated at C-1, but fructose is a notable exception. This sugar may be transported by more than one PTS system and may be phosphorylated at C-1 or C-6 depending upon the system in question. The overall reaction normally requires the participation of 4 different proteins, a divalent cation (normally Mg^{2+}) and a phospholipid; and can be represented as follows:

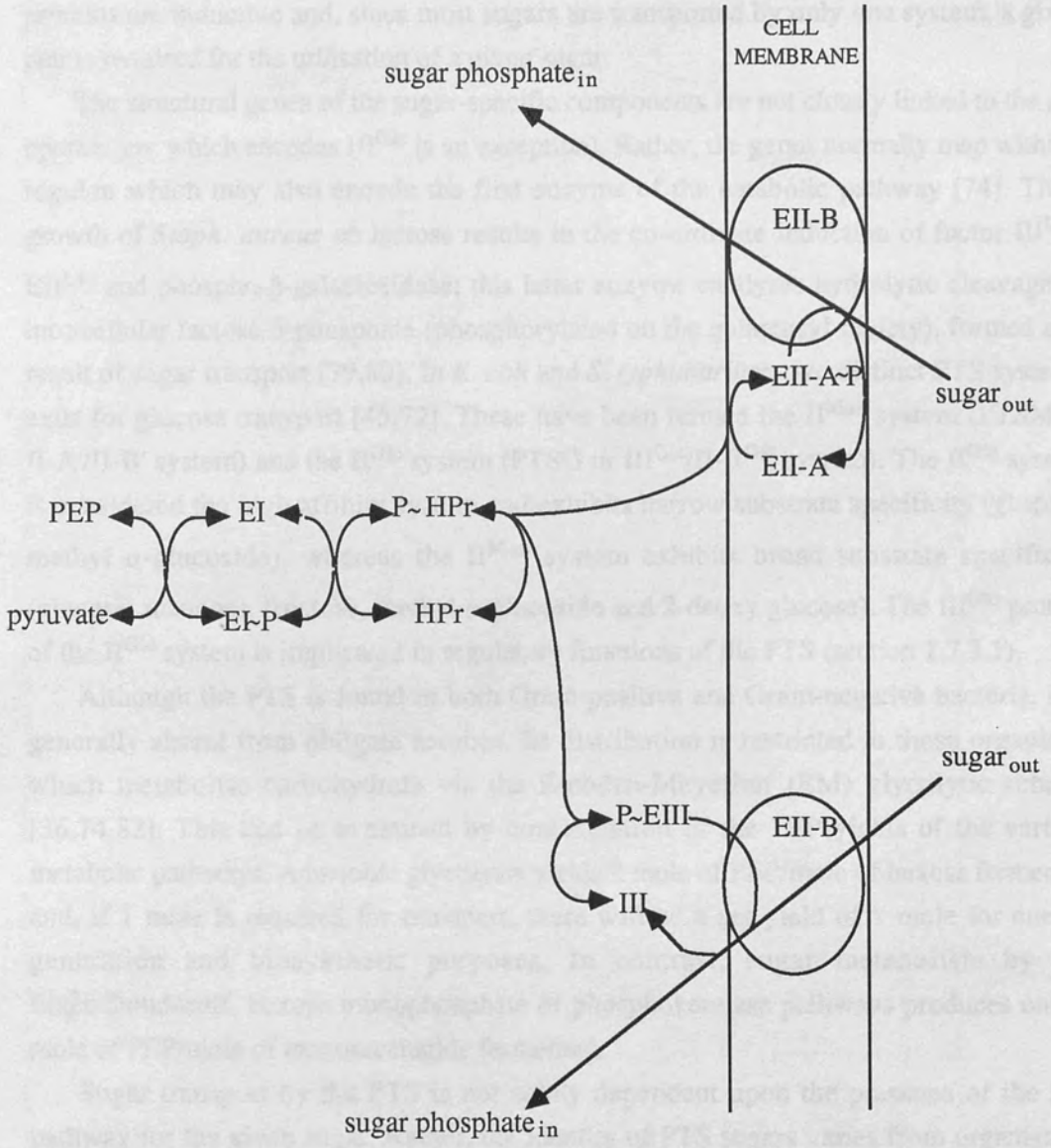
$$sugar_{out} + PEP_{in} \text{ -------> } sugar\text{-}phosphate_{in} + pyruvate_{in} \quad (\Delta G^{\circ\prime} = -11.4 \text{ kcal/mol})$$

A more detailed description of the reaction scheme is given in Fig. 3. The 4 proteins of the PTS are termed enzyme I (EI), histidine-containing phosphocarrier protein (HPr), enzyme II-A (EII-A) (or enzyme III (EIII), depending upon the system) and enzyme II-B (EII-B). Only EI and EII-B are true enzymes, the other two proteins act as substrates for these enzymes. Phosphoryl group transfer occurs sequentially from PEP to EI, onto HPr, then to EII-A (or EIII) and finally to the sugar; this last step is catalysed by the II-B protein (which is not itself phosphorylated). Translocation of the sugar substrate occurs concomitantly with its phosphorylation. The high phosphate transfer potential of PEP is conserved in the sequence until the translocation step.

Enzyme I and HPr (*pts I* and *pts H* gene products, respectively) are referred to as the general proteins of the system, since they are both required for the transport and phosphorylation of all sugar substrates [77,78]. They are usually found in the soluble fraction of cell extracts and their primary function is to couple metabolic energy to the sugar specific components of the system. They are often considered to be constitutively produced, but their levels do fluctuate depending on the PTS sugar in the growth medium; variations of up to 3-fold have been reported [36,72].

The remaining two proteins of the PTS are sugar specific and, together with lipid and a divalent cation, form the enzyme II complex. At least one of these proteins is an integral membrane component (EII-B) and the other protein may be either membrane-associated (EII-A) or cytoplasmic (EIII or factor III). Enzyme II-B functions as the sugar receptor protein of the system, since it both recognises and binds the substrate, whereas the II-A (or

Figure 3: The bacterial phosphoenolpyruvate: sugar phosphotransferase system



EI - enzyme I
HPr - histidine-containing phosphocarrier protein
EIII - enzyme or factor III
EII-A - the II-A protein
EII-B - the II-B protein
~ P- denotes a high energy phosphate group

Adapted from [76]

III) protein functions as a sugar-specific energy coupling protein. Sugar specificity is normally denoted in the form of superscripts; an enzyme II complex specific for sugar X would be represented by II-A^X/II-B^X (or III^X/II-B^X) [72]. In general, the sugar-specific proteins are inducible and, since most sugars are transported by only one system, a given pair is required for the utilisation of a given sugar.

The structural genes of the sugar-specific components are not closely linked to the *pts* operon (*crr* which encodes III^{Glc} is an exception). Rather, the genes normally map within a regulon which may also encode the first enzyme of the catabolic pathway [74]. Thus, growth of *Staph. aureus* on lactose results in the co-ordinate induction of factor III^{Lac}, EII^{Lac} and phospho-β-galactosidase; this latter enzyme catalyses hydrolytic cleavage of intracellular lactose 6-phosphate (phosphorylated on the galactosyl moiety), formed as a result of sugar transport [79,80]. In *E. coli* and *S. typhimurium*, two distinct PTS systems exist for glucose transport [45,72]. These have been termed the II^{Man} system (PTSM or II-A'/II-B' system) and the II^{Glc} system (PTSG or III^{Glc}/II-B^{Glc} system). The II^{Glc} system is considered the high affinity system and exhibits narrow substrate specificity (glucose, methyl α-glucoside), whereas the II^{Man} system exhibits broad substrate specificity (glucose, mannose, fructose, methyl α-glucoside and 2-deoxy glucose). The III^{Glc} protein of the II^{Glc} system is implicated in regulatory functions of the PTS (section 1.7.3.1).

Although the PTS is found in both Gram-positive and Gram-negative bacteria, it is generally absent from obligate aerobes. Its distribution is restricted to those organisms which metabolise carbohydrate via the Embden-Meyerhof (EM) glycolytic scheme [36,74,82]. This can be explained by consideration of the PEP yields of the various metabolic pathways. Anaerobic glycolysis yields 2 mole of PEP/mole of hexose fermented and, if 1 mole is required for transport, there will be a net yield of 1 mole for energy generation and biosynthetic purposes. In contrast, sugar metabolism by the Entner-Doudoroff, hexose monophosphate or phosphoketolase pathways produces only 1 mole of PEP/mole of monosaccharide fermented.

Sugar transport by the PTS is not solely dependent upon the presence of the EM pathway for the given sugar. Rather, the identity of PTS sugars varies from organism to organism (Table 7). Data are somewhat scarce regarding sugar transport systems in the clostridia and no data are available on lactose transport in this genus.

Several factors have been identified which may be important in determining whether a given organism transports a given sugar by a PTS system [74]. The PTS is an energetically favourable uptake system on two accounts: the sugar is transported and prepared for metabolism in one step and phosphorylation essentially traps the substrate within the cell. Andrews and Lin (1976) have compared β-galactoside utilisation in several bacteria [39]. Those organisms which possess a PTS mechanism (*Str. lactis*, *Staph. aureus*), are able to grow at lower substrate concentrations than those organisms which possess a proton-linked system (*E. coli*, *K. aerogenes*) and are therefore more efficient scavengers of the sugar. The main advantage of the proton-linked system is the ability to

Table 7: Distribution of the PTS in selected bacteria.

Organism	PTS sugars	Non-PTS sugars
E. coli	glucose mannose fructose glucosamine N-acetylglucosamine N-acetylmannosamine hexitols β-glucosides	lactose melibiose maltose α-glycerophosphate glycerol hexose phosphates arabinose ribose xylose gluconate galactose
S. typhimurium	as for *E. coli*	as for *E. coli*, but not lactose
Staph. aureus	as for *E. coli* plus: lactose sucrose galactose trehalose melibiose melezitose pentoses	maltose glycerol
Cl. pasteurianum	glucose fructose sucrose sorbitol mannitol	galactose gluconate
Cl.thermocellum	fructose mannitol	
Cl. perfringens	glucose mannose	

Sugars transported by mechanisms such as facilitated diffusion, ion gradient-dependent systems and binding-protein systems are grouped together as non-PTS sugars. Adapted from [72,75,83-86].

use a wide range of substrates. This increased versatility is safeguarded by the presence of an efflux pathway which permits the removal of non-metabolisable substrates [39]. Saier has drawn attention to a further important advantage of the PTS [74]; the PTS can regulate non-PTS uptake systems (section 1.7.3.1) and this feature permits the preferential uptake of PTS sugars. Unnecessary energy expenditure can be avoided if easily metabolisable sugars (such as monosaccharides) are taken up by PTS systems, and more complex sugars (such as disaccharides) are taken up by non-PTS systems. In fact, most organisms which possess the PTS also transport glucose by this system [87].

1.5 Glycolytic Pathways.

Bacterial metabolism of glucose to pyruvate can proceed by four major pathways: the Embden-Meyerhof (EM) pathway, the hexose monophosphate pathway, the Entner-Doudoroff pathway and the phosphoketolase pathway [88]. The presence and relative participation of each of these pathways is normally species-dependent. In the case of *Cl. perfringens*, both enzymological and radiolabelling studies indicate that the major (and most likely sole) route of glucose metabolism is the EM pathway [89,90].

The possible routes whereby lactulose may enter this main-line glycolytic pathway are considered below, with particular reference to lactose metabolism in bacteria.

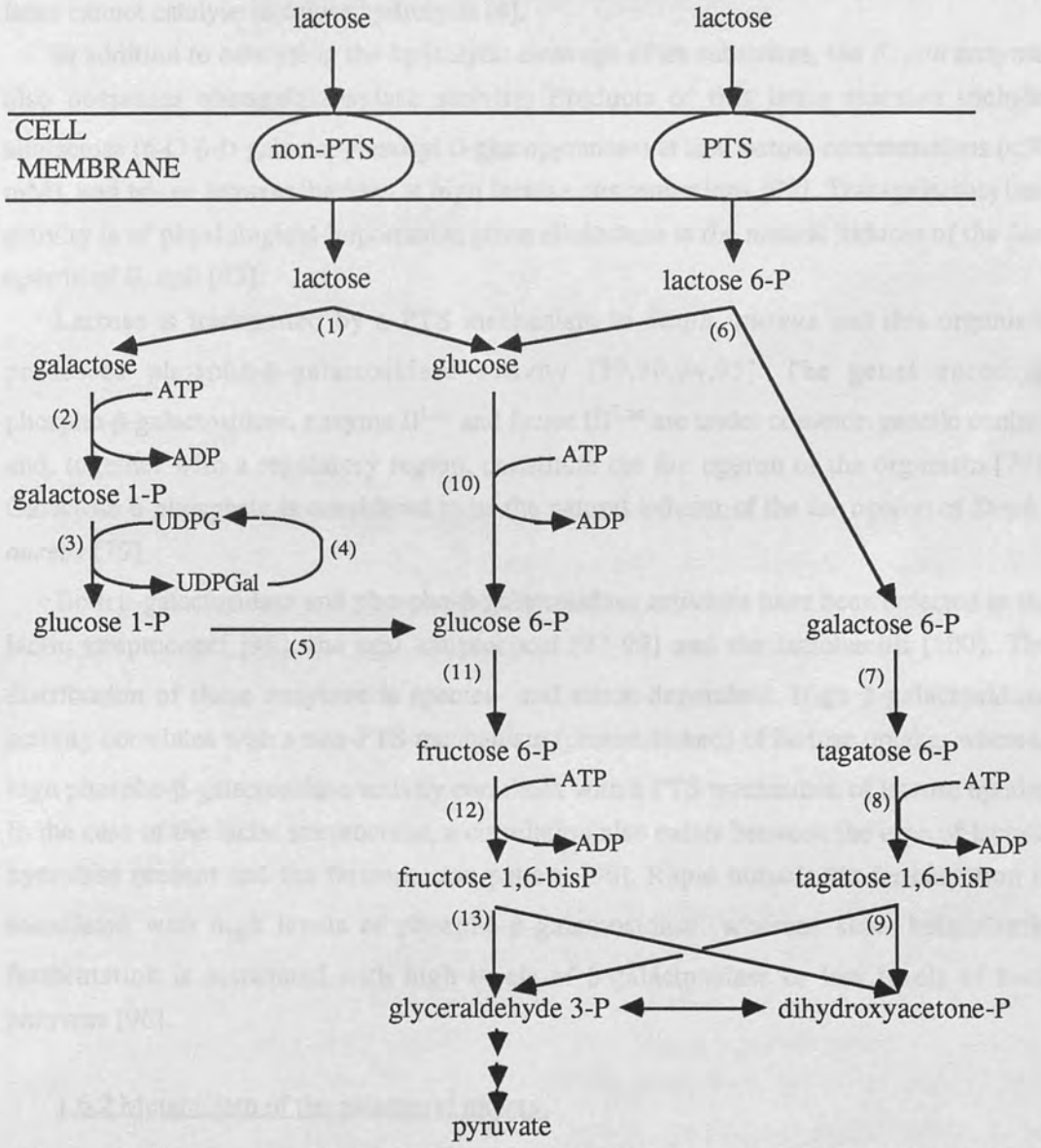
1.6 Lactose Metabolism.

Lactose metabolism has been studied in detail in a diverse range of bacterial species. The most common routes of catabolism are depicted in Fig. 4. It is apparent that the catabolic pathways employed are in part determined by the mechanism of sugar transport. Uptake by a non-PTS mechanism requires a hydrolase (β-galactosidase) specific for the free form of the disaccharide and the operation of the Leloir pathway. Uptake by a PTS-mechanism requires a hydrolase (phospho-β-galactosidase) specific for the phosphorylated form of the disaccharide and the operation of the tagatose 6-phosphate pathway. Phosphorylation of the glucosyl moiety of lactose, concomitant with transport, has not been described to-date, therefore, an ATP-dependent kinase will be required to initiate metabolism of this moiety irrespective of the transport mechanism.

1.6.1 Lactose hydrolysis.

β-Galactosidase activity has been detected in a diverse range of bacteria, including representatives of the Enterobacteriaceae, Pseudomonadaceae, Neisseriaceae, Bacillaceae and Propionobacteriaceae [91]. The enzyme from *E. coli* has been purified and is well characterised (reviewed in [91]). It is relatively specific with regard to the glycone (galactoside) moiety of the substrate and the nature of the glycosidic linkage

Figure 4: Routes of lactose metabolism in prokaryotes.



Key to enzymes

(1) β -galactosidase
(2) galactokinase
(3) galactose 1-P uridylyl transferase
(4) UDPgalactose 4-epimerase
(5) phosphoglucomutase
(6) phospho-β-galactosidase
(7) galactose 6-P isomerase
(8) tagatose 6-P kinase
(9) tagatose 1,6-bisP aldolase
(10) hexokinase
(11) glucose 6-P isomerase
(12) phosphofructokinase
(13) fructose 1,6-bisP aldolase

P denotes a phosphate group.

(β-configuration preferred), but is relatively non-specific with regard to the aglycone moiety. This may be another sugar residue, an alkyl group or an aryl group, but its structure does influence the kinetics of hydrolysis [91]. Therefore, the bacterial enzyme appears to possess broader substrate specificity than the human intestinal enzyme, since the latter cannot catalyse lactulose hydrolysis [4].

In addition to catalysing the hydrolytic cleavage of its substrates, the *E. coli* enzyme also possesses transgalactosylase activity. Products of this latter reaction include allolactose (6-O-β-D-galactopyranosyl D-glucopyranose) at low lactose concentrations (<50 mM), and tri- or tetrasaccharides at high lactose concentrations [92]. Transgalactosylase activity is of physiological importance, since allolactose is the natural inducer of the *lac* operon of *E. coli* [93].

Lactose is transported by a PTS mechanism in *Staph. aureus* and this organism possesses phospho-β-galactosidase activity [79,80,94,95]. The genes encoding phospho-β-galactosidase, enzyme II^{Lac} and factor III^{Lac} are under common genetic control and, together with a regulatory region, constitute the *lac* operon of the organism [79]. Galactose 6-phosphate is considered to be the natural inducer of the *lac* operon of *Staph. aureus* [79].

Both β-galactosidase and phospho-β-galactosidase activities have been detected in the lactic streptococci [96], the oral streptococci [97-99] and the lactobacilli [100]. The distribution of these enzymes is species- and strain-dependent. High β-galactosidase activity correlates with a non-PTS mechanism (proton-linked) of lactose uptake, whereas high phospho-β-galactosidase activity correlates with a PTS mechanism of lactose uptake. In the case of the lactic streptococci, a correlation also exists between the type of lactose hydrolase present and the fermentation pattern [96]. Rapid homolactic fermentation is associated with high levels of phospho-β-galactosidase, whereas slow heterolactic fermentation is associated with high levels of β-galactosidase or low levels of both enzymes [96].

1.6.2 Metabolism of the galactosyl moiety.

In *E. coli*, the galactosyl moiety of lactose is metabolised via the Leloir pathway and, following phosphoglucomutase catalysed conversion of glucose 1-phosphate to glucose 6-phosphate, enters the glycolytic pathway (Fig. 4) [101]. The three enzymes of the Leloir pathway (galactokinase, galactose 1-phosphate uridylyl transferase and UDPgalactose 4-epimerase) are encoded within a single polycistronic operon (the *gal* operon), which is induced by free galactose [102].

The tagatose 6-phosphate pathway is required for the metabolism of the galactosyl moiety of lactose in *Staph. aureus* [103]. Although this pathway is analogous to the three steps of the EM pathway which result in the conversion of glucose 6-phosphate to triose

phosphates (Fig. 4), both genetic and enzymological studies indicate that the two pathways are separate and distinct [103-107]. The three enzymes of the tagatose 6-phosphate pathway are encoded within a single operon (the *tag* operon) [104], and are specifically induced by growth on galactose or lactose [101,105-107].

Enzymes of both the Leloir pathway and the tagatose 6-phosphate pathway have been reported in oral and lactic streptococci [101,108]. In these bacteria, the distribution of the two pathways is both species- and strain-dependent, as is the mechanism of lactose hydrolysis.

1.6.3 Metabolism of the glucosyl and fructosyl moieties of disaccharides.

The ATP-dependent hexose kinase activities of bacteria exhibit a high degree of substrate specificity and are therefore named accordingly: glucokinase (EC 2.7.1.2), mannokinase (EC 2.7.1.7), fructokinase (EC 2.7.1.4) and mannofructokinase (Table 8). These enzymes are normally found in the soluble fraction of cell extracts and they phosphorylate their substrates at C-6 (ATP:hexose 6-phosphotransferases).

The ATP-dependent hexose kinases are required for the metabolism of non-PTS hexose and intracellular free hexose generated as a result of disaccharide cleavage. Thus, although the glucokinase of *E. coli* does not play any appreciable role in the metabolism of exogenous free glucose, it is required for the efficient utilisation of lactose [115,116]. The main physiological role of the hexose kinase activities of oral streptococci is also thought to be the metabolism of disaccharide moieties [111,117,118].

The clostridial enzymes have not been studied in any great detail. Glucose-grown cells of *Cl. perfringens* [89] and *Cl. thermocellum* [119] contain glucokinase activity, and the enzyme exhibits negligible specificity for fructose and mannose. In the case of *Cl. perfringens*, the enzyme does not appear to play a significant role in the metabolism of exogenous free glucose, since this sugar is transported by a PTS mechanism [85]. Phosphorylation of exogenous free fructose also appears to be catalysed by a PTS system in a variety of saccharolytic clostridia (*Cl. thermocellum*, *Cl. pasteurianum*, *Cl. roseum*, *Cl. rubrum* and *Cl. butyricum*) [86,120]. The system phosphorylates fructose at C-1, and the resulting fructose 1-phosphate is metabolised to fructose 1,6-bisphosphate by an inducible fructose 1-phosphate kinase (ATP:fructose 1-phosphate 6-phosphotransferase). Sucrose-grown cells of *Cl. pasteurianum* also contain high levels of this latter enzyme [120], suggesting that PTS-mediated phosphorylation is required for the metabolism of the fructosyl moiety of the disaccharide.

1.7 Regulation of Sugar Utilisation.

When *E. coli* is presented with a mixture of glucose and lactose, a biphasic growth pattern (diauxie) is observed [121]. Utilisation of lactose does not take place until all of the glucose has been removed from the medium. A lag period occurs between glucose

Table 8: Characteristics of bacterial ATP-dependent hexose kinase activity.

Organism	Hexose kinase	Substrate	K_m (mM)	Comments	Ref.
K. aerogenes	GK	glucose glucosamine	0.08 0.40	constitutive	[109]
	FK	fructose	0.30	highly specific, inducible	[110]
Str. mutans	GK	glucose	1.40	highly specific, expandable	[111]
	MFK	fructose mannose	0.63 0.37	expandable	[112]
E. coli	GK	glucose	ND	highly specific, constitutive	[113]
	MFK	mannose fructose glucosamine glucose 2-deoxyglucose	0.20 0.30 5.00 10.00 10.00	constitutive	[114]

GK - glucokinase
FK - fructokinase
MFK - mannofructokinase

ND - no data available.

exhaustion and the commencement of lactose utilisation, which corresponds to the time required for induction of the lactose catabolic system [121]. Since glucose can exert this effect on the utilisation of a wide range of other sugars, the phenomenon has been termed the 'glucose effect' [121,122].

Three distinct glucose effects have been identified [102,121,122]:

a) Catabolite repression.

Inclusion of glucose in the growth medium results in the repression of inducible catabolic enzyme synthesis. Repression is normally severe (but not complete) and permanent. This effect can be brought about by any readily metabolisable sugar and has been attributed to high levels of catabolites (unidentified) of the repressing sugar.

b) Transient repression.

This effect is kinetically distinct from catabolite repression and occurs when glucose (or any repressing sugar) is added to cells already growing on a second carbon source. Induction of catabolic enzyme synthesis is completely repressed for a transient period (0.5-1.0 cell doubling time; transient repression) and then resumes at a rate characteristic for growth on the repressing sugar (permanent repression). Transient repression can be brought about by non-metabolisable analogues of the repressing sugar.

c) Inducer exclusion.

The presence of a repressing sugar (or its non-metabolisable analogue) prevents the uptake of an inducing sugar and thereby indirectly inhibits catabolic operon expression.

The contribution of inducer exclusion to repressive effects can be assessed using regulatory mutants constitutive for the given system under study [102]. In the case of the *gal* operon of *E. coli*, the only glucose effect appears to be inducer exclusion (no glucose effect in *gal* constitutive mutants), whereas in the case of the *lac* operon of this organism, the extent of catabolite and transient repression is identical in both inducible and *lac* constitutive strains [102]. Inducer exclusion is considered further in the section dealing with the regulation of sugar transport (section 1.7.3).

1.7.1 Cyclic AMP, catabolite gene expression and catabolite repression.

In 1965, Makman and Sutherland reported the presence of adenosine 3',5'-monophosphate (cyclic AMP or cAMP) in *E. coli* and demonstrated a depletion in intracellular levels of this nucleotide in response to glucose [123]. Subsequent studies by Pastan and Perlman showed that addition of cAMP to *E. coli* could both stimulate β-galactosidase synthesis in cells cultured in a non-glucose medium and relieve glucose-mediated repression of this enzyme [124]. They proposed that glucose repression is mediated via a decrease in the cellular levels of the nucleotide.

Further work has shown that cAMP plays an important role in catabolite gene expression, acting as a positive effector of transcription initiation [102,121,125,126]. The nucleotide does not exert this effect directly, but requires the participation of a receptor protein (catabolite gene activator protein (CAP) or cAMP receptor protein (CRP)). Only one

form of this protein is produced per *E. coli* cell (*crp* gene product) and it requires the binding of cAMP for biological activity. Once activated, the protein binds to specific regions of the DNA helix and stimulates the transcription of mRNA molecules. The mechanism of transcription stimulation is unclear, it may be mediated by either destabilisation of the DNA helix (promotion of open complex formation) or direct protein-protein interaction with RNA polymerase [102].

The involvement of cAMP in catabolite repression is not fully understood. According to the hypothesis of Pastan and Perlman, cAMP acts as the exclusive mediator of transient and permanent repression, therefore, cellular cAMP levels should be correlated with the extent of repression [124]. Although one group has produced convincing evidence for the existence of such a correlation [127], others have produced conflicting results. This discrepancy may be due to difficulties in the measurement of intracellular cAMP levels [102,121].

The possible target sites whereby cAMP levels may be regulated are: synthesis (catalysed by adenylate cyclase), degradation (catalysed by cAMP-phosphodiesterase) and excretion. Present evidence suggests that the latter two mechanisms do not play an important role, therefore, adenylate cyclase is thought to be the major regulatory site [121,124-126,128].

The regulation of adenylate cyclase is exceedingly complex and not yet fully understood. Most work has been conducted on the *E. coli* enzyme, which is membrane-bound, and conditions which interfere with the integrity of the membrane often alter its regulation. Effectors of the enzyme include a variety of transport systems (non-PTS and PTS systems) which inhibit activity [74,128-131], and the CAP protein which both represses synthesis and inhibits activity [102].

Attempts to demonstrate a direct relationship between adenylate cyclase activity and intracellular cAMP levels have yielded paradoxical results [102,128]. A correlation does exist, but cAMP levels are inversely related to *in vitro* adenylate cyclase activity. This may be explained by changes in the level of synthesis of the protein when grown on different carbon sources [128]. On a poor carbon source, inhibition of the enzyme will be minimal, cAMP levels will be high and enzyme synthesis may be repressed. Conversely, on a good carbon source, inhibition of the enzyme will be maximal, cAMP levels will be low and enzyme synthesis may be derepressed, thereby allowing a rapid rate of cAMP synthesis upon exhaustion of the carbon source from the medium.

1.7.2 Catabolite modulator factor and catabolite repression.

Irrespective of the mechanism(s) of regulation of cAMP levels, evidence is accumulating which suggests that the cAMP-CAP complex may only play an indirect role in the phenomenon of catabolite repression [102,128]. The most direct evidence is summarised below:

a) Pseudorevertants which exhibit pleiotropic carbohydrate-positive character have been isolated from *cya* (adenylate cyclase gene) mutants (pleiotropic carbohydrate-negative) by a variety of second site mutations e.g. the Sigma subunit of RNA polymerase (*alt* mutants) or the CAP protein (becomes active in the absence of cAMP). Utilisation of carbohydrate by such pseudorevertants is catabolite repressible.

b) Double mutants, defective in both CAP protein and rho protein (a termination factor), exhibit pleiotropic carbohydrate-negative character. Carbohydrate-positive pseudorevertants, which are altered in a RNA polymerase subunit, are still subject to catabolite repression.

c) Certain Gram-positive bacteria, such as *B. megaterium*, do not contain cAMP but exhibit catabolite repression.

d) A high exogenous concentration of cAMP (200-1000 times the intracellular concentration) is required for the relief of catabolite and transient repression and, under certain growth conditions, catabolite repression can still persist in the presence of exogenous cAMP.

Ullmann and Danchin (1983) have postulated that catabolite repression may be mediated by a negative effector acting at the level of gene expression [102]. This effector may be displaced from its site of action by the CAP-cAMP complex, and its synthesis/degradation may be controlled by the level of catabolites in the cell so that its concentration is high under conditions of strong catabolite repression and low under conditions of weak catabolite repression. A possible mediator has been isolated (termed catabolite modulator factor) and, although it remains poorly characterised, it does exhibit many of the required properties [102].

1.7.3 Regulation of sugar transport.

Catabolite gene expression can also be regulated by control of the intracellular levels of inducer. Indeed, a variety of mechanisms have been described whereby sugar transport system activity may be regulated.

1.7.3.1 PTS-mediated regulation of non-PTS uptake systems.

Genetic studies with *E. coli* and *S. typhimurium* have shown that two classes of *ptsH* and *ptsI* mutants can be isolated; these are termed 'leaky' or 'tight' mutants according to the residual levels of the respective PTS proteins [78,132,133]. 'Tight' mutants are unable to grow on both PTS and non-PTS sugars (glycerol, maltose, melibiose and lactose), whereas 'leaky' mutants can grow on non-PTS sugars, but growth is hypersensitive to repression by PTS sugars.

Inhibition of non-PTS sugar uptake is due to the repression of catabolic enzyme synthesis and does not require extensive uptake of the PTS sugar. It can be overcome by removal of the PTS sugar from the medium or by further mutation [78,132,133]. The

general *crr* mutation maps adjacent to the *pts HI* operon and results in general relief from repression, whereas the specific *crr* mutation maps close to (or within) the structural genes of a given non-PTS sugar uptake system and results in relief for that sugar only.

An allosteric regulatory model has been proposed in an attempt to explain the above findings, coupled with the co-ordinate effects of PTS sugars on adenylate cyclase activities (section 1.7.1 and Fig. 5) [72,74,122,130,131]. The central regulatory protein (RPr; general *crr* gene product) is in equilibrium with the general PTS proteins and can be phosphorylated by direct transfer from phospho-HPr. The free form binds to a regulatory site (specific *crr* gene product) on the non-PTS permease and inhibits transport, whereas the phosphorylated form binds to a regulatory site on adenylate cyclase and activates the enzyme.

In wild-type cells, under conditions of energy (PEP) proficiency, the phosphorylated forms of EI, HPr and RPr will predominate and both adenylate cyclase and non-PTS uptake systems will be active. Addition of a readily utilisable PTS sugar will drain phosphoryl groups from the general PTS proteins and, ultimately, phospho-RPr, resulting in inhibition of non-PTS uptake systems and deactivation of adenylate cyclase.

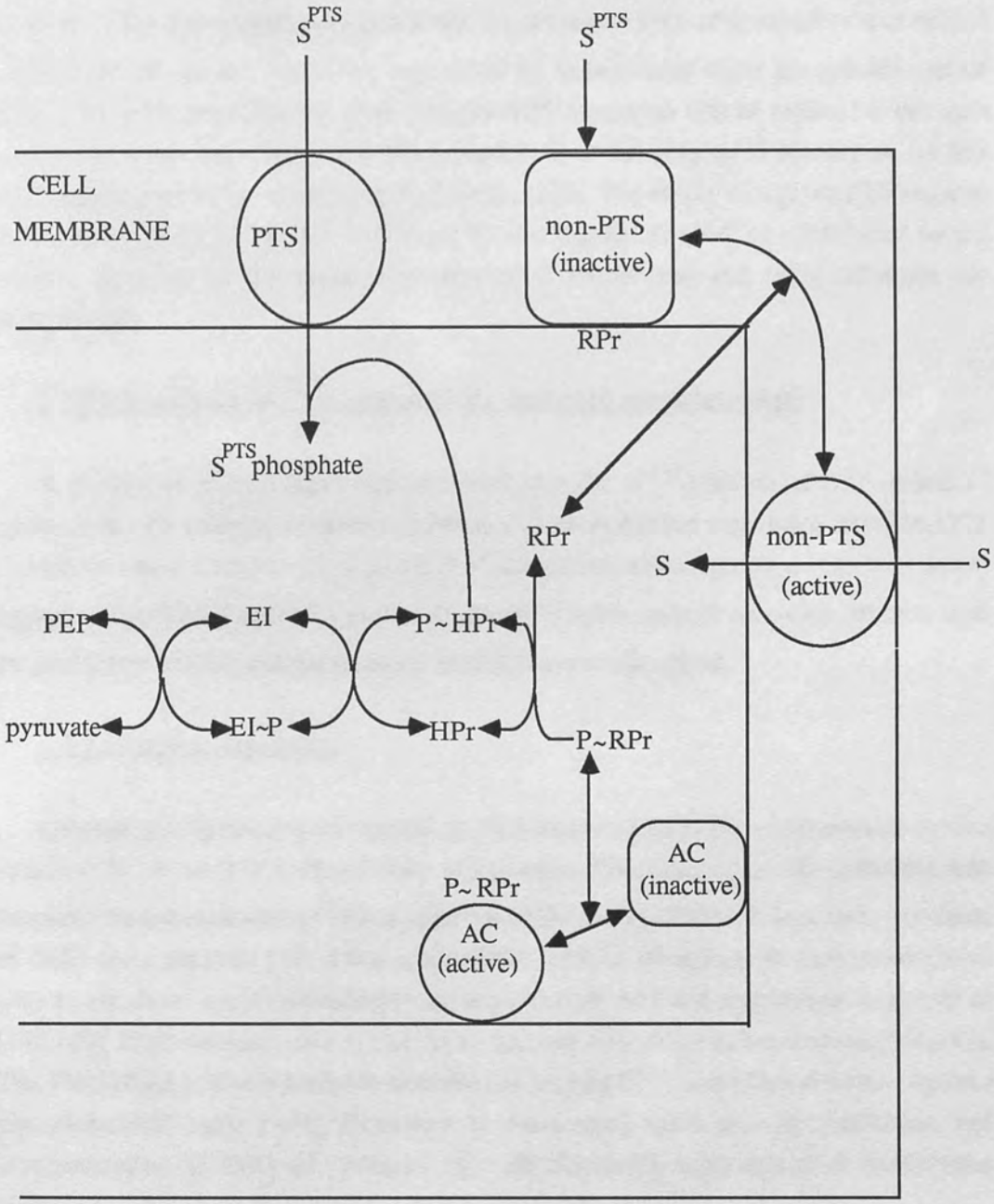
A considerable amount of experimental evidence supports this allosteric model. The RPr protein has been identified as factor III^{Glc} [134,135] and a direct interaction between this protein and the lactose carrier protein of *E. coli* has been reported [136]. Only the dephosphorylated form of factor III^{Glc} will bind, and there is a requirement for a galactoside substrate [136]. Experiments with *E. coli* vesicles, or proteoliposomes reconstituted with purified carrier protein, have demonstrated inhibition of galactoside transport in response to purified III^{Glc} [136,137]. The Gram-positive organism, *B. subtilis*, also possesses a soluble factor III^{Glc}, and preliminary experiments indicate the operation of a similar allosteric mechanism [138].

1.7.3.2 Regulation by intracellular sugar phosphate.

In *E. coli*, glucose can inhibit the uptake of the PTS sugar fructose [139]. Inhibition is enhanced in mutants restricted in the metabolism of intracellular glucose 6-phosphate, and glucose 6-phosphate can also inhibit fructose uptake in cells constitutive for the hexose phosphate uptake system. Inhibition can be relieved by mutation of the II-B^{Glc} protein, or a mutation which maps close to (or within) the gene encoding enzyme II^{Fru} (*cif* mutation). On the basis of these findings, it has been postulated that the inhibitory effect of glucose is mediated by intracellular sugar phosphate binding to a regulatory site on the fructose uptake system [139].

This mechanism may apply to a variety of transport systems (PTS and non-PTS) in a wide range of organisms [36,122]. In the case of Gram-positive bacteria (*Staph. aureus*, *B. subtilis*), inhibition of glycerol uptake by PTS sugars has been attributed to a similar mechanism [140].

Figure 5: Allosteric regulatory model for PTS-mediated, co-ordinate regulation of non-PTS sugar transport systems and adenylate cyclase.



S^{PTS} - sugar substrate of the PTS
S - non-PTS sugar substrate
EI - enzyme I of the PTS
HPr - histidine-containing phosphocarrier protein
RPr - putative regulatory protein
AC - adenylate cyclase

~ P- denotes a high energy phosphate bond

Adapted from [76].

1.7.3.3 Regulation of PTS systems via competition for phospho-HPr.

Methyl α-glucoside inhibition of fructose uptake in *S. typhimurium* is enhanced when cellular energy levels are depleted or when enzyme I levels are decreased ('leaky' enzyme I mutants) [122]. These conditions also result in a decreased level of accumulation of methyl α-glucoside phosphate, therefore, regulation by intracellular sugar phosphates cannot explain these findings. Rather, since phospho-HPr formation will be reduced under such conditions, it has been suggested that competition of the enzyme II complexes for this intermediate may be the underlying mechanism [122]. The ability of a given PTS sugar to regulate the uptake of another PTS sugar by this mechanism will be determined by the relative amounts of the respective enzyme II complexes and their affinities for phospho-HPr.

1.7.3.4 Regulation of PTS systems by the energised membrane state.

A variety of studies have demonstrated that the II^{Glc} systems of *E. coli* and *S. typhimurium* are subject to negative regulation by the energised membrane state [36,122]. Conditions which stimulate the production of an electrochemical gradient of protons across the membrane inhibit methyl α-glucoside uptake, whereas conditions which interfere with the production of this gradient stimulate methyl α-glucoside uptake.

1.7.3.5 Inducer expulsion.

Glucose and lactose are transported by PTS mechanisms in *Str. pyogenes* and certain strains of *Str. lactis* [141,142]. Addition of a glucose-PTS substrate to cells preloaded with thiomethyl β-D-galactoside (TMG; a substrate of the lactose-PTS) elicits a rapid expulsion of TMG from the cells [141,142]. Intracellular TMG 6-phosphate is dephosphorylated prior to expulsion, and dephosphorylation requires both ATP and a glycolytic intermediate [142,143]. Non-metabolisable analogues of glucose will only elicit exclusion [141,142]. The TMG efflux pathway has been identified as enzyme II^{Lac}, and efflux does not require a phosphorylated sugar [144]. Expulsion is much more rapid than accumulation, and reaccumulation of TMG will proceed once the displacing sugar has been metabolised [141].

1.8 Aims.

Lactulose metabolism by human intestinal bacteria is poorly documented in the literature. Several investigations have been concerned with the identification of bacterial groups which may be responsible for metabolism in vivo [33-35] and, apart from quantitation of *in vitro* fermentation products [33], no detailed studies have been made of

metabolic pathways. The major aim of this Ph.D. project was to initiate studies in this area.

The organism of choice for this investigation was the Gram-positive, anaerobic bacterium *Cl. perfringens*. Preliminary screening studies in the Department had shown that batch cultures of this organism actively metabolise lactulose and exhibit a classical utilisation pattern [33,145]. Areas of investigation were restricted to sugar uptake and subsequent pathways of intermediary metabolism, since fermentation product formation had already been studied in detail [145].

The present study was restricted by the unavailability of radiolabelled lactulose and this required the use of 'cold' procedures. The main aims of the project were:

a) Determination of the pathways of lactulose metabolism by enzyme assay and fluorimetric determination of metabolites.

b) Assay of sugar uptake and identification of the transport mechanism. It was envisaged that a spectrophotometric technique could be employed to monitor sugar uptake. This procedure measures changes in cell size (due to plasmolysis and deplasmolysis) when cells are presented with a hypertonic solution of the compound under study. Identification of the type of transport system in operation would be accomplished by the use of inhibitors which specifically interfere with mechanisms of energy coupling.

c) Control of lactulose utilisation. It was intended to investigate the effect of glucose on lactulose utilisation in *Cl. perfringens* and, if any effect was present, to determine the underlying mechanism.

d) Investigation of lactulose metabolism in continuous cultures of *Cl. perfringens*. All the above work was to be conducted on cells cultured under batch conditions and the results of such studies may be of limited value in extrapolation to the *in vivo* situation. It was therefore intended to repeat certain aspects of the work using an 'open' culture system, such as the chemostat.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials.

General laboratory chemicals were purchased from BDH Ltd and were of AnalaR grade wherever possible. Fine chemicals were obtained from the Sigma (London) Chemical Company Ltd. All radiochemicals were supplied by Amersham International PLC and gases were purchased from the British Oxygen Company.

The organism of study, *Clostridium perfringens*, was obtained from Dr. A. Vince (University College Medical School, London) and had been isolated from human faeces. Crystalline lactulose was kindly donated by Duphar BV (Amsterdam, Holland). Details of any other special chemicals or equipment are given in the text (section 2.2).

2.2 Methods.

2.2.1 Growth Media.

2.2.1.1 Blood agar.

Blood agar base number 2 (Oxoid; 40 g) was dissolved in distilled water (930 ml) and autoclaved. After cooling to 45-50 °C, human blood (70 ml) was added and plates were immediately poured.

2.2.1.2 Simmonds citrate agar.

Simmonds citrate agar powder (Oxoid; 23 g) was dissolved in distilled water (1 l), autoclaved and plates were poured after cooling.

2.2.1.3 Peptone-yeast basal medium (BM).

This was a modified form of the medium described by Holdeman *et al.* (1977) [146]. The following constituents were mixed together per litre of medium:

Proteose peptone number 3 (Difco)	10 g
Yeast extract (Difco)	10 g
Salts solution (section 2.2.1.3.1)	40 ml
Resazurin solution (1 mg/ml)	1 ml
Sodium thioglycollate	0.5 g
Sodium formaldehyde sulphoxylate	0.3 g
Distilled water	950 ml

The mixture was boiled, whilst stirring, to dissolve the components. After cooling, hemin-Vitamin K solution (10 ml) (section 2.2.1.3.2) was added, followed by cysteine hydrochloride (0.5 g), and the pH was adjusted to 7.2 with sodium hydroxide (1 M).

2.2.1.3.1 Salts solution.

Composition per litre:	
Calcium chloride (dihydrate)	0.25 g
Magnesium sulphate	0.2 g
Dipotassium hydrogen orthophosphate	1 g
Potassium dihydrogen orthophosphate	1 g
Sodium hydrogen carbonate	10 g

The $CaCl_2$ and $MgSO_4$ were dissolved in distilled water (300 ml), and to this was added the remaining salts in distilled water (500 ml), whilst stirring. The volume was made up to 1 l with distilled water, and this stock solution was stored at 4 °C.

2.2.1.3.2 Hemin-Vitamin K solution.

Two stock solutions were prepared:

Solution A consisted of a 0.5% (w/v) solution of menadione in ethanol (95% (v/v)). The solution was sterilised by membrane filtration.

Solution B was prepared by dissolving hemin (Sigma bovine type I; 50 mg) in sodium hydroxide (1 M; 1 ml) and the volume was made up to 100 ml with distilled water. The solution was sterilised by autoclaving.

Both stock solutions were stored at 4 °C, and the working solution was freshly prepared by mixing 1 part of solution A with 100 parts of solution B.

2.2.1.4 Storage medium.

Cooked meat medium (Oxoid; 0.6 g) was added to BM (10 ml) and autoclaved.

2.2.2 Sterilisation.

If the material withstood heat, then autoclaving at 121 °C, 15 psi for 20 min was routinely employed. Heat-labile material was sterilised by membrane filtration using 'Nuflow' cellulose acetate filters (Oxoid; 0.22 μm pore size, 25 mm diameter) and Swinnex-25 filter holders (Millipore).

2.2.3 Culture Techniques.

Strict aseptic techniques were observed throughout all culture procedures.

2.2.3.1 Culture conditions.

The organism was routinely cultured at 37 °C, under anaerobic conditions, using a gas jar system. This system basically consisted of a converted Tefal pressure cooker and palladium catalysts. The catalysts were heated at 100 °C for 12 h prior to use, and the gas mixture consisted of $N_2/CO_2/H_2$ (80/10/10%). A biological indicator of anaerobiosis was included in each incubation; *Pseudomonas aeruginosa* streaked on Simmonds citrate agar. All media were pregassed overnight prior to inoculation.

2.2.3.2 Stock cultures.

These were prepared by culturing the organism for 24 h in storage medium. An equal volume of sterile glycerol (30% (v/v)) was added before storage at -20 °C or under liquid nitrogen.

2.2.3.3 Experimental cultures - batch growth.

Stock cultures were firstly checked for contamination by streaking onto blood agar plates. After incubation for 24 h, a loopful of bacteria was transferred to BM and cultured for a further 12 h. This culture was then used for inoculation of the appropriate experimental medium (5% (v/v) inoculum).

2.2.3.4 Continuous culture.

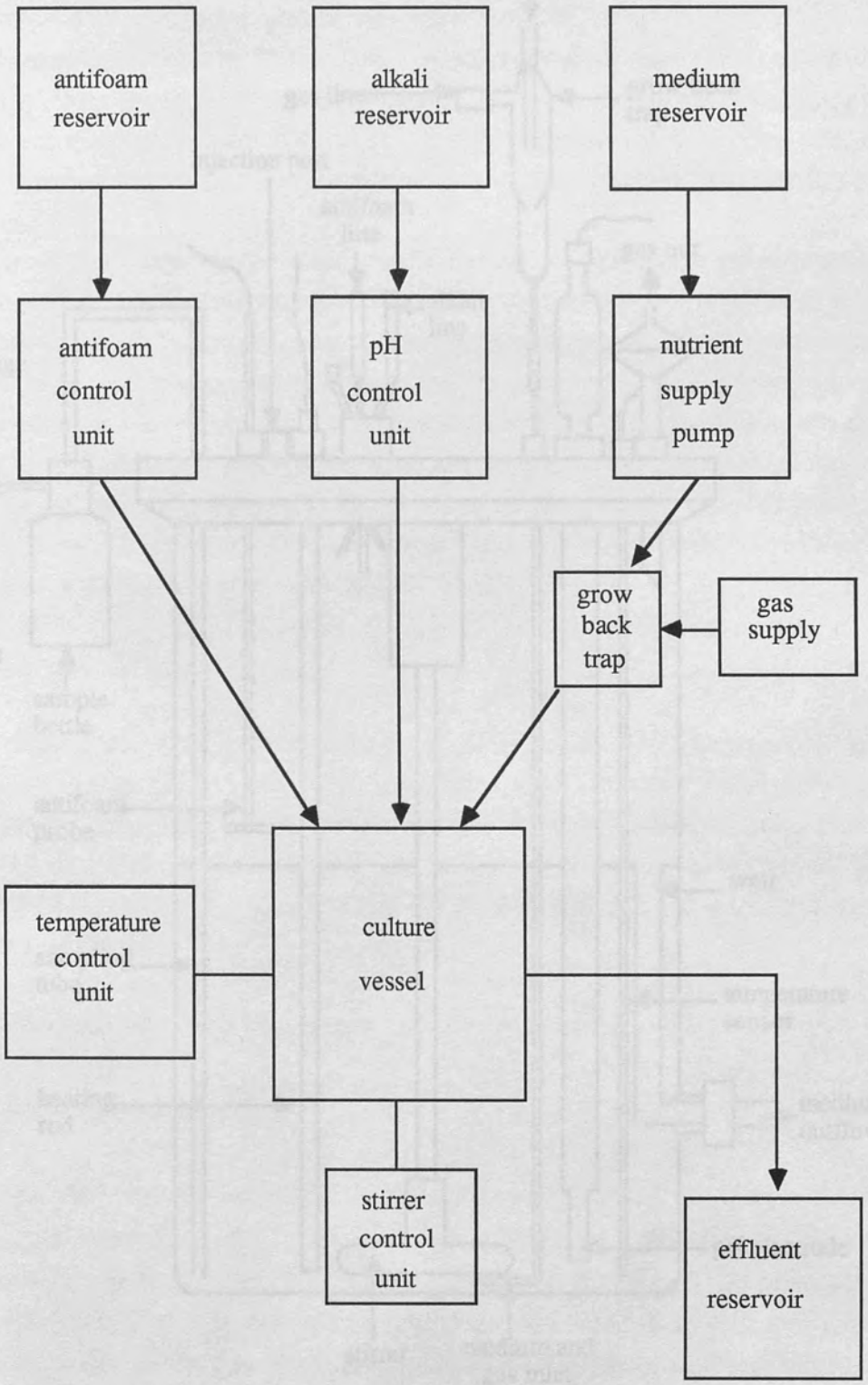
The system used was a LH Engineering 500 Series III modular fermenter of 500 ml working volume. The apparatus essentially consisted of a culture vessel, nutrient supply and drainage systems, a gas supply system and various control modules (stirrer, pH, temperature and antifoam units) (Figs 6 and 7).

Fresh medium (BM + 5 mM lactulose) was supplied to the culture vessel by means of a calibrated peristaltic pump (Watson-Marlow model 50L; tubing internal diameter of 1.6 mm) and the culture volume was kept constant by an overflow arrangement (weir). Actual flow rates were determined by measurement of the spent medium volume collected over a given period of time. A glass anti grow-back device was positioned between the medium pump and the culture vessel to prevent contamination of the nutrient reservoir.

Anaerobic conditions were maintained in the culture vessel by supply of N_2/CO_2 (97/3%) at a flow rate of approx. 2 ml/min. The gas supply was connected to the culture vessel via the anti grow-back tube and was sterilised by passage through a cotton wool filter. The L-shaped inlet pipe was directed towards the magnetic stirrer, in order to ensure immediate mixing of incoming gas and fresh medium.

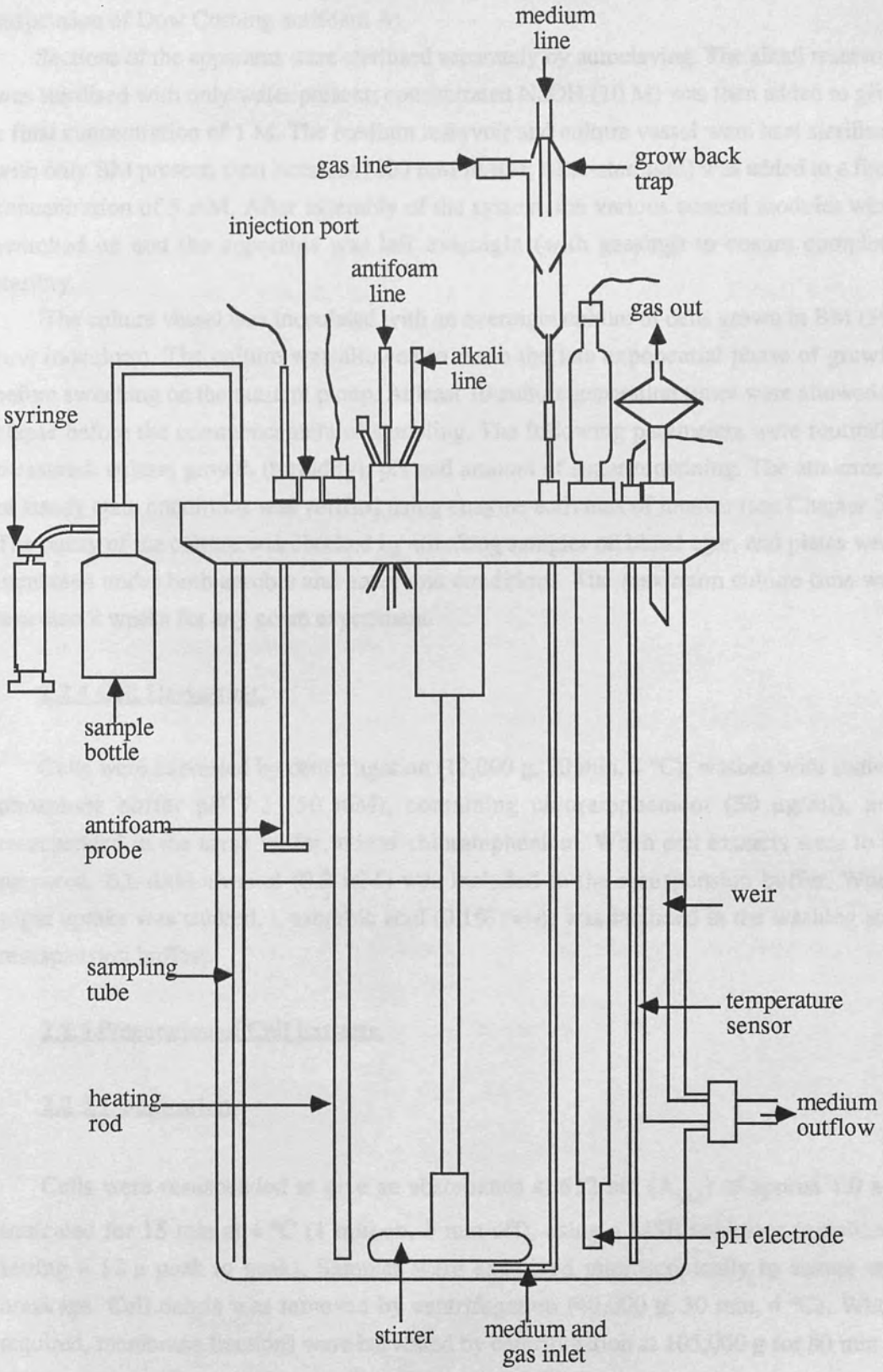
The magnetic drive unit was set at an agitation speed of 300 rpm, temperature was maintained at 37 °C and the culture pH set at 7.0 ± 0.1 by automatic addition of NaOH (1

Figure 6: General layout of the continuous culture apparatus.



Flow of gas and liquid additions is indicated by the arrows.

Figure 7: The continuous culture vessel.



Not to scale.

M). The actual pH of the culture was checked periodically using freshly removed samples. Foaming of the culture was controlled by the automatic addition of antifoam (20% (w/v) suspension of Dow Corning antifoam A).

Sections of the apparatus were sterilised separately by autoclaving. The alkali reservoir was sterilised with only water present; concentrated NaOH (10 M) was then added to give a final concentration of 1 M. The medium reservoir and culture vessel were heat sterilised with only BM present, then lactulose (200 mM in BM; filter-sterilised) was added to a final concentration of 5 mM. After assembly of the system, the various control modules were switched on and the apparatus was left overnight (with gassing) to ensure complete sterility.

The culture vessel was inoculated with an overnight culture of cells grown in BM (5% (v/v) inoculum). The culture was allowed to reach the late exponential phase of growth before switching on the nutrient pump. At least 10 culture generation times were allowed to elapse before the commencement of sampling. The following parameters were routinely measured: culture growth (turbidity), pH and amount of sugar remaining. The attainment of steady state conditions was verified using enzyme activities of interest (see Chapter 5). The purity of the culture was checked by streaking samples on blood agar, and plates were incubated under both aerobic and anaerobic conditions. The maximum culture time was less than 2 weeks for any given experiment.

2.2.4 Cell Harvesting.

Cells were harvested by centrifugation (12,000 **g**, 20 min, 4 °C), washed with sodium phosphate buffer pH 7.3 (50 mM), containing chloramphenicol (50 μg/ml), and resuspended in the same buffer, minus chloramphenicol. When cell extracts were to be prepared, D,L-dithiothreitol (0.2 mM) was included in the resuspension buffer. When sugar uptake was studied, L-ascorbic acid (0.1% (w/v)) was included in the washing and resuspension buffers.

2.2.5 Preparation of Cell Extracts.

2.2.5.1 Sonication.

Cells were resuspended to give an absorbance at 612 nm (A_{612}) of approx 1.0 and sonicated for 15 min at 4 °C (1 min on, 1 min off), using a MSE sonicator (amplitude setting = 12 μ peak to peak). Samples were examined microscopically to ensure cell breakage. Cell debris was removed by centrifugation (40,000 **g**, 30 min, 4 °C). When required, membrane fractions were harvested by centrifugation at 105,000 **g** for 80 min (4 °C).

2.2.5.2 Lysis treatment.

Lysis mixture consisted of:

Sodium dodecyl sulphate (10% (w/v))	1 vol
Manganous sulphate (0.02 M)	1 vol
Toluene	1 vol
2-Mercaptoethanol	5 vol

Lysis mixture (250 µl) was added to cells (1 ml; A_{612} of approx 1.0) and, after vortex mixing for 15 s, whole cells were removed by centrifugation (12,000 **g**, 20 min, 4 °C).

2.2.6 Growth Analyses.

2.2.6.1 Turbidity.

Culture growth was routinely monitored by measurement of the absorbance at 612 nm (A_{612}) .

2.2.6.2 Dry weight determination.

Cells were harvested by centrifugation in preweighed tubes and washed once with buffer. The resulting pellets were lyophilised and the tubes reweighed. Determinations were carried out in triplicate.

A calibration curve, relating turbidity to culture dry wt, was constructed using cultures grown in basal medium supplemented with lactose (20 mM) or lactulose (20 mM) (Fig. 8). This curve was routinely used for the calculation of dry wt, however, since the curve deviated from linearity at A_{612} >0.8 absorbance units, dense cultures (or cell suspensions) were diluted appropriately.

Culture growth rates were calculated using the formula:

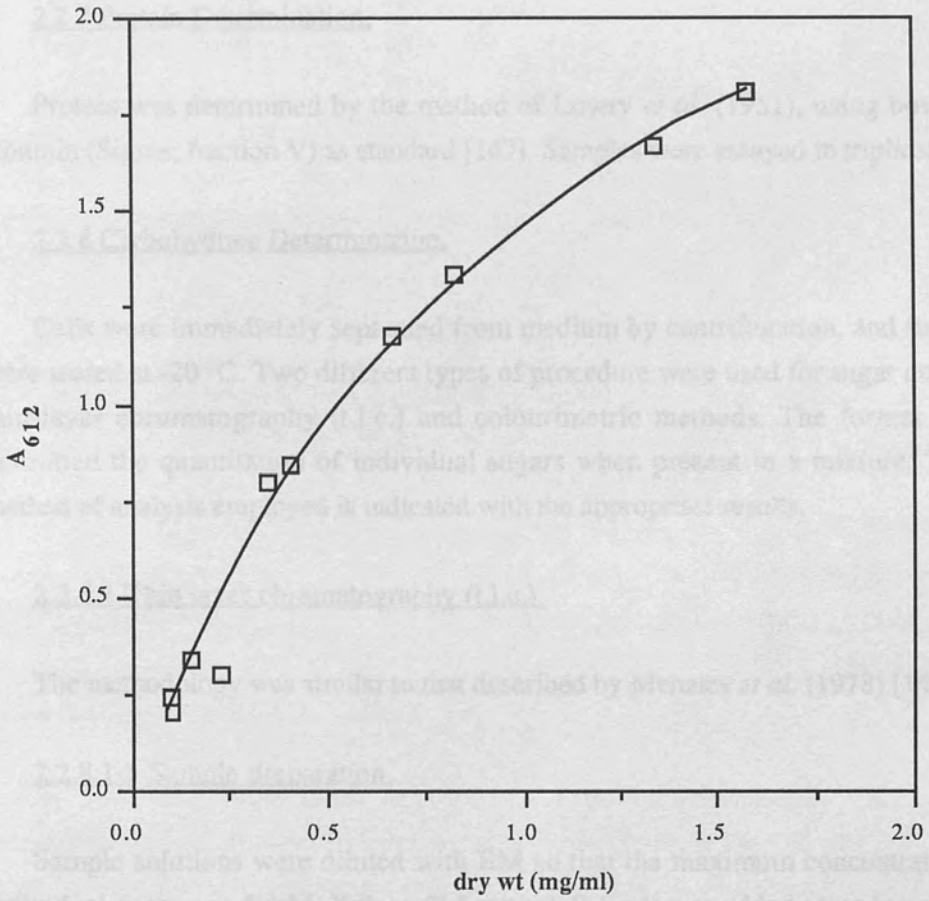
$$\mu = 2.303 \, (\log x_{t2} - \log x_{t1}) / (t_2 - t_1)$$

Where:

μ	is the specific growth rate (h^{-1}).
x_{t1} and x_{t2}	are dry wt contents (mg/ml) of the culture at times t_1 and t_2, respectively.

Plots of log dry wt versus time, during the exponential phase of growth, yielded straight lines of slope μ / 2.303. Mean generation times (MGT; units h) were calculated

Figure 8: Calibration curve relating culture growth (turbidity) to culture dry weight.



using the formula:

$MGT = 0.693 / \mu$.

2.2.6.3 Viable cell count.

Sterile BM was used for preparation of dilution tubes. Aliquots (0.1 ml) of diluted culture were spread onto blood agar plates (in triplicate) and immediately incubated to reduce the risk of cell death due to aerobiosis.

2.2.7 Protein Determination.

Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin (Sigma; fraction V) as standard [147]. Samples were assayed in triplicate.

2.2.8 Carbohydrate Determination.

Cells were immediately separated from medium by centrifugation, and supernatants were stored at -20 °C. Two different types of procedure were used for sugar quantitation: thin-layer chromatography (t.l.c.) and colourimetric methods. The former procedure permitted the quantitation of individual sugars when present in a mixture. The actual method of analysis employed is indicated with the appropriate results.

2.2.8.1 Thin layer chromatography (t.l.c.).

The methodology was similar to that described by Menzies *et al.* (1978) [148].

2.2.8.1.1 Sample preparation.

Sample solutions were diluted with BM so that the maximum concentration of any individual sugar was 5 mM. Xylose (7.5 mg/ml; 0.1 ml) was added as an internal marker to aliquots (0.9 ml) of samples, followed by Zerolit DMF (BDH; acetate form). The desalting resin was added so that it occupied approx. 60% of the combined volume and, after shaking for 3 min, samples were centrifuged (2000 g, 5 min). The clear supernatants were used for sample application.

Standard solutions (25, 50, 75,and 100% maximum sample concentration) were prepared in BM and treated as for samples.

2.2.8.1.2 Sample application.

Plastic-backed, silica gel plates (Schleicher and Schull, type F1500; 20x20 cm) were

cut into half and each portion was marked out to accommodate 12 applications (Fig. 9). Aliquots (2 µl) of samples or standards were applied as 1.1 cm uniform bands using a 5 µl Hamilton syringe. Each sample solution was applied in duplicate and plates were allowed to air dry prior to development.

2.2.8.1.3 Plate development.

Plates were rolled into cylinders (silica facing inwards), inserted into 250 ml beakers and developed in the following solvent system:

Ethyl acetate	60 ml
Pyridine	30 ml
Glacial acetic acid	10 ml
Water	10 ml

Each plate was subjected to two solvent developments and plates were allowed to dry in the fume cupboard for 30 min between runs. After the final development, plates were left in the fume cupboard overnight to remove any traces of pyridine.

2.2.8.1.4 Colour reaction.

The locating reagent was prepared by dissolving 4-aminobenzoic acid (7 g) in methanol (approx. 400 ml), to which was added orthophosphoric acid (specific gravity of 1.75; 17.5 ml) and the volume was made up to 500 ml with methanol.

A dipping chamber was used to apply the reagent to the plates. The colour was developed by heating in an oven at 120 °C for 10 min. The oven possessed a small motor attachment for rotation of plates, thereby allowing uniform colour development.

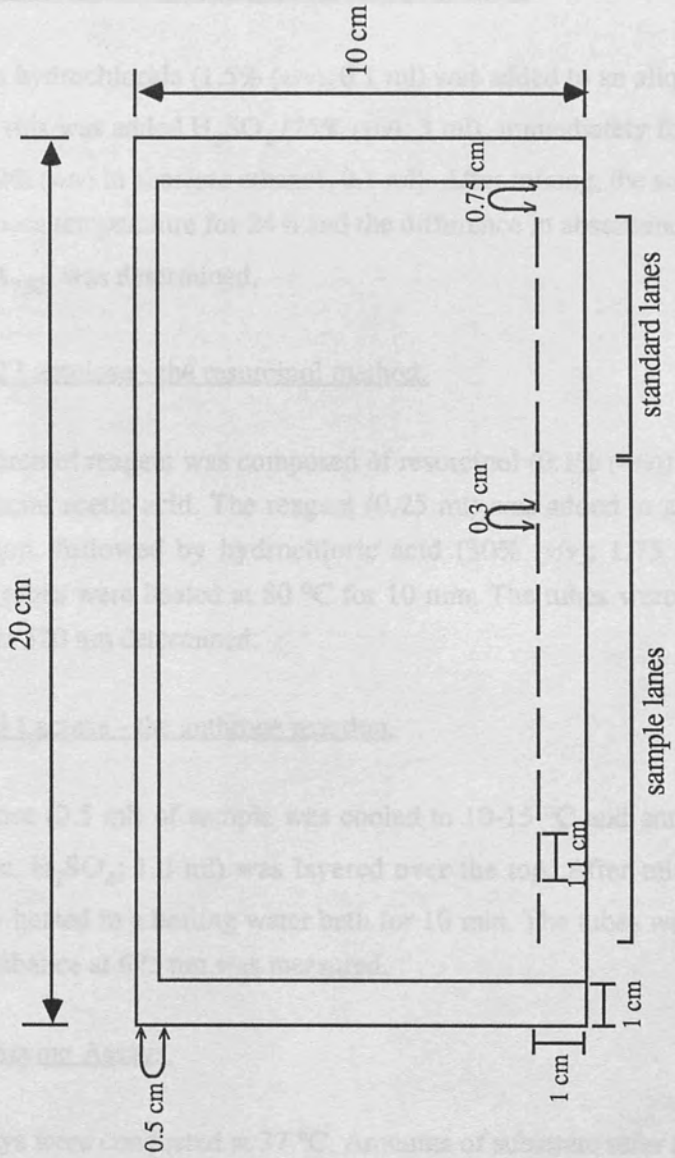
2.2.8.1.5 Sample quantitation.

A Joyce-Loebl chromoscan double-beam recording densitometer was used for zone measurement. Each chromatogram was cut into sample lanes and the machine was zeroed using a blank area of the chromatogram. Peak heights were determined from the scans and sample concentrations were calculated using calibration curves. These curves were constructed by plotting the peak height ratio versus sugar concentration, where:

peak height ratio = peak height of sugar (mm) / peak height of internal marker (mm)

The lowest sample concentration detected was 0.05 mM, which corresponded to a 1 mm peak height on the chart paper.

Figure 9: Preparation of the t.l.c. plate for sample application.



2.2.8.2 Colourimetric procedures.

The methods used were essentially the same as those described by Ashwell (1957) [149]. Standard curves (10-50 μg/ml sugar) were determined with each batch of samples, and assays were normally conducted in triplicate.

The cysteine-carbazole was initially used for lactulose determination, but because of the long incubation time (24 h) required for quantitative determination, this was replaced by the resorcinol method.

2.2.8.2.1 Lactulose - the cysteine-carbazole reaction.

Cysteine hydrochloride (1.5% (w/v); 0.1 ml) was added to an aliquot of sugar solution (0.5 ml). To this was added H_2SO_4 (75% (v/v); 3 ml), immediately followed by carbazole reagent (0.12% (w/v) in absolute ethanol; 0.1 ml). After mixing, the solutions were allowed to stand at room temperature for 24 h and the difference in absorbance at 560 nm and 750 nm (A_{560} - A_{750}) was determined.

2.2.8.2.2 Lactulose - the resorcinol method.

The resorcinol reagent was composed of resorcinol (0.1% (w/v)) and thiourea (0.25% (w/v)), in glacial acetic acid. The reagent (0.25 ml) was added to an aliquot (0.5 ml) of sugar solution, followed by hydrochloric acid (30% (v/v); 1.75 ml). After thorough mixing, the tubes were heated at 80 °C for 10 min. The tubes were then cooled and the absorbance at 520 nm determined.

2.2.8.2.3 Lactose - the anthrone reaction.

An aliquot (0.5 ml) of sample was cooled to 10-15 °C and anthrone reagent (0.2% (w/v) in conc. H_2SO_4; 1.0 ml) was layered over the top. After mixing, the tubes were immediately heated in a boiling water bath for 10 min. The tubes were then cooled in ice and the absorbance at 625 nm was measured.

2.2.9 Enzyme Assays.

All assays were conducted at 37 °C. Amounts of substrate refer to final concentration in the assay. One unit of enzyme activity is defined as the amount of enzyme required to catalyse the conversion of 1 μmol of substrate, per min, under the assay conditions specified. Details of linking enzymes are given in Table 9.

Table 9: Details of linking enzymes.

Trivial name	EC number	Source
Aldolase	4.1.2.13	rabbit muscle (type I)
Alkaline phosphatase	3.1.3.1	bovine intestinal mucosa (type VII-S)
β-Galactose dehydrogenase	1.1.1.48	*Pseudomonas fluorescens*
Glucose 6-phosphate dehydrogenase	1.1.1.49	baker's yeast (type VII)
α-Glycerophosphate dehydrogenase	1.1.1.8	rabbit muscle (type I)
Hexokinase	2.7.1.1	yeast (type C130)
Phosphoglucomutase	2.7.5.1	rabbit muscle
Phosphoglucose isomerase	5.3.1.9	yeast (type III)
Triose phosphate isomerase	5.3.1.1	rabbit muscle (type III)

All enzymes were purchased from the Sigma Chemical Co. (London) Ltd.

2.2.9.1 β-Galactosidase (EC 3.2.1.23).

The assay used for β-galactosidase was that described in a Sigma product information sheet (product number G-6008). Assays (1.5 ml) contained:

Sodium phosphate buffer pH 7.3	87 mM
Magnesium chloride (hexahydrate)	1 mM
2-Mercaptoethanol	112 mM
Cell extract	0.1 ml
o-nitrophenyl-β-D-galactopyranoside (ONPG)	2.27 mM

The assay mixture was preincubated for 3 min at 37 °C, prior to ONPG addition, and the subsequent increase in absorbance at 410 nm was measured.

2.2.9.2 Phospho-β-galactosidase (EC 3.2.1.85).

Assays (0.75 ml) were conducted in microcuvettes, and were essentially the same as for β-galactosidase except that ONPG was replaced with ONPG 6-phosphate (di(cyclohexylamine) salt).

2.2.9.3 Galactokinase (EC 2.7.1.6).

The assay used was adapted from the method of Dey (1980) [150]. Assays (0.2 ml) contained:

Sodium phosphate buffer pH 7.3	30 mM
Bovine serum albumin	1.5 mg
2-Mercaptoethanol	3 mM
Magnesium chloride (hexahydrate)	1.5 mM
Sodium fluoride	3.75 mM
D-[1-^{14}C]-Galactose	0.2 μCi; 0.5 mM
ATP (disodium salt)	2.5 mM
Cell extract	50 μl

A sampling manifold (Millipore, model 1225), which could accommodate up to 12 discs, aided sample processing. Aliquots (20 μl) of incubation mixture were removed at time intervals (normally every 4 min, up to a maximum of 20 min) and spotted onto cellulose discs (Whatman, type DE81). Each disc was immediately washed with water (200 ml), dried and then prepared for liquid scintillation counting (section 2.2.10). Control incubations (minus ATP) served as assay blanks.

2.2.9.4 Galactose 1-phosphate uridylyl transferase (EC 2.7.7.12).

The procedure of Bisset and Anderson was followed [101]. Assays (0.72 ml) contained:

Glycylglycine buffer pH 7.5	62.5 mM
Magnesium chloride (hexahydrate)	6.94 mM
Galactose 1-phosphate (dipotassium salt)	0.69 mM
UDPglucose (UDPG; sodium salt)	0.69 mM
$NADP^+$ (sodium salt)	0.69 mM
Cell extract	50 µl

Nonlimiting glucose 6-phosphate dehydrogenase and phosphoglucomutase.

The reaction was started by the addition of UDPG and the absorbance at 340 nm monitored. Blanks were minus UDPG or galactose 1-phosphate.

2.2.9.5 UDPgalactose 4-epimerase (EC 5.1.3.2).

Assays (1.0 ml) were conducted using the method of Maxwell (1957) [151] and contained:

Glycine buffer pH 8.9	0.1 M
NAD^+	1 mM
Cell extract	50 µl
UDPgalactose (UDPgal; sodium salt)	0.3 mM

Nonlimiting UDPG dehydrogenase (see section 2.2.9.6.1).

The UDPgal was added to start the reaction and changes in the absorbance at 340 nm followed. Blanks were minus UDPgal.

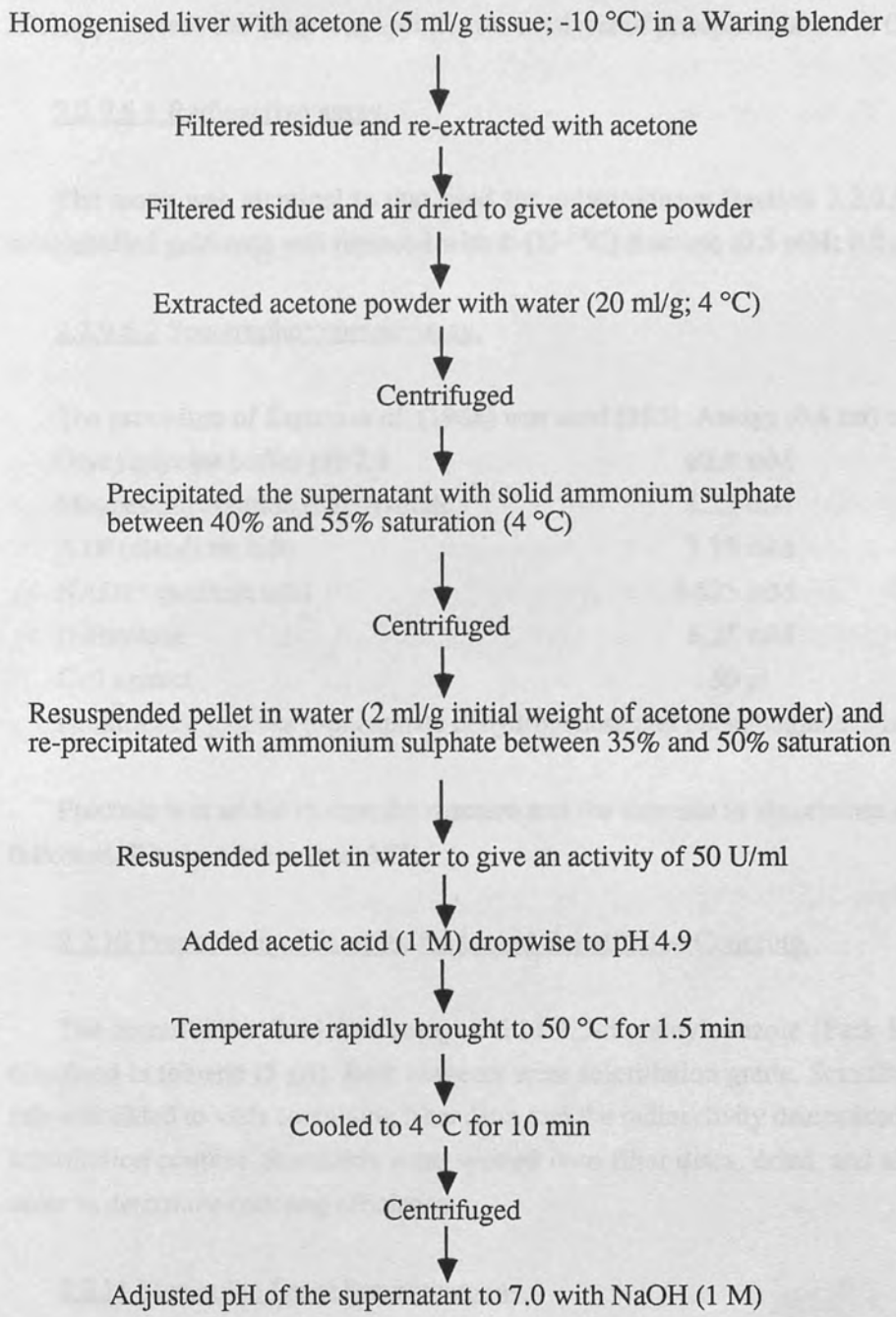
2.2.9.5.1 Preparation of UDPG dehydrogenase.

The enzyme was prepared from calf liver using a published procedure (Fig. 10) [152]. The final preparation was found to be free of any UDPgalactose 4-epimerase activity and was stable when stored at -20 °C for up to 1 month. The enzyme was assayed using the method of Strominger *et al.* (1957) [152]. Assays (1.5 ml) contained:

Glycine buffer pH 8.7	0.1 M
NAD^+	1 mM
UDPG (sodium salt)	0.2 mM
Cell extract	50 µl

The UDPG was added to start the reaction and the increase in absorbance at 340 nm followed.

Figure 10: Preparation of UDPG dehydrogenase from calf liver.



All centrifugation steps were at 9000g for 20 min (4 °C)

1 U of activity is the amount of enzyme required to convert 1 µmol substrate/min.

2.2.9.6 Fructokinase.

Radioactive and spectrophotometric procedures were employed. The former permitted the assay of an enzyme catalysing phosphorylation at positions C-1 (ATP:D-fructose 1-phosphotransferase; EC 2.7.1.3) or C-6 (ATP:D-fructose 6-phosphotransferase; EC 2.7.1.4), whereas the latter was specific for catalysis of phosphorylation at C-6.

2.2.9.6.1 Radioactive assay.

The assay was identical to that used for galactokinase (section 2.2.9.4) except that radiolabelled galactose was replaced with D-[U-^{14}C]-fructose (0.5 mM; 0.2 μCi).

2.2.9.6.2 Spectrophotometric assay.

The procedure of Sapico *et al.* (1968) was used [153]. Assays (0.8 ml) contained:

Glycylglycine buffer pH 7.5	62.5 mM
Magnesium chloride (hexahydrate)	6.25 mM
ATP (disodium salt)	3.13 mM
$NADP^+$ (sodium salt)	0.625 mM
D-Fructose	6.25 mM
Cell extract	50 μl

Nonlimiting glucose 6-phosphate dehydrogenase and phosphoglucose isomerase.

Fructose was added to start the reaction and the increase in absorbance at 340 nm was followed. Blanks were minus ATP.

2.2.10 Preparation of Samples for Liquid Scintillation Counting.

The scintillation fluid was composed of 2,5-diphenyloxazole (Park Scientific Ltd) dissolved in toluene (5 g/l). Both reagents were scintillation grade. Scintillation fluid (10 ml) was added to vials containing filter discs and the radioactivity determined using a liquid scintillation counter. Standards were spotted onto filter discs, dried, and also counted in order to determine counting efficiency.

2.2.11 Metabolite Level Determinations.

Metabolite levels were determined fluorimetrically using enzymes, or a series of enzymes, linked to the formation or degradation of NAD(P)H. A Locarte single-sided fluorimeter (Model Mk 4) with an attached chart recorder was used. Excitation and emission wavelengths were set at 340-380 nm and 440 nm, repectively, by the use of filters.

2.2.11.1 Extraction of metabolites.

Cells were collected by rapid filtration onto Millipore filters (47 mm diameter, 0.8 μm pore size) and the filters were immediately immersed in perchloric acid (0.6 N; 10 ml) at 4 °C. Approx. 20 mg dry wt cells were collected in total. After 20 min, precipitated protein was removed by centrifugation (20,000 **g**, 10 min, 4 °C) and the extract was neutralised with KOH (1 M). The potassium perchlorate precipitate was removed by a further centrifugation step (20,000 **g**, 10 min, 4 °C), and the supernatant was then concentrated by lyophilisation and resuspension in distilled water (1 ml).

2.2.11.2 Assay procedures.

The imidazole used in the preparation of buffers was of fluorimetric grade (Sigma). The change in fluorescence due to the addition of enzyme was taken into account when calculating results. Thus, a second amount of enzyme was added to a series of samples after the appropriate reaction was complete, and the mean background increase was determined. For a change in fluorescence to represent a real result, it had to be greater than the fluorescence change due to enzyme alone plus 3x the standard deviation of this background increase. Standards were assayed with each batch of samples, and metabolite concentrations were calculated from standard curves. All assays were conducted at 25 °C. Details of linking enzymes are given in Table 9, and amounts of substrates in assays refer to final concentrations.

2.2.11.2.1 Galactose, galactose 1-phosphate and galactose 6-phosphate.

Fluorimetric determination of galactose and its phosphates was performed using a modified procedure of that described by Thomas *et al.* (1980) [154]. Assays (1.2 ml) contained:

Imidazole/HCl buffer pH 7.5	50 mM
NAD^+	10 μM
Cell extract	50-100 μl

Sequential addition of β-galactose dehydrogenase (0.25 U) and alkaline phosphatase (1 U) permitted the determination of free galactose and total galactose phosphate (galactose 1-phosphate + galactose 6-phosphate), respectively.

The galactose phosphate species present was determined on the basis of acid lability [155]. Cell extracts were hydrolysed in HCl (0.1 M) (0.9 ml cell extract + 0.1 ml 1 M HCl) at 100 °C for 15 min and then neutralised with NaOH (0.1 M). Galactose 1-phosphate is completely hydrolysed under these conditions, whereas galactose 6-phosphate is stable. The acid hydrolysed samples were then assayed as described above. Any fluorescence change observed in the presence of galactose dehydrogenase will originate from both free intracellular galactose and acid labile galactose 1-phosphate. In

contrast, any fluorescence change observed upon the subsequent addition of alkaline phosphatase will result from acid stable galactose 6-phosphate.

2.2.11.2.2 Fructose, fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate.

The assay procedure was based on the method of Thompson (1979) [118]. Two separate assays were performed on each sample; one allowed the determination of the sugar phosphates only, whereas the other permitted the determination of free fructose plus fructose 6-phosphate. Assays (1.85 ml) contained:

Imidazole/HCl buffer pH 7.5	60 mM
Magnesium chloride (hexahydrate)	6 mM
$NADP^+$ (sodium salt)	6 μM
Cell extract	100 μl

Sequential addition of glucose 6-phosphate dehydrogenase (4 U), phosphoglucose isomerase (4 U) and phosphoglucomutase (4 U) permitted quantitation of glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate, respectively.

The assay was then repeated in the presence of ATP (disodium salt; 6 μM), hexokinase (4.4 U) and glucose 6-phosphate dehydrogenase (4 U). Fructose plus fructose 6-phosphate were determined from the fluorescence change after addition of phosphoglucose isomerase (4 U), and free fructose was calculated after correction for fructose 6-phosphate levels.

2.2.11.2.3 Fructose 1,6-bisphosphate (FDP), dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P).

The procedure of Collins and Thomas (1974) was used [156]. Assays (1.64 ml) contained:

Imidazole/HCl buffer pH 7.0	64 mM
NADH (disodium salt)	6.4 mM
Cell extract	100 μl

Sequential addition of α-glycerophosphate dehydrogenase (0.4 U), triose phosphate isomerase (1.2 U) and FDP aldolase (0.6 U) allowed the determination of DHAP, GA3P and FDP, respectively.

2.2.12 Assay of Sugar Utilisation in a Buffered Incubation System.

Exponential phase cells were harvested, washed twice with sodium phosphate buffer pH 7.0 (50 mM), containing L-ascorbic acid (0.1% (w/v)) and resuspended in this buffer to yield a dense suspension (approx 3.8 mg dry wt/ml or $5x10^9$ cells/ml). Ascorbate was included in buffers in order to maintain a suitable oxido-reduction potential [84] and, when

necessary, cell suspensions were stored at 4 °C under N_2/CO_2 (95/5%), but only for brief periods.

Cell suspensions (6 ml) were incubated with sugar solution (8.76 mM in resuspension buffer; 3 ml) at 37 °C, under an atmosphere of N_2/CO_2 (95/5%). Samples were removed periodically (usually over a 1 h period, see Chapter 4) and, after cooling in an ice bath, were immediately centrifuged (2000 g, 20 min, 4 °C) to remove cells. Supernatants were diluted 1:20 in water and stored at -20 °C prior to sugar assay. Lactulose was assayed by the resorcinol method (section 2.2.8.2.2), and lactose was assayed by the anthrone reaction (section 2.2.8.2.3).

The inhibitors (carbonyl)N,N'-dicyclohexylcarbodiimide (DCCD) and cyanide *m*-chlorophenylhydrazone (CCCP) were dissolved in absolute ethanol, and aliquots (45 µl) were added to incubations so that the final ethanol concentration was 0.5% (v/v). Details of inhibitor concentrations are given in the text (Chapter 4).

2.2.13 Protoplast Formation.

Formation of *Cl. perfringens* protoplasts was attempted with lysozyme (mucopeptide N-acetylmuramyl-hydrolase; EC 3.2.1.17; Sigma grade I from chick egg white), using the method of Chassy and Giuffrida (1980) [157]. Cells were washed with, and then resuspended in, Tris/HCl buffer pH 8.2 (10 mM), to give an A_{612} value of approx. 1.0. Incubation mixes contained the following components in buffer:

Cell suspension	1 vol
Osmotic stabiliser	2 vol
Lysozyme solution	1 vol

The osmotic stabiliser was polyethylene glycol (PEG; Carbowax 20,000; 24% (w/v)). The amount of lysozyme added varied from experiment to experiment (see Chapter 4) and was normally calculated as a function of the bacterial dry wt content of incubations. Incubations were performed at 37 °C, under anaerobic conditions (N_2/CO_2, 95/5%) and samples were removed at intervals for examination by phase-contrast microscopy (magnification x200). The % population changed in morphology was estimated after observation of 3 random fields. If changes were less than 10%, values were approximated to the nearest 1%, whereas when changes were greater than this value they were approximated to the nearest 10%. These studies were therefore only semi-quantitative.

RESULTS

CHAPTER 3: PATHWAYS OF INTERMEDIARY METABOLISM

3.1 Introduction.

Metabolism of disaccharides normally proceeds via inducible pathways in batch cultures of bacteria. In the case of lactose, correlations exist between the sugar transport mechanism, the galactohydrolase responsible for catalysing the first step in intracellular metabolism and the subsequent metabolic pathway of the galactosyl moiety (Introduction, section 1.6). Thus, transport of lactose by a non-PTS system is normally associated with high levels of β-galactosidase and Leloir pathway enzymes, whereas transport of lactose by a PTS system normally correlates with high levels of phospho-β-galactosidase and tagatose 6-phosphate pathway enzymes.

Determination of the uptake mechanism would therefore prove a useful starting point in identifying the possible pathways of lactulose metabolism. However, assay of sugar transport normally requires a radioactive substrate and, since radiolabelled lactulose was not available for this project, an alternative procedure needed to be developed. Instead, the pathways of lactulose metabolism in *Cl. perfringens* were initially investigated by assay of both enzymes and metabolites of possible catabolic pathways.

3.2 Results.

3.2.1 Assay of Enzyme Activities.

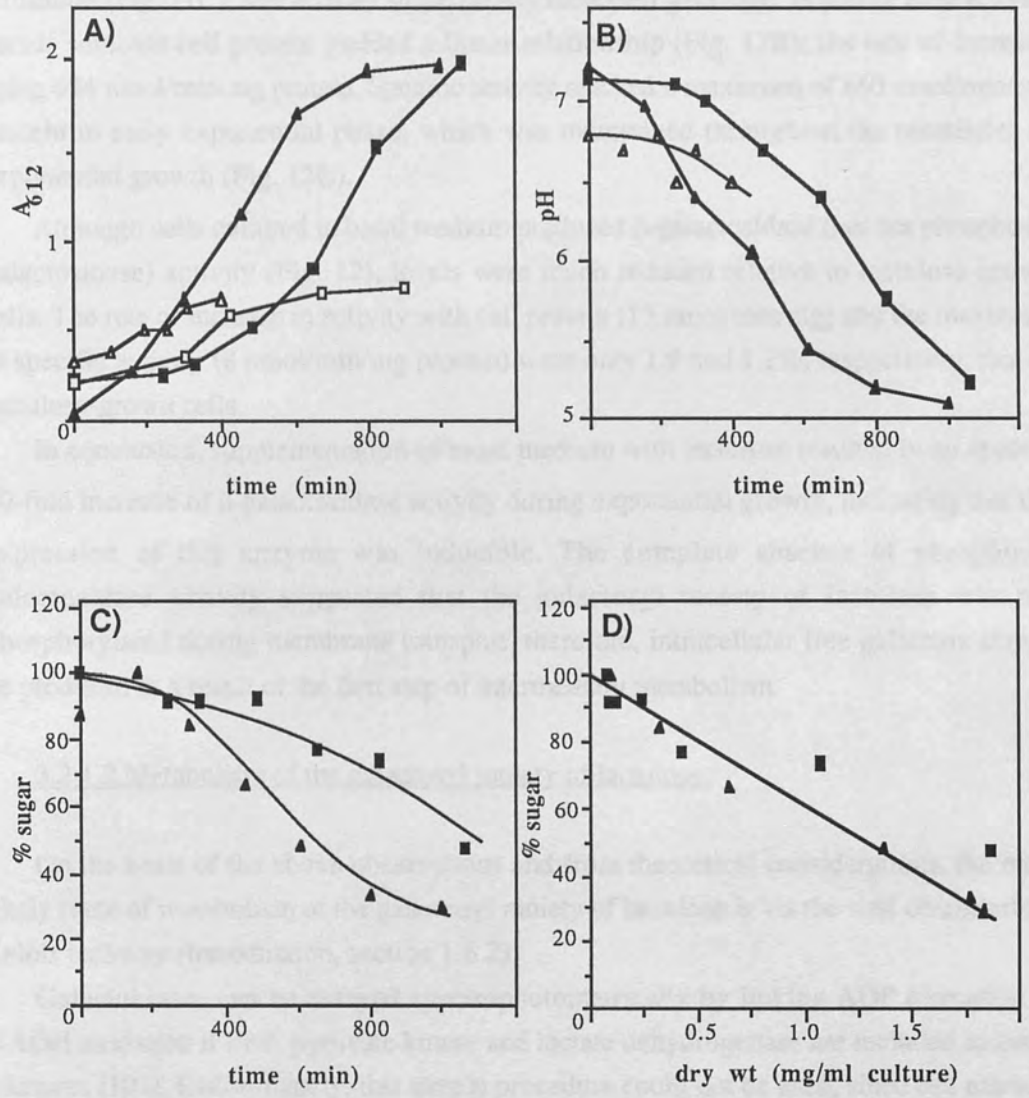
3.2.1.1 Identity of the galactohydrolase.

Batch cultures of *Cl. perfringens* were grown in basal medium alone or basal medium supplemented with lactulose (20 mM). Samples were periodically removed and the following parameters determined: growth (A_{612}), culture pH, medium lactulose concentration (by t.l.c.; Materials and Methods, section 2.2.8.1), β-galactosidase and phospho-β-galactosidase activities. Enzyme assays were performed using the chromogenic substrates ONPG and ONPG 6-phosphate (Materials and Methods, sections 2.2.9.1 and 2.2.9.2, respectively), and cell extracts were prepared by sonication (Materials and Methods, section 2.2.5.1).

Cells grew considerably faster in medium supplemented with lactulose (culture LL1) than unsupplemented medium (culture BM1) (Fig. 11A). Mean generation times (MGT) of 2.54 h and 4.78 h, respectively, were calculated from log dry wt versus time plots (Materials and Methods, section 2.2.6.2; data not shown). Growth in lactulose-containing medium coincided with the initiation of lactulose utilisation (Fig. 11C) and acidification of the culture medium (Fig. 11B). Lactulose was the only carbohydrate detected by t.l.c. in all samples; suggesting the absence of any significant mechanism of extracellular cleavage of the disaccharide or excretion of the constituent monosaccharides.

Growth in the presence of lactulose resulted in the production of β-galactosidase

Figure 11: Growth, medium pH and sugar utilisation profiles of cells cultured in basal medium alone or basal medium supplemented with lactulose (20 mM).



Growth media:

■ - basal medium + lactulose (culture LL1).

▲ - basal medium + lactulose (culture LL2).

△ - basal medium (culture BM2).

□ - basal medium (culture BM1).

activity (Fig. 12), whereas phospho-β-galactosidase could not be detected at any stage of culture growth, from lag to late exponential phase. In a separate experiment (culture LL2), samples were also taken in stationary phase and phospho-β-galactosidase activity was still undetectable.

The appearance of β-galactosidase (Fig. 12A) coincided with the initiation of lactulose utilisation (Fig. 11C), and activity subsequently increased with time. A plot of total activity versus sonicant cell protein yielded a linear relationship (Fig. 12B); the rate of increase being 694 nmol/min/mg protein. Specific activity reached a maximum of 660 nmol/min/mg protein in early exponential phase, which was maintained throughout the remainder of exponential growth (Fig. 12C).

Although cells cultured in basal medium produced β-galactosidase (but not phospho-β-galactosidase) activity (Fig. 12), levels were much reduced relative to lactulose-grown cells. The rate of increase in activity with cell protein (13 nmol/min/mg) and the maximum in specific activity (8 nmol/min/mg protein) were only 1.9 and 1.2%, respectively, that of lactulose-grown cells.

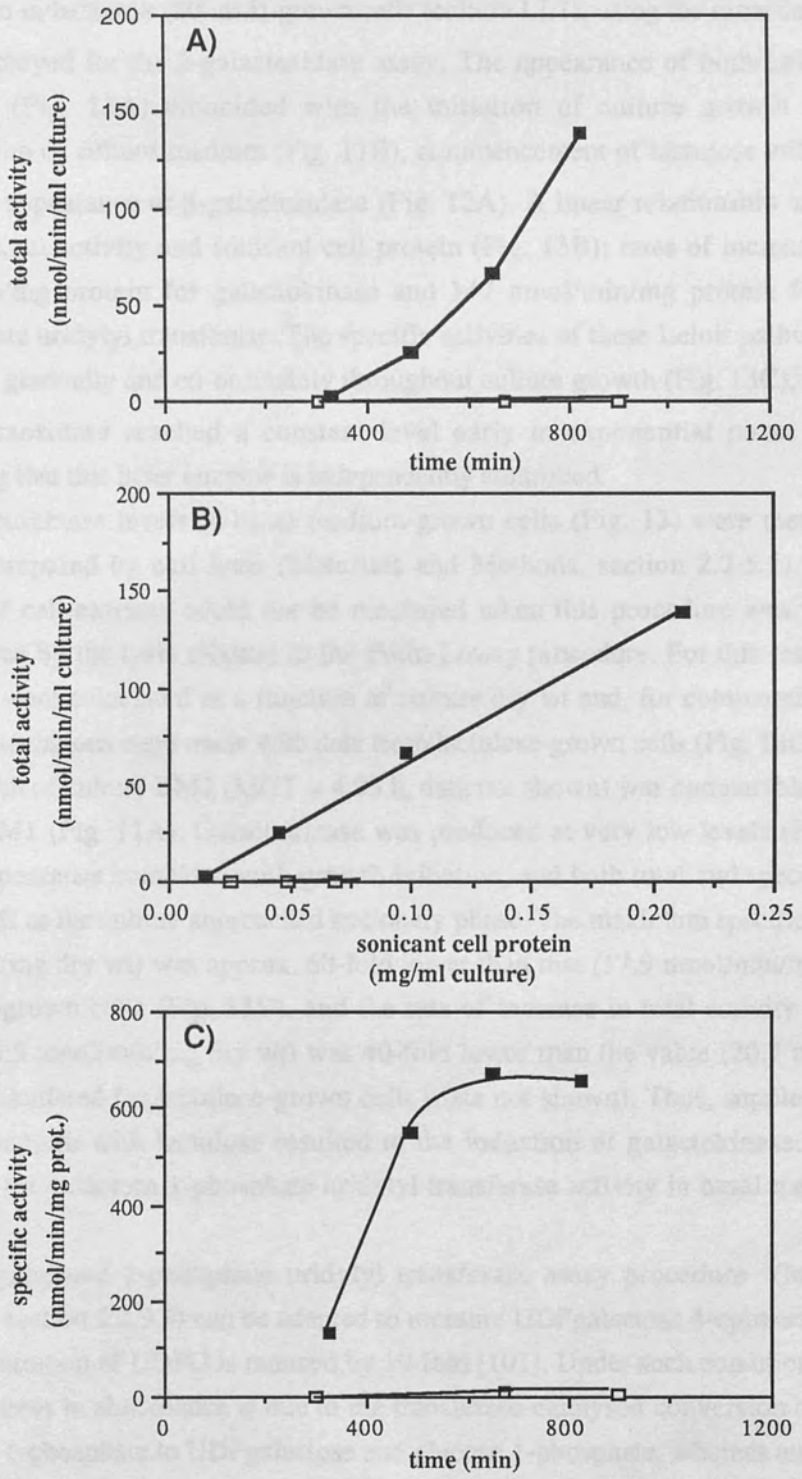
In conclusion, supplementation of basal medium with lactulose resulted in an approx. 50-fold increase of β-galactosidase activity during exponential growth, indicating that the expression of this enzyme was inducible. The complete absence of phospho-β-galactosidase activity suggested that the galactosyl moiety of lactulose was not phosphorylated during membrane transport, therefore, intracellular free galactose should be produced as a result of the first step of intermediary metabolism.

3.2.1.2 Metabolism of the galactosyl moiety of lactulose.

On the basis of the above observations and from theoretical considerations, the most likely route of metabolism of the galactosyl moiety of lactulose is via the well characterised Leloir pathway (Introduction, section 1.6.2).

Galactokinase can be assayed spectrophotometrically by linking ADP formation to NADH oxidation if PEP, pyruvate kinase and lactate dehydrogenase are included in assay mixtures [101]. Unfortunately, this simple procedure could not be used, since cell extracts of *Cl. perfringens* contained significant NADH oxidase activity. In a preliminary experiment, exponential phase, lactulose (20 mM)-grown cells were found to contain an activity of approx. 90 nmol/min/mg protein, and dilution of cell extracts, in order to reduce this background reaction, resulted in undetectable galactokinase activity. Previous attempts by other investigators to remove NADH oxidase activity have proved unsuccessful [145], therefore, an alternative, non-spectrophotometric method was employed. This procedure measures the formation of galactose phosphate from [1-^{14}C]-galactose and ATP (Materials and Methods, section 2.2.9.3). Galactose 1-phosphate uridylyl transferase activity was assayed by a spectrophotometric procedure, using $NADP^+$-dependent glucose 6-phosphate dehydrogenase as a linking enzyme (Materials and Methods, section 2.2.9.4), since no

Figure 12: βGalactosidase activity in cells cultured in basal medium alone or basal medium supplemented with lactulose (20 mM).



Growth media:

■ - basal medium + lactulose (20 mM) (culture LL1)

□ - basal medium (culture BM1).

interference from NADH oxidase was observed.

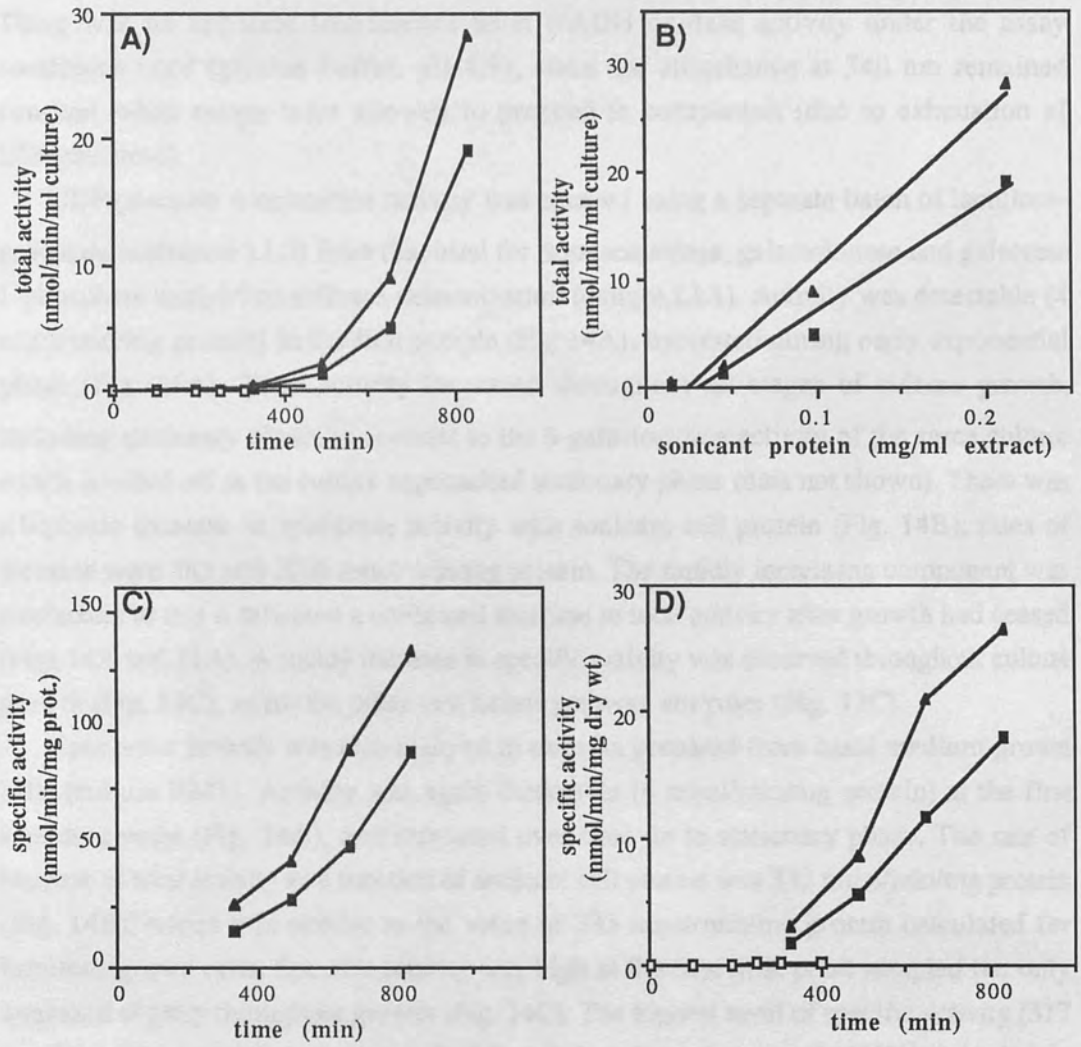
Galactokinase and galactose 1-phosphate uridylyl transferase activities were determined in lactulose (20 mM)-grown cells (culture LL1), using the same cell extracts as those employed for the β-galactosidase assay. The appearance of both Leloir pathway activities (Fig. 13A) coincided with the initiation of culture growth (Fig. 11A), acidification of culture medium (Fig. 11B), commencement of lactulose utilisation (Fig. 11C) and appearance of β-galactosidase (Fig. 12A). A linear relationship was observed between total activity and sonicant cell protein (Fig. 13B); rates of increase were 99.3 nmol/min/mg protein for galactokinase and 147 nmol/min/mg protein for galactose 1-phosphate uridylyl transferase. The specific activities of these Leloir pathway enzymes increased gradually and co-ordinately throughout culture growth (Fig. 13C), whereas that of β-galactosidase reached a constant level early in exponential phase (Fig. 12C), suggesting that this latter enzyme is independently controlled.

Galactokinase levels in basal medium-grown cells (Fig. 13) were measured using extracts prepared by cell lysis (Materials and Methods, section 2.2.5.2). The protein content of cell extracts could not be measured when this procedure was used, due to interference by the lysis mixture in the Folin-Lowry procedure. For this reason, specific activities were calculated as a function of culture dry wt and, for comparative purposes, similar calculations were made with data from lactulose-grown cells (Fig. 13D).

Growth of culture BM2 (MGT = 4.95 h, data not shown) was comparable with that of culture BM1 (Fig. 11A). Galactokinase was produced at very low levels (Figs 13A and D), its appearance coincided with growth initiation, and both total and specific activities levelled off as the culture approached stationary phase. The maximum specific activity (0.3 nmol/min/mg dry wt) was approx. 60-fold lower than that (17.9 nmol/min/mg dry wt) of lactulose-grown cells (Fig. 13D), and the rate of increase in total activity with culture growth (0.5 nmol/min/mg dry wt) was 40-fold lower than the value (20.7 nmol/min/mg dry wt) calculated for lactulose-grown cells (data not shown). Thus, supplementation of culture medium with lactulose resulted in the induction of galactokinase. No data are available for galactose 1-phosphate uridylyl transferase activity in basal medium-grown cells.

The galactose 1-phosphate uridylyl transferase assay procedure (Materials and Methods, section 2.2.9.4) can be adapted to measure UDPgalactose 4-epimerase activity if the concentration of UDPG is reduced by 10-fold [101]. Under such conditions, any initial rapid increase in absorbance is due to the transferase-catalysed conversion of UDPG and galactose 1-phosphate to UDPgalactose and glucose 1-phosphate, whereas any subsequent increase in absorbance is dependent upon the epimerase-catalysed reconversion of UDPgalactose to UDPG [101]. Use of this procedure is only applicable under conditions when transferase activity is not rate limiting. Indeed, levels of epimerase and transferase activity were found to be similar when the assay procedure was performed on lactulose (20 mM)-grown cells of *Cl. perfringens*. In total, assays were performed on 6 different cell extracts and epimerase activity was found to be 73.9 ± 15.0% (mean ± standard deviation)

Figure 13: Galactokinase and galactose 1-phosphate uridylyl transferase activities in cells cultured in basal medium ± lactulose (20mM).



Enzymes

■ galactokinase

▲ galactose 1-phosphate uridylyl transferase.

Closed symbols - lactulose (20 mM)-grown cells

Open symbols - basal medium-grown cells

that of transferase activity (data not shown).

It was therefore preferable to use an alternative assay system, independent of galactose 1-phosphate uridylyl transferase activity. This procedure (Materials and Methods, section 2.2.9.5) employed NAD^+-dependent UDPG dehydrogenase activity as the linking enzyme. There was no apparent interference from NADH oxidase activity under the assay conditions used (glycine buffer, pH 8.9), since the absorbance at 340 nm remained constant when assays were allowed to proceed to completion (due to exhaustion of UDPgalactose).

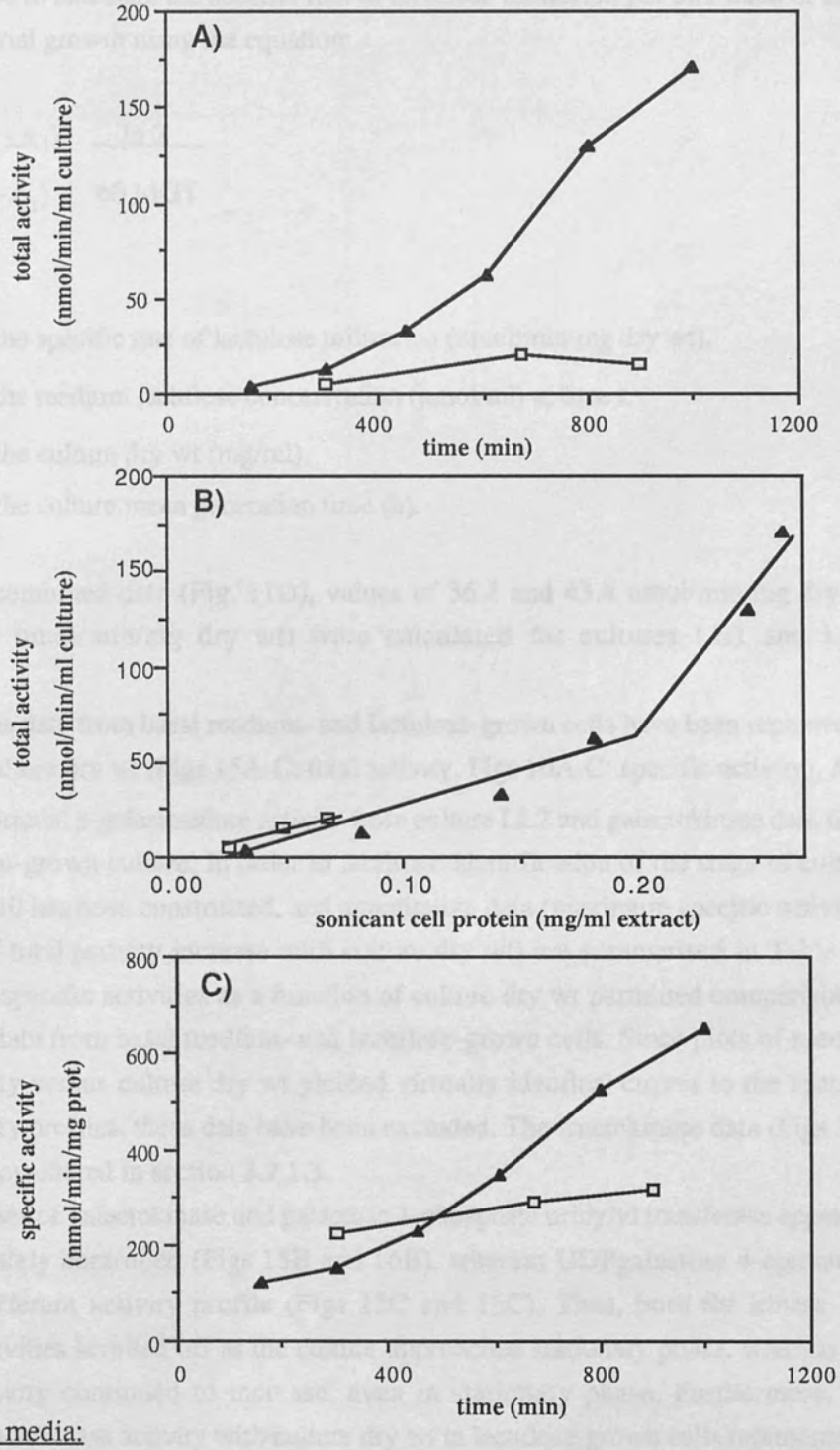
UDPgalactose 4-epimerase activity was assayed using a separate batch of lactulose-grown cells (culture LL2) from that used for β-galactosidase, galactokinase and galactose 1-phosphate uridylyl transferase determination (culture LL1). Activity was detectable (4 nmol/min/mg protein) in the first sample (Fig 14A), harvested during early exponential phase (Fig. 11A). Total activity increased throughout all stages of culture growth, including stationary phase, in contrast to the β-galactosidase activity of the same culture which levelled off as the culture approached stationary phase (data not shown). There was a biphasic increase in epimerase activity with sonicant cell protein (Fig. 14B); rates of increase were 383 and 2000 nmol/min/mg protein. The rapidly increasing component was artefactual in that it reflected a continued increase in total activity after growth had ceased (Figs 14A and 11A). A steady increase in specific activity was observed throughout culture growth (Fig. 14C), as for the other two Leloir pathway enzymes (Fig. 13C).

Epimerase activity was also assayed in extracts prepared from basal medium-grown cells (culture BM1). Activity was again detectable (6 nmol/min/mg protein) at the first sampling point (Fig. 14A), and increased over time up to stationary phase. The rate of increase in total activity as a function of sonicant cell protein was 332 nmol/min/mg protein (Fig. 14B), which was similar to the value of 383 nmol/min/mg protein calculated for lactulose-grown cells. Specific activity was high at the first time point sampled but only increased slightly throughout growth (Fig. 14C). The highest level of specific activity (317 nmol/min/mg protein) was approx. half that in lactulose-grown cells (653 nmol/min/mg protein).

It should be noted that differences existed between the growth characteristics of cultures LL1 and LL2 (Fig. 11A). The lag period of culture LL2 (160 min) was much shorter than that of culture LL1 (350 min), and differences also existed between growth rates; MGT were 2.13 h and 2.54 h, respectively (data not shown). The reasons for these differences are unclear, but one possible factor may have been the physiological state of the inoculum. The inoculum used for culture LL1 was taken from a stationary phase culture, whereas that for culture LL2 was taken from an exponential phase culture. Other possible explanations include: variation in the initial state of anaerobiosis of the culture media and slight differences in composition between batches of basal medium.

Due to the growth differences, it followed that comparison of the enzyme data on a time basis would be invalid. However, consideration of the data as function of culture growth (dry wt) should remedy the discrepancy. In order to illustrate this point, sugar

Figure 14: UDPgalactose 4-epimerase activity in cells cultured in basal medium ± lactulose (20 mM).



Growth media:

▲ basal medium + lactulose (20 mM) (culture LL2)

□ basal medium (culture BM1)

utilisation data from cultures LL1 and LL2 have been plotted together as a function of time (Fig. 11C) and dry wt (Fig. 11D).

It is possible to calculate the specific rate of lactulose utilisation per unit mass of cells during exponential growth using the equation:

$$q_{LL} = \frac{(s_{t2} - s_{t1})}{(x_{t2} - x_{t1})} \cdot \frac{\ln 2}{60\ MGT}$$

Where:

q_{LL} is the specific rate of lactulose utilisation (nmol/min/mg dry wt).

s_t is the medium lactulose concentration (nmol/ml) at time t.

x_t is the culture dry wt (mg/ml).

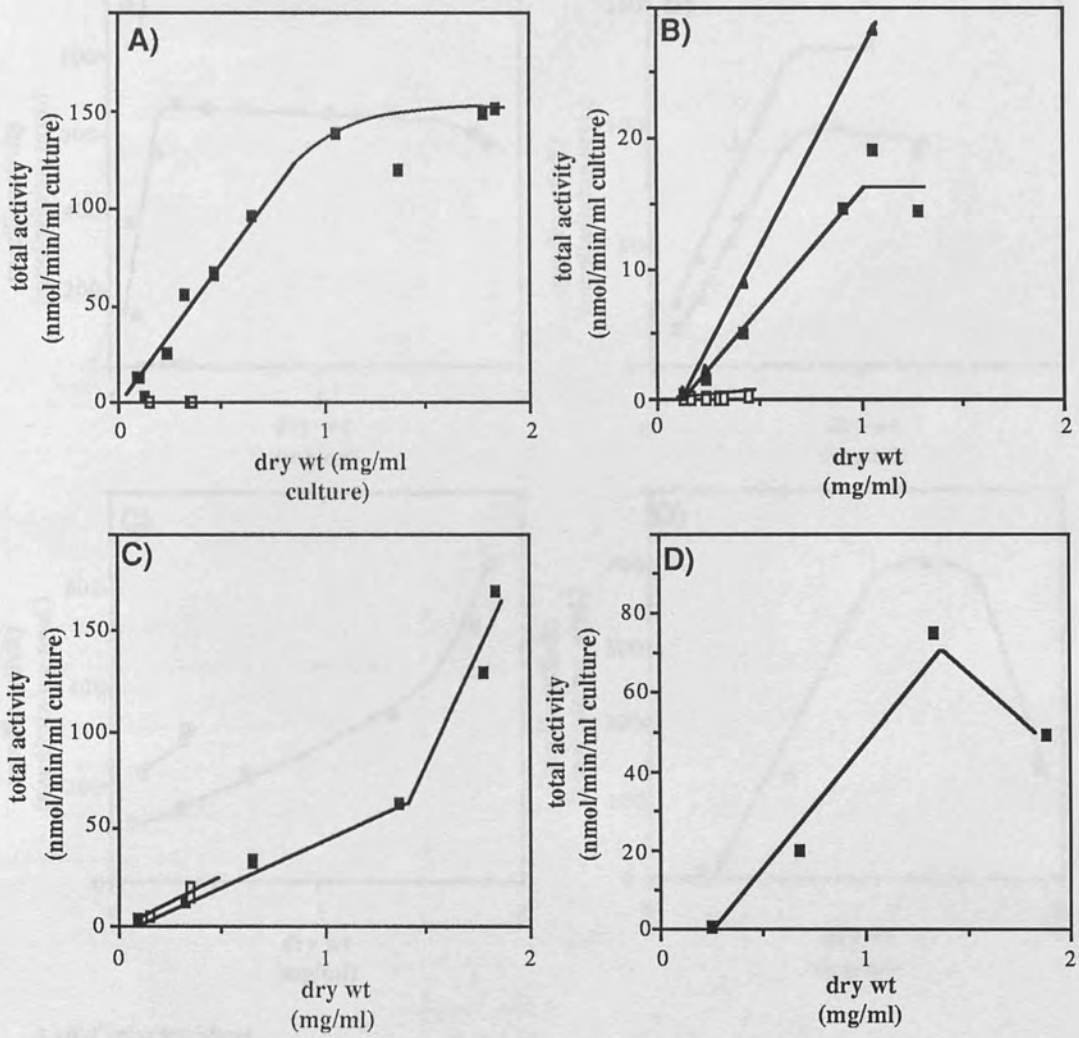
MGT is the culture mean generation time (h).

From the combined data (Fig. 11D), values of 36.4 and 43.4 nmol/min/mg dry wt (mean = 39.9 nmol/min/mg dry wt) were calculated for cultures LL1 and LL2, respectively.

All previous data from basal medium- and lactulose-grown cells have been replotted as a function of culture dry wt (Figs 15A-C: total activity, Figs 16A-C: specific activity). Also included is additional β-galactosidase activity from culture LL2 and galactokinase data from a third lactulose-grown culture. In order to facilitate identification of the stage of culture growth, Table 10 has been constructed, and quantitative data (maximum specific activities and the rate of total activity increase with culture dry wt) are summarised in Table 11. Calculation of specific activities as a function of culture dry wt permitted comparison of galactokinase data from basal medium- and lactulose-grown cells. Since plots of specific (dry wt) activity versus culture dry wt yielded virtually identical curves to the specific (protein) activity profiles, these data have been excluded. The fructokinase data (Figs 15D and 16D) are considered in section 3.2.1.3.

The activities of galactokinase and galactose 1-phosphate uridylyl transferase appeared to be co-ordinately controlled (Figs 15B and 16B), whereas UDPgalactose 4-epimerase exhibited a different activity profile (Figs 15C and 16C). Thus, both the kinase and transferase activities levelled off as the culture approached stationary phase, whereas the epimerase activity continued to increase, even in stationary phase. Furthermore, the increase in galactokinase activity with culture dry wt in lactulose-grown cells represented a massive induction of the enzyme (approx. 30-fold) (Table 11), whereas epimerase activity appeared to be constitutively produced (Figs 15C and 16C, Table 11). The activity profiles of β-galactosidase (Figs 15A and 16A) were distinct from those of the Leloir pathway enzymes and supplementation of growth media with lactulose (20 mM) resulted in an 80-fold induction of the enzyme (Table 11).

Figure 15: Comparison of enzyme data from cells cultured in basal medium ± lactulose (20 mM) - total activities.



A) β-Galactosidase

B) Galactokinase (■) and galactose 1-phosphate uridylyl transferase (▲)

C) UDPgalactose 4-epimerase

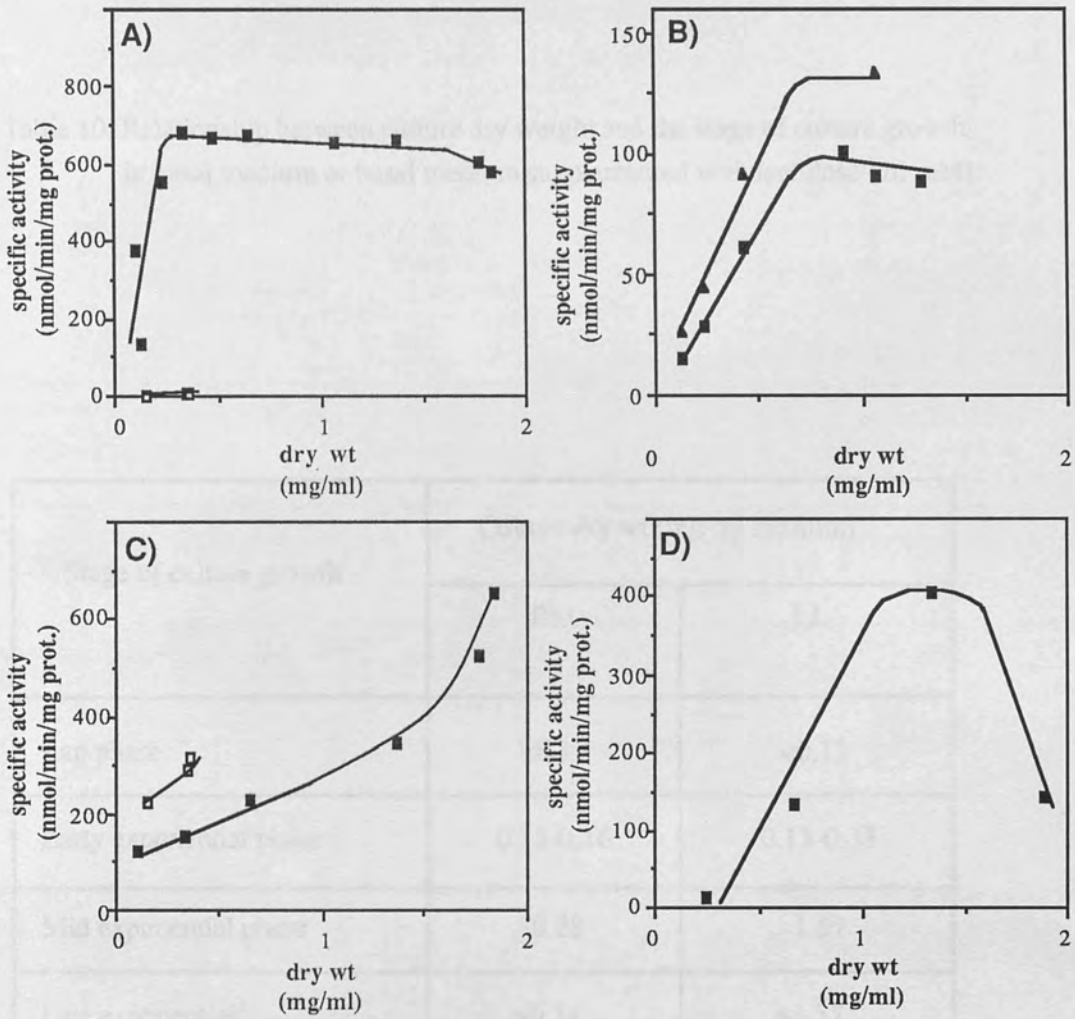
D) Fructokinase

Growth media:

Closed symbols - basal medium + lactulose (20 mM)

Open symbols - basal medium alone

Figure 16: Comparison of enzyme data from cells cultured in basal medium ± lactulose (20 mM) - specific activities.



A) β-Galactosidase

B) Galactokinase (■) and galactose 1-phosphate uridylyl transferase (▲)

C) UDPgalactose 4-epimerase

D) Fructokinase

Growth media:

Closed symbols - basal medium + lactulose (20 mM)

Open symbols - basal medium alone.

Table 10: Relationship between culture dry weight and the stage of culture growth in basal medium or basal medium supplemented with lactulose (20 mM).

Stage of culture growth	Culture dry wt (mg/ml medium)	
	BM	LL
Lag phase	<0.13	<0.13
Early exponential phase	0.13-0.16	0.13-0.33
Mid exponential phase	0.28	1.07
Late exponential/ stationary phase	>0.34	>1.77

BM - basal medium

LL - basal medium + lactulose (20 mM).

Table 11: Summary of enzyme data from cells grown in basal medium alone or basal medium supplemented with lactulose (20 mM).

Enzyme	Growth medium	Rate of activity increase (nmol/min/ mg dry wt)	Maximum specific activity (nmol/min/ mg protein)	Maximum specific activity (nmol/min/ mg dry wt)
β-Galactosidase	BM	2	8	2
	LL	165	684	171
Galactokinase	BM	0.50*	ND	0.34*
	LL	15.58	100.76	17.91
Galactose 1-P uridylyl transferase	BM	ND	ND	ND
	LL	30	134	26
UDPgalactose 4-epimerase	BM	58	317	43
	LL	47	653	93

BM - basal medium

LL - basal medium + lactulose (20 mM)

ND - not determined

*Cell extracts were prepared by lysis mix treatment. All other cell extracts were prepared by sonication.

3.2.1.3 Metabolism of the fructosyl moiety.

Phosphorylation of the glucosyl moiety of lactose, concomitant with transport, has not been described to-date. Glucokinase is therefore required for metabolism irrespective of the mechanism of lactose transport. By analogy, the most likely route of metabolism of the fructosyl moiety of lactulose is via fructokinase catalysed conversion to fructose 6-phosphate.

Initial attempts to assay fructokinase activity employed a procedure similar to that used for galactokinase (Materials and Methods, section 2.2.9.6.1). No activity could be detected in sonicant supernatants or membrane fractions prepared from mid exponential, lactulose (20 mM)-grown cells. However, positive results were obtained when a spectrophotometric assay (Materials and Methods, section 2.2.9.6.2) was employed. This procedure is more specific than the radioactive method, since it measures the formation of fructose 6-phosphate. It should be noted that the increase in absorbance could not be attributed to fructose 1-phosphate formation followed by isomerisation to fructose 6-phosphate, since addition of fructose 1-phosphate to assay mixtures did not produce any stimulatory effect.

Fructokinase activity was determined throughout culture growth in basal medium supplemented with lactulose (20 mM). Total activity and specific activity profiles have been plotted as a function of culture dry wt (Figs 15D and 16D) for comparison with the other enzyme data. Both total and specific activities increased gradually throughout exponential phase and then decreased as the culture entered stationary phase. The rate of increase in total activity was calculated as 63 nmol/min/mg dry wt (Fig. 15D) or 166 nmol/min/mg protein (data not shown) and the maximum specific activity was 400 nmol/min/mg protein (Fig. 16D) or 56 nmol/min/mg dry wt (data not shown).

The activity profiles of fructokinase were similar to those of galactokinase, since maximum values were reached in mid exponential phase. Fructokinase activity subsequently declined rapidly, whereas galactokinase activity remained relatively stable (Figs 15B and 16B), however, levels of the latter enzyme were not assayed in stationary phase. Similarities in the galactokinase and fructokinase profiles are not unexpected, since the substrates for these enzymes (galactose and fructose, respectively) are simultaneously produced by β-galactosidase action on intracellular lactulose.

3.2.2 Assay of Lactulose Metabolites.

Sensitive fluorimetric procedures were used to determine metabolite levels. Metabolites were assayed indirectly, as a function of NAD(P)(H) oxidation/reduction, by the addition of appropriate substrate-specific linking enzymes.

In early experiments, assays were performed directly on neutralised cell extracts, prepared by perchlorate treatment (Materials and Methods, section 2.2.11.1). Difficulties were encountered with regard to assay sensitivity for certain metabolites, therefore, extracts were concentrated 5-fold by lyophilisation/reconstitution in water in subsequent

experiments. All metabolites were assayed in samples taken from different stages of culture growth in basal medium ± lactulose (20 mM) and, for most metabolites, data were obtained from several cultures.

Metabolite levels can be expressed as a function of either the intracellular (protoplast) volume or culture dry wt, with the former being more commonly used. In the present study, the latter convention was followed, since the intracellular volume of *Cl. perfringens* was not determined.

3.2.2.1 Galactose, galactose 1-phosphate and galactose 6-phosphate.

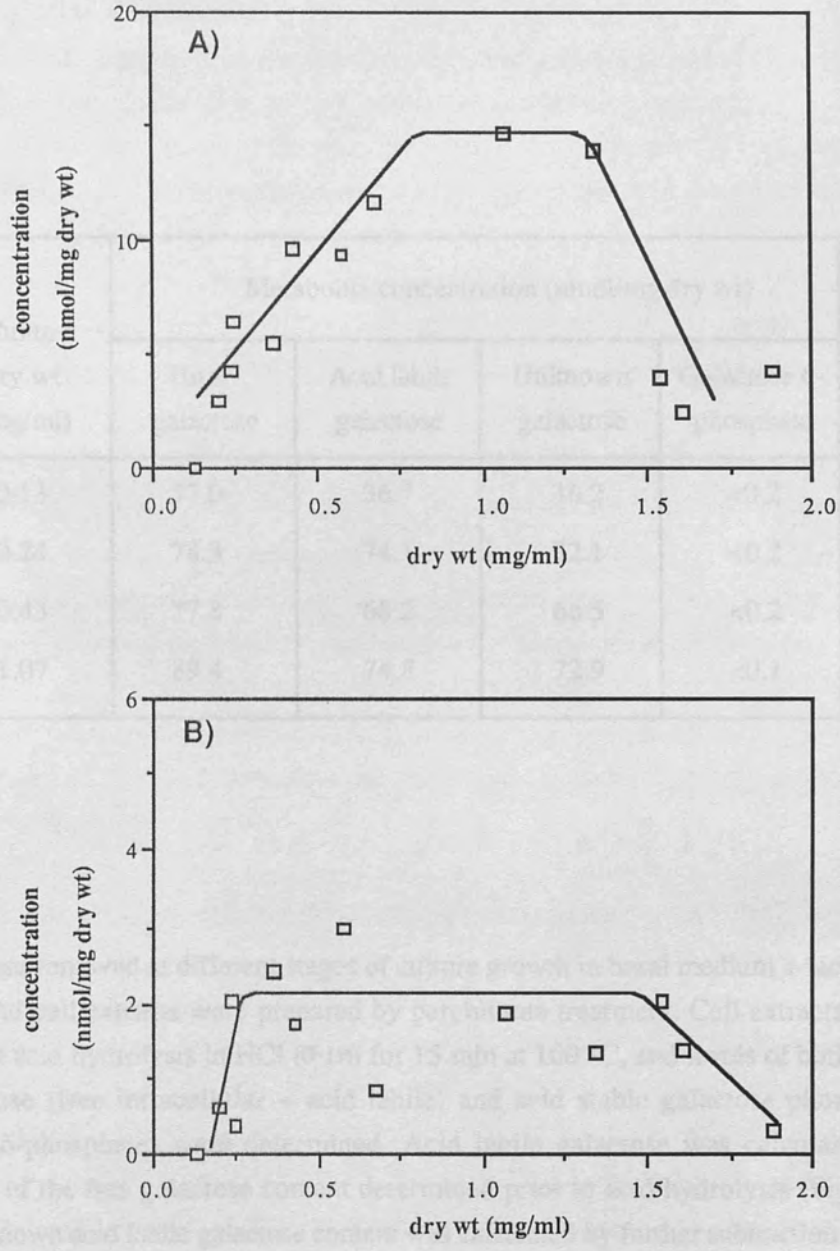
Intracellular levels of galactose and total galactose phosphates (galactose 1-phosphate + galactose 6-phosphate) were determined in 3 separate cultures of lactulose-grown cells (combined data in Fig. 17, see Table 10 for clarification of growth stages). Both metabolites were detectable in early exponential phase cells, but not lag phase cells (<2.0 nmol/mg dry wt for galactose and <0.8 nmol/mg dry wt for total galactose phosphate). Levels of galactose reached a maximum of ca. 14 nmol/mg dry wt in mid exponential phase and subsequently declined rapidly as the culture approached stationary phase, whereas total galactose phosphate levels reached a maximum of approx. 2 nmol/mg dry wt in early exponential phase which was maintained until late exponential phase. It was notable that the increase in galactokinase and galactose 1-phosphate uridylyl transferase specific activities (Fig. 16B) paralleled the increase in galactose levels.

Attempts were made to identify the species of galactose phosphate present. Differences in acid lability between galactose 1-phosphate (acid labile) and galactose 6-phosphate (acid stable) [155] have been exploited by several workers for this purpose [154,158]. Thus, galactose 1-phosphate is normally determined as free galactose in acid hydrolysed cell extracts (after correction for free galactose present prior to hydrolysis), and sequential addition of alkaline phosphatase permits the determination of galactose 6-phosphate. However, the use of a similar approach in the present investigation (Materials and Methods, section 2.2.11.2.1) produced equivocal results.

When galactose dehydrogenase was added to acid hydrolysed samples, the fluorescence increase exceeded that which could be accounted for by free galactose plus total galactose phosphate, assuming that all of the galactose phosphate was present as the acid labile species (Table 12). Preliminary studies with standard solutions of galactose 1-phosphate and galactose 6-phosphate had indicated that alkaline phosphatase addition to the galactose assay system resulted in quantitative recovery (> 95%) of these compounds, therefore, the discrepancy could not be attributed to the underestimation of total galactose phosphate levels in unhydrolysed samples. Rather, cell extracts must have contained a substantial pool of acid labile galactose, distinct from galactose 1-phosphate.

The level of the unknown metabolite was relatively high in the first sample assayed (lag phase) and a maximum of approx. 70 nmol/mg dry wt was reached in early exponential phase (Table 12). This level was maintained until mid exponential phase, but

Figure 17: Galactose and galactose phosphate levels in cells cultured in basal medium supplemented with lactulose (20 mM).



A) Galactose

B) Total galactose phosphate.

Table 12: Assay of free galactose and galactose phosphate in acid hydrolysed samples.

Culture dry wt (mg/ml)	Metabolite concentration (nmol/mg dry wt)			
	Total galactose	Acid labile galactose	Unknown galactose	Galactose 6-phosphate
0.13	37.0	36.7	36.2	<0.2
0.24	78.3	74.1	72.1	<0.2
0.43	77.8	68.2	66.5	<0.2
1.07	89.4	74.8	72.9	<0.1

Samples were removed at different stages of culture growth in basal medium + lactulose (20 mM) and cell extracts were prepared by perchlorate treatment. Cell extracts were subjected to acid hydrolysis in HCl (0·1M) for 15 min at 100 °C, and levels of both total free galactose (free intracellular + acid labile) and acid stable galactose phosphate (galactose 6-phosphate) were determined. Acid labile galactose was calculated by subtraction of the free galactose content determined prior to acid hydrolysis (Fig. 17), and the unknown acid labile galactose content was calculated by further subtraction of the total galactose phosphate level of unhydrolysed samples (Fig. 17). Galactose 6-phosphate was undetectable in all samples, values refer to detection limits.

no assays were performed on late exponential/stationary phase cells. The profile of this metabolite was similar to the specific activity profile of β-galactosidase (Fig. 16). The most likely identity of this unknown compound is lactulose. Unfortunately no attempt was made to determine intracellular levels of this disaccharide, therefore, this proposal remains conjectural. In retrospect, determination of lactulose could have been attempted using commercial β-galactosidase, purified from a bacterial source such as *E. coli*, in conjunction with the galactose assay system. Sequential addition of alkaline phosphatase would have also permitted the quantitation of any lactulose phosphate, produced as a result of PTS-mediated phosphorylation of the galactosyl moiety during membrane transport.

It was significant that no net increase in fluorescence resulted from the addition of alkaline phosphatase to assays containing acid hydrolysed extracts (Table 12). Calculation of detection limits as a percentage of total galactose phosphate levels in unhydrolysed extracts yielded values of: 38.8, 10.9, 13.7 and 7.1% for culture dry wts of 0.13, 0.24, 0.43 and 1.07 mg/ml, respectively. With the exception of the lag phase sample, if galactose 6-phosphate was present, then at best it could only represent a small fraction of the total galactose phosphate pool. Thus, by a process of elimination, galactose 1-phosphate appears to be the only species of galactose phosphate present, which is consistent with the absence of phospho-β-galactosidase in lactulose-grown cells (section 3.2.1.1). Certain refinements could have been made to the assay procedure to permit direct quantitation of galactose 1-phosphate levels. Indeed, the use of ion exchange resins, prior to acid hydrolysis, may have removed contaminating acid labile material.

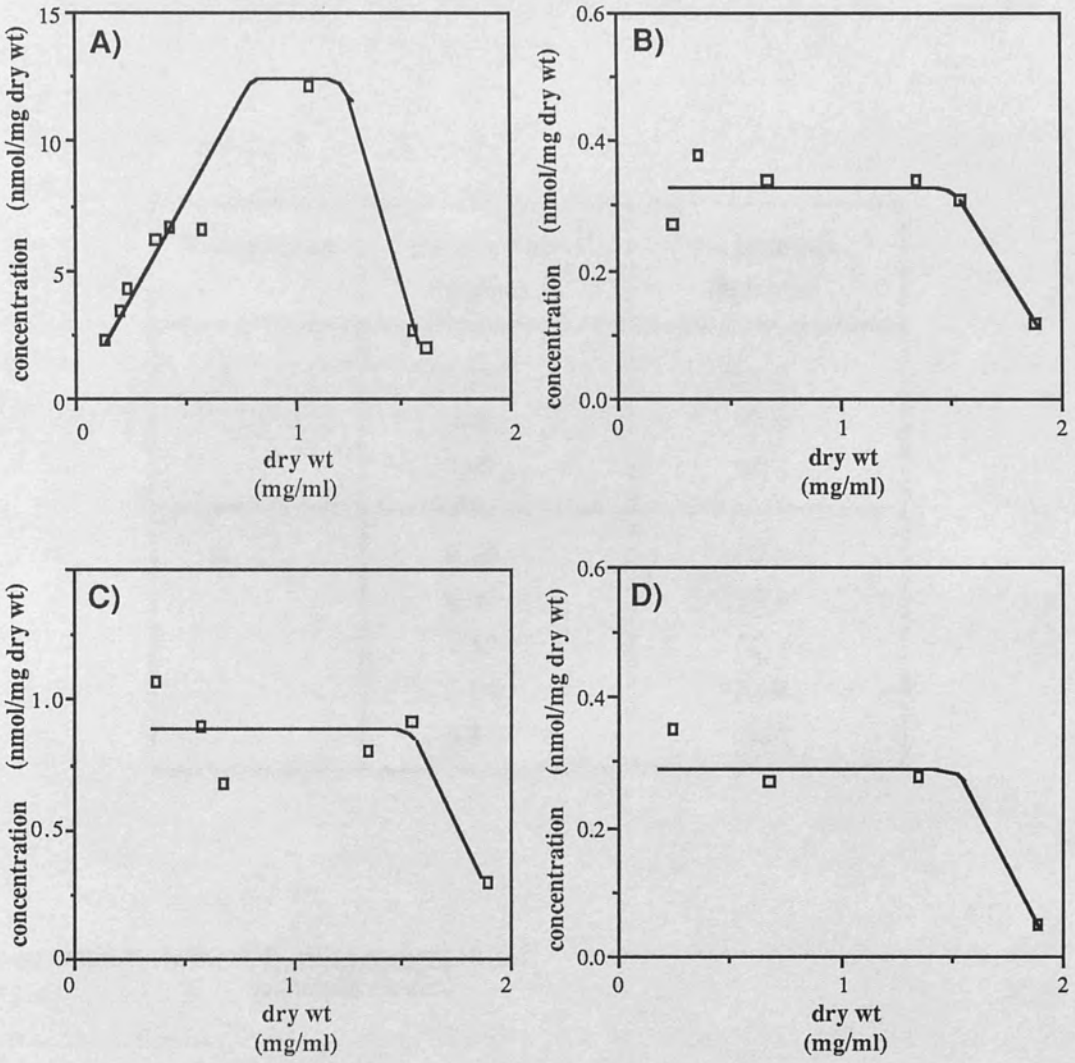
Assay of galactose was also attempted in basal medium-grown cells, harvested at different stages of culture growth, but was undetectable (<0.5 nmol/mg dry wt) in all samples assayed.

3.2.2.2 Fructose, fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate levels.

Intracellular free fructose was detectable in all samples of lactulose-grown cells assayed (Fig. 18A), but was absent (<0.3 nmol/mg dry wt) from basal medium-grown cells (early exponential to stationary phase samples; data not shown). The presence of fructose indicates that, at least, part of the intracellular lactulose was not phosphorylated on the fructose moiety, prior to β-galactosidase cleavage. The profile of this hexose (Fig. 18A) was strikingly similar to that of free galactose (Fig. 17A) and, in order to illustrate this point, fructose levels are expressed as a percentage of galactose levels (Table 13). Values ranged from ca. 110% in early exponential phase to ca. 75% in late exponential phase. This indicates some degree of co-ordinate control over the metabolism of these two hexosyl moieties.

Lactulose-grown cells contained lower levels of fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate (Figs 18B, C and D, respectively). The presence of these latter two metabolites provides further evidence for the involvement of the Leloir

Figure 18: Fructose, fructose 6-phosphate and glucose phosphate levels in cells cultured in basal medium supplemented with lactulose (20 mM).



A) Fructose

B) Fructose 6-phosphate

C) Glucose 6-phosphate

D) Glucose 1-phosphate.

Table 13: Relationship between fructose and galactose levels in cells grown in basal medium supplemented with lactulose (20 mM).

Culture code	Culture dry wt (mg/ml)	Concentration ratio (%)
A	0.24	103.8
	0.43	70.4
	1.07	83.3
B	0.20	117.1
	0.37	115.3
	0.57	71.1
	1.54	68.4
	1.61	84.2

$$\text{concentration ratio} = \frac{\text{fructose content}}{\text{galactose content}} \times 100$$

pathway in metabolism of the galactosyl moiety (Fig. 4). Fructose 6-phosphate may be formed as a result of either direct phosphorylation of the free fructose moiety (catalysed by fructokinase) or subsequent metabolism of glucose 6-phosphate by the EM pathway. Fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate remained constant throughout the exponential phase of growth at levels of 0.3, 0.9 and 0.3 nmol/mg dry wt, respectively, and only declined when the culture approached stationary phase (Figs 18B-D). Fructose 6-phosphate and glucose 6-phosphate were also assayed in basal medium-grown cells but were undetectable (<0.1 nmol/mg dry wt) in all samples (early exponential to stationary phase cells).

3.2.2.3 Fructose 1,6-bisphosphate (FDP), dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P).

Triose phosphates were only detectable in lactulose-grown cells from early exponential phase, whereas FDP was found throughout culture growth (Table 14). There appeared to be a peak in the level of this metabolite in early exponential phase (Table 14). Neither FDP nor triose phosphate was detectable in basal medium-grown cells (Table 14).

3.2.3 Control of Galactokinase Activity.

Other studies have shown that the β-galactosidase activity of *Cl. perfringens* is subject to a 'glucose effect' [145]. It was of interest, therefore, to determine if the galactokinase activity of this organism was subject to similar regulatory control.

3.2.3.1 Variation in galactokinase activity with carbon source.

In order to determine the inducer specificity of galactokinase, assays were performed on sonicant extracts prepared from cells cultured in basal medium ± one of the following sugars: glucose, fructose, galactose, lactose or lactulose (5 mM final concentration). Cells were harvested at late exponential phase and specific activities calculated as a function of sonicant cell protein. Assays were normally performed in duplicate and, in most cases, data were available from two separate cultures (Table 15).

Taking basal medium alone as a reference point, supplementation with glucose resulted in significantly lower levels of galactokinase, whereas supplementation with lactose or lactulose resulted in significantly higher levels of activity ($p < 0.005$ in all cases, by Student t-test). Levels in lactose- or lactulose-supplemented media were not significantly different from each other ($p > 0.1$). Insufficient data were available from fructose- or galactose-grown cells to permit a statistical evaluation of these results, however, the duplicate values from each culture were reproducible. The following rank order of inducing ability can be calculated for the various sugars, using the specific activity of basal medium-grown cells as a reference:

Table 14: Assay of glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and fructose 1,6-bisphosphate in cells grown in basal medium or basal medium supplemented with lactulose (20 mM).

Culture dry weight (mg/ml)	Metabolite level (nmol/mg dry wt)		
	Glyceraldehyde 3-phosphate	Dihydroxyacetone phosphate	Fructose 1,6-bisphosphate
BM			
0.16	<2.1	<2.6	<1.1
0.28	<0.6	<0.8	<0.3
0.35	<0.7	<0.9	<0.4
0.36	<0.7	<0.9	<0.4
LL			
0.25	1.3	1.0	1.7
0.68	<2.4	<3.0	2.4
1.34	<1.0	<1.3	1.1
1.88	<0.5	<0.6	1.2

BM - cells grown in basal medium

LL - cells grown in basal medium +lactulose (20 mM).

Table 15: Specific activities of galactokinase in cells grown in basal medium supplemented with different sugars.

Carbon source	Specific activity (nmol/min/mg protein)		
	Replicate	Mean	Standard deviation
None	4.54 4.79 5.49 6.04	5.22	0.68
Glucose	1.63 1.42 1.41 1.41	1.47	0.11
Fructose	2.48 2.87	2.68	
Galactose	9.38 11.63	10.51	
Lactose	39.10 27.54 30.67	32.44	5.98
Lactulose	32.84 37.03 42.54 31.34	35.94	5.02

Final sugar concentrations were 5 mM. Cells were harvested at the late exponential phase of culture growth and extracts were prepared by sonication.

lactulose = lactose > galactose > none > fructose > glucose
6.9 = 6.2 > 2.0 > 1.0 > 0.5 > 0.3

Although the measurement of specific activities is a useful indicator of enzyme induction, it does not take into account any possible influence of growth rate on enzyme expression. This is particularly pertinent to the above results, since the differences in specific activity were only slight. A more appropriate method is to compare the differential rates of enzyme synthesis, determined from plots of total activity versus culture dry wt.

Galactokinase activities were measured throughout culture growth in medium supplemented with different sugars. In these and subsequent experiments reported in section 3.2.3.2, cell extracts were prepared by lysis mix treatment. This procedure was considerably less time-consuming than sonication, thereby permitting the simultaneous processing of a large number of culture samples. Specific activities were calculated as a function of culture dry wt, due to lysis mix interference with the protein assay procedure.

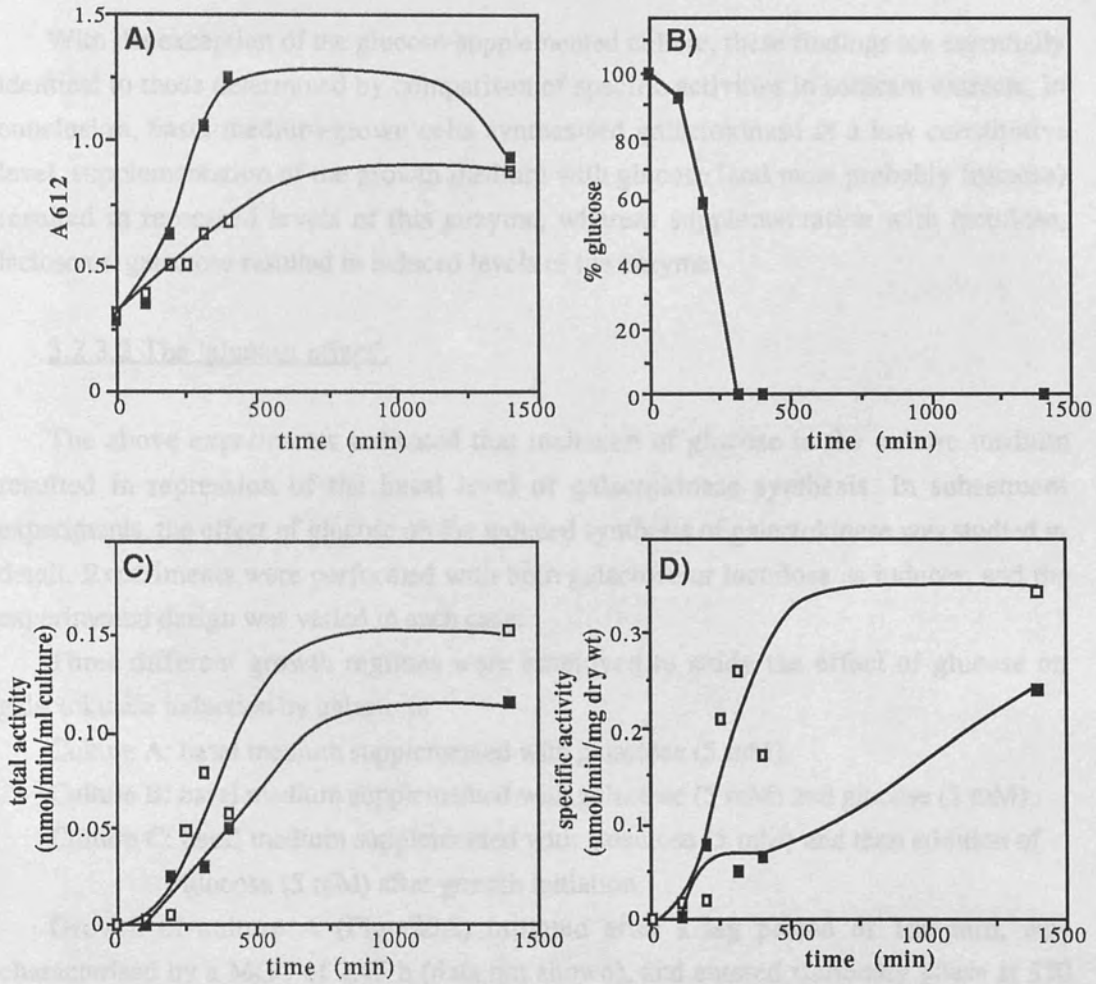
Although galactokinase activities in basal medium-grown cells have been presented earlier (section 3.2.1.2), they are also depicted together with activities in glucose (5 mM)-supplemented cells (Fig. 19), for comparative purposes. Culture growth in glucose-supplemented medium (MGT = 1.47 h, data not shown) was much more rapid than that in basal medium alone (MGT = 4.95 h, data not shown). Glucose was rapidly utilised by the culture (Fig. 19B), the specific rate of glucose utilisation was calculated as 87.5 nmol/min/mg dry wt (data not shown). Glucose exhaustion from the medium (Fig. 19B) preceded the entry of the culture into stationary phase (Fig. 19A).

Although the time courses of total activity were similar (Fig. 19C), specific activities varied considerably (Fig. 19D). Comparison of the maximum specific activities during exponential growth revealed that the value for glucose-grown cells (0.08 nmol/min/mg dry wt) was 4.4-fold lower than that for basal medium-grown cells (0.35 nmol/min/mg dry wt). This relationship is consistent with the findings from sonicated cell extracts. However, when the differential rates of enzyme synthesis were compared, the repressive effect of medium glucose appeared to be more pronounced. Absolute values, calculated during the exponential phase of growth (data not shown), were 0.07 and 0.50 nmol/min/mg dry wt for glucose- and basal medium-grown cells, respectively; representing a 7.1-fold repression of galactokinase synthesis by medium glucose.

In the case of glucose-supplemented cultures, both total (Fig. 19C) and specific (Fig. 19D) activities appeared to increase during stationary phase, after sugar exhaustion from the medium (Fig. 19B). These increases in activity may have resulted from the removal of a repressing compound; produced as a direct result of glucose metabolism.

Differential rates of galactokinase synthesis were also measured in cells cultured in basal medium supplemented with lactulose (5 mM) or galactose (5 mM). These cultures are considered in detail below (section 3.2.3.2), however, respective differential rates of galactokinase synthesis were 3.32 and 1.42 nmol/min/mg dry wt (Figs 23C and 21C). Comparison of the differential rates of galactokinase synthesis, using basal medium-grown

Figure 19: Galactokinase activities in cells cultured in basal medium ± glucose (5mM).



Growth media:

□ - basal medium

■ - basal medium + glucose (5mM)

cells as a reference, permits the following rank order of inducing ability to be calculated:

lactulose > galactose > none > glucose
6.6 > 2.8 > 1 > 0.1

With the exception of the glucose-supplemented culture, these findings are essentially identical to those determined by comparison of specific activities in sonicant extracts. In conclusion, basal medium-grown cells synthesised galactokinase at a low constitutive level, supplementation of the growth medium with glucose (and most probably fructose) resulted in repressed levels of this enzyme, whereas supplementation with lactulose, lactose or galactose resulted in induced levels of the enzyme.

3.2.3.2 The 'glucose effect'.

The above experiments indicated that inclusion of glucose in the culture medium resulted in repression of the basal level of galactokinase synthesis. In subsequent experiments, the effect of glucose on the induced synthesis of galactokinase was studied in detail. Experiments were performed with both galactose or lactulose as inducer, and the experimental design was varied in each case.

Three different growth regimes were employed to study the effect of glucose on galactokinase induction by galactose:

Culture A: basal medium supplemented with galactose (5 mM).

Culture B: basal medium supplemented with galactose (5 mM) and glucose (5 mM).

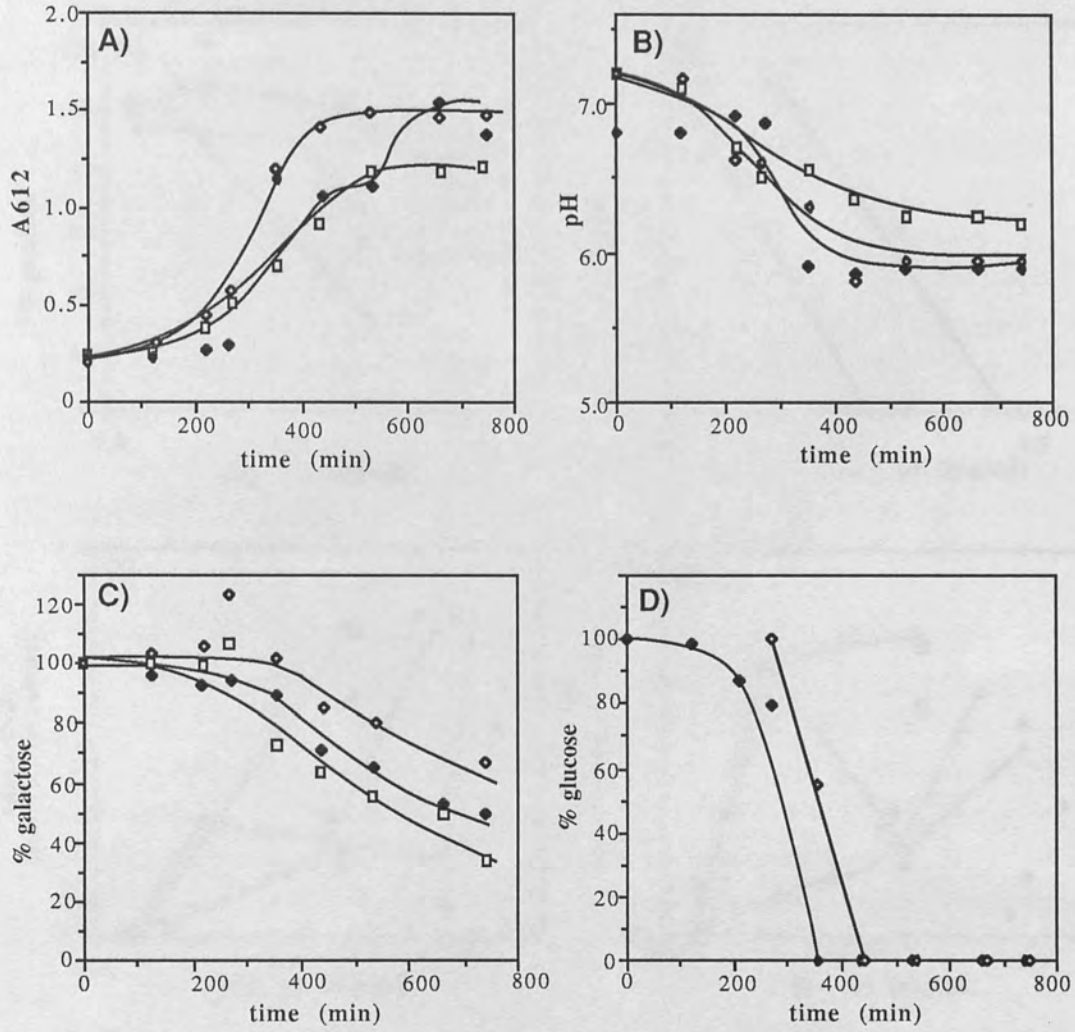
Culture C: basal medium supplemented with galactose (5 mM) and then addition of glucose (5 mM) after growth initiation.

Growth of culture A (Fig. 20A) initiated after a lag period of 150 min, was characterised by a MGT of 2.45 h (data not shown), and entered stationary phase at 550 min. Both acidification of the culture medium (Fig. 20B) and sugar utilisation (Fig. 20C) followed a similar pattern, with continued galactose utilisation evident in stationary phase. The specific rate of galactose utilisation was calculated as 10.5 nmol/min/mg dry wt (data not shown). The differential rate of galactokinase synthesis was calculated as 1.42 nmol/min/mg dry wt (Fig. 21C; as mentioned in section 3.2.3.1), and the maximum level in specific activity was 1.17 nmol/min/mg dry wt (Fig. 21D).

When *Cl. perfringens* was presented with a mixture of glucose and galactose (culture B) a diauxic growth curve resulted (Fig. 20A). Growth initiated after a slightly longer lag period of 250 min, relative to culture A, and proceeded rapidly (MGT = 1.36 h, data not shown) up to 360 min. There then followed a second lag period (360-540 min), which preceded a short growth phase, and the culture finally entered stationary phase at 660 min. The MGT of this second growth phase was estimated as 2.58 h, but this value is unreliable due to the lack of data points (Fig. 20A).

The sugar utilisation data (Figs 20C and D) demonstrated a preferential utilisation of

Figure 20: Effect of glucose on galactose utilisation - growth and sugar utilisation data.



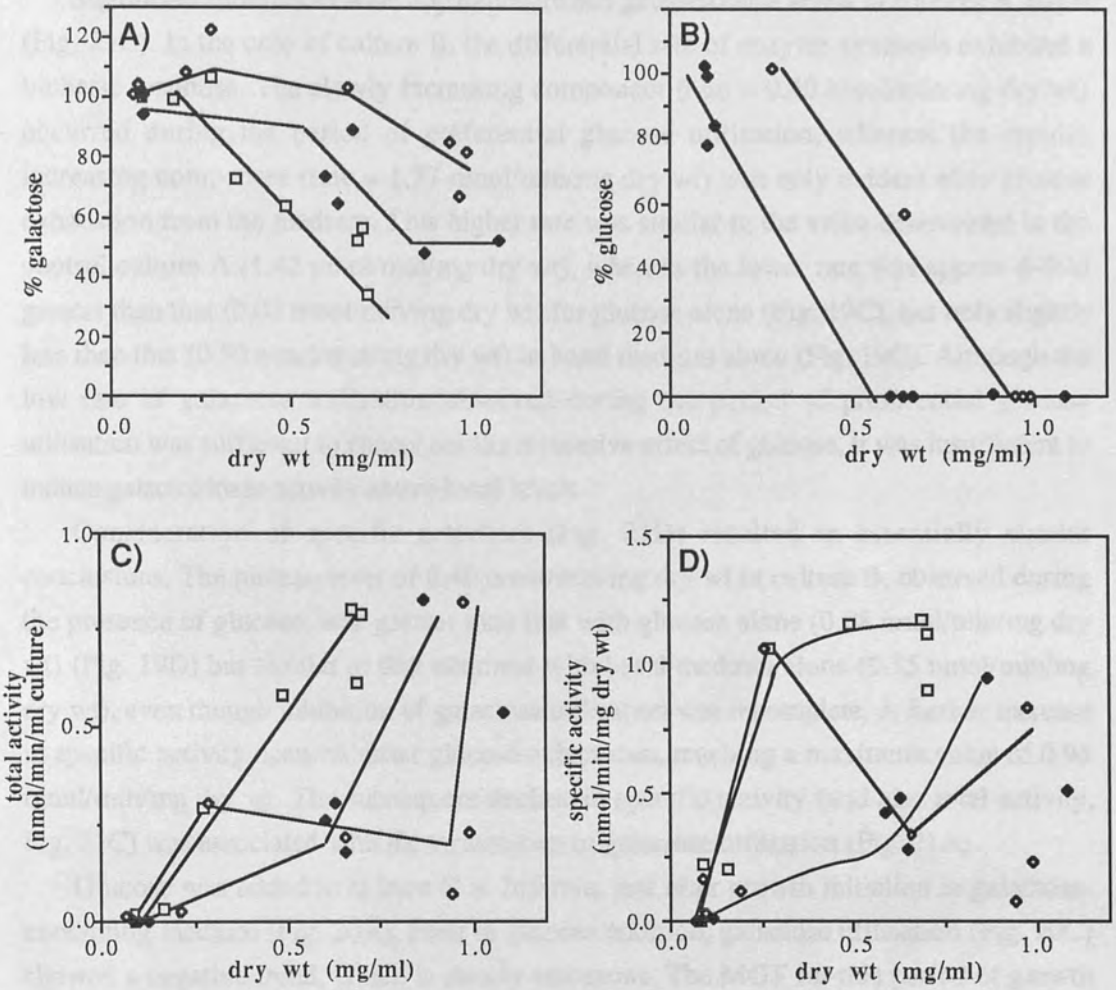
Growth media:

□ basal medium + galactose (5 mM) (culture A)

◆ basal medium + galactose (5 mM) + glucose (5 mM) (culture B)

◇ basal medium + galactose (5 mM), then glucose (5 mM) added at 265 min (culture C).

Figure 21: Effect of glucose on galactokinase induction by galactose.



Growth media:

- ▫ basal medium + galactose (5 mM) (culture A)
- ◆ basal medium + galactose + glucose (5 mM) (culture B)
- ◇ basal medium + galactose (5 mM), then glucose (5 mM) added at 265 min (culture C)

glucose. Glucose utilisation initiated at 120 min (Fig. 20D) and was complete by 360 min (Fig. 20D). Glucose was not used exclusively in preference to galactose; the galactose content of the medium had decreased by 12% at the point of glucose exhaustion (Figs 20C and 21A). However, galactose utilisation in culture B resumed at a high rate once glucose was exhausted from the medium and was apparent during the interphasic growth lag.

Significant differences were apparent between galactokinase levels in cultures A and B (Fig. 21C). In the case of culture B, the differential rate of enzyme synthesis exhibited a biphasic response. The slowly increasing component (rate = 0.40 nmol/min/mg dry wt) occurred during the period of preferential glucose utilisation, whereas the rapidly increasing component (rate = 1.77 nmol/min/mg dry wt) was only evident after glucose exhaustion from the medium. This higher rate was similar to the value determined in the control culture A (1.42 nmol/min/mg dry wt), whereas the lower rate was approx 6-fold greater than that (0.07 nmol/min/mg dry wt) for glucose alone (Fig. 19C), but only slightly less than that (0.50 nmol/min/mg dry wt) in basal medium alone (Fig. 19C). Although the low rate of galactose utilisation observed during the period of preferential glucose utilisation was sufficient to cancel out the repressive effect of glucose, it was insufficient to induce galactokinase activity above basal levels.

Consideration of specific activities (Fig. 21D) resulted in essentially similar conclusions. The plateau level of 0.40 nmol/min/mg dry wt in culture B, observed during the presence of glucose, was greater than that with glucose alone (0.08 nmol/min/mg dry wt) (Fig. 19D) but similar to that obtained with basal medium alone (0.35 nmol/min/mg dry wt), even though inhibition of galactose utilisation was incomplete. A further increase in specific activity occurred after glucose exhaustion, reaching a maximum value of 0.94 nmol/min/mg dry wt. The subsequent decline in specific activity (and also total activity, Fig. 21C) was associated with the termination of galactose utilisation (Fig. 21A).

Glucose was added to culture C at 265 min, just after growth initiation in galactose-containing medium (Fig. 20A). Prior to glucose addition, galactose utilisation (Fig. 20C) showed a negative trend, which is clearly erroneous. The MGT for this period of growth was estimated as 1.91 h (data not shown) and, since this value is similar to that for culture A, it would suggest that galactose was actually being used to support culture growth. Glucose was used immediately upon its addition to culture C (Fig. 20D), resulting in an increased growth rate (Fig. 20A) (MGT = 1.12 h, data not shown). The presence of glucose resulted in reduced galactose utilisation, relative to the control culture.

Although sugar utilisation was comparable for cultures B and C, a major difference existed with respect to the growth data in that diauxie was not apparent in culture C (Fig. 20A). Growth completely ceased after glucose exhaustion at 440 min, possibly due to adverse culture conditions.

Moderate galactokinase activity was present at the point of glucose addition to culture C (Figs 21C and D). The effect of glucose addition was to result in a decrease in total activity, which was reversed upon glucose exhaustion from the medium (Fig. 21C). The differential rates of galactokinase synthesis were estimated as: +3.1 nmol/min/mg dry wt

prior to glucose addition and -0.4 nmol/min/mg dry wt during glucose utilisation (Fig. 21C). This latter decrease presumably resulted from turnover, or possibly inhibition, of enzyme activity, therefore, the effect of glucose on galactokinase activity was more severe in culture C relative to culture B. After glucose exhaustion from culture C, galactokinase increased at a rate of 12.1 nmol/min/mg dry wt (Fig. 21C). The rapidity of this increase was artefactual since it represented an increase in total activity without any concomitant increase in culture mass (Fig. 20A).

Specific activity in culture C initially increased prior to glucose addition, and levels were similar to those in the control culture A (Fig. 21D). A severe decrease was observed upon glucose addition, due to enzyme degradation/inhibition and dilution by cell division. After removal of glucose from the medium, levels increased to near control values, and then decreased, concomitant with the termination of galactose utilisation (Fig. 21A).

A slightly different experimental design was employed when the effect of glucose on galactokinase induction by lactulose was studied, in order to determine if cAMP was involved in the mediation of glucose effects. Three different culture regimes were studied:

Culture A - basal medium supplemented with lactulose (5 mM).

Culture B - basal medium supplemented with lactulose (5 mM), to which was added glucose (5 mM), after growth initiation.

Culture C - as for culture B, except cAMP (5 mM) was added together with glucose (5 mM).

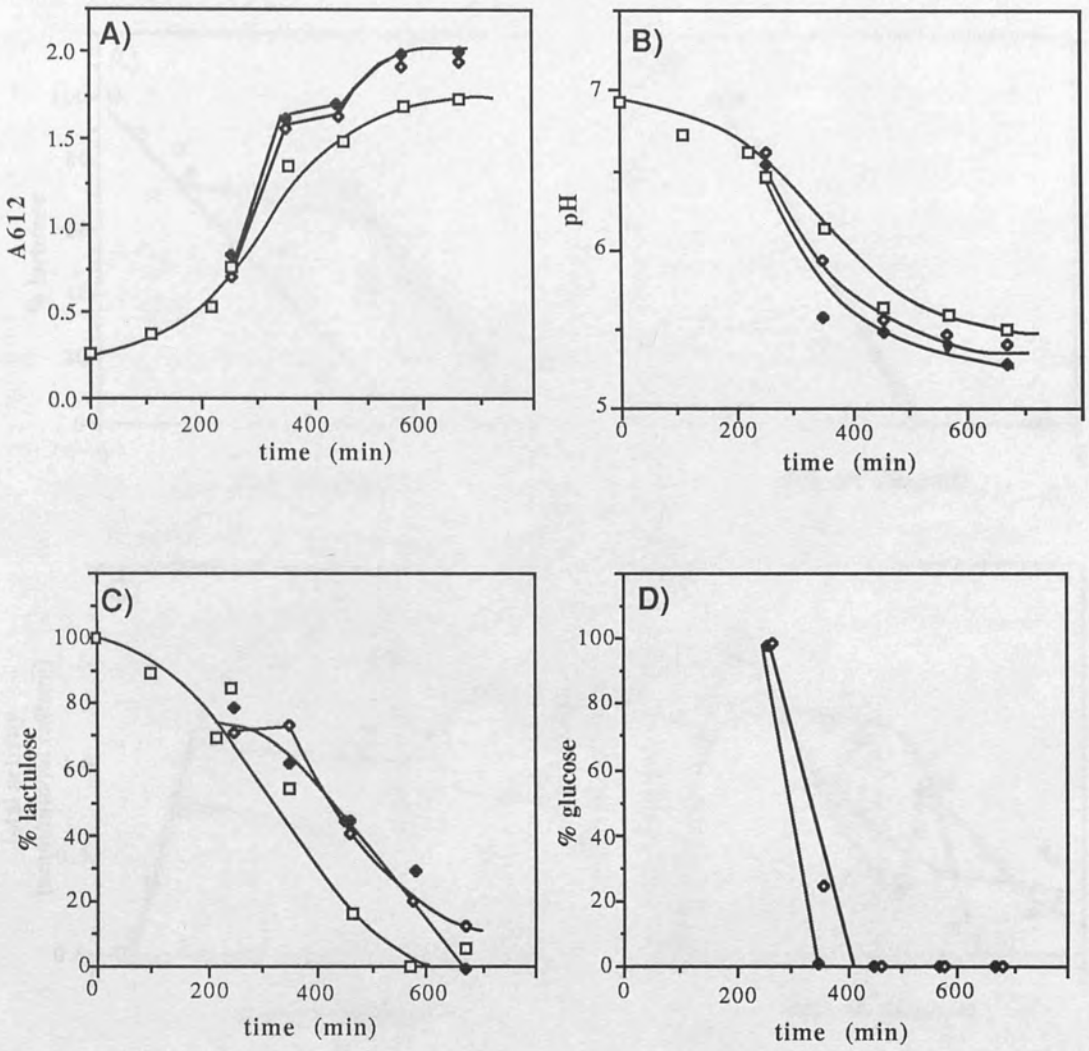
The additions to cultures B and C were made at 255 min, just prior to sample removal.

The addition of glucose, or glucose plus cAMP, to cells growing on lactulose resulted in diauxie (Fig. 22A). Glucose was immediately used in both cultures, and this resulted in an increased growth rate. Thus, prior to the addition of glucose, or glucose plus cAMP, MGT were 2.31 h for culture A and 2.43 h for cultures B and C, whereas after medium additions MGT decreased to values of 1.16 h and 1.19 h for B and C, respectively (data not shown). The exhaustion of glucose from B and C (Fig. 22D) was followed by a short lag period in growth of approx. 100 min (Fig. 22A), and this then resumed at greatly reduced rates (MGT were estimated as 4.21 h and 4.72 h, respectively), however, the cultures were approaching stationary phase.

Glucose was used immediately upon addition to cultures B and C (Figs 22D and 23B): specific rates of glucose utilisation were almost identical at 61.5 and 61.4 nmol/min/mg dry wt, respectively (data not shown). Glucose resulted in a severe inhibition of lactulose utilisation, which was particularly apparent when data were plotted as a function of culture dry wt (Fig. 23A), and cAMP did not relieve this effect. Slight differences were evident between cultures B and C in that lactulose utilisation appeared to be inhibited to a greater degree in the presence of cAMP, however, interpretation of these data is compromised by the lack of data points. The utilisation of lactulose resumed in both B and C, immediately upon glucose removal from the medium (Figs 23A and B), therefore, the interphasic growth lag could not be attributed to inhibition of lactulose utilisation.

Levels of galactokinase total activity increased throughout growth in the control culture

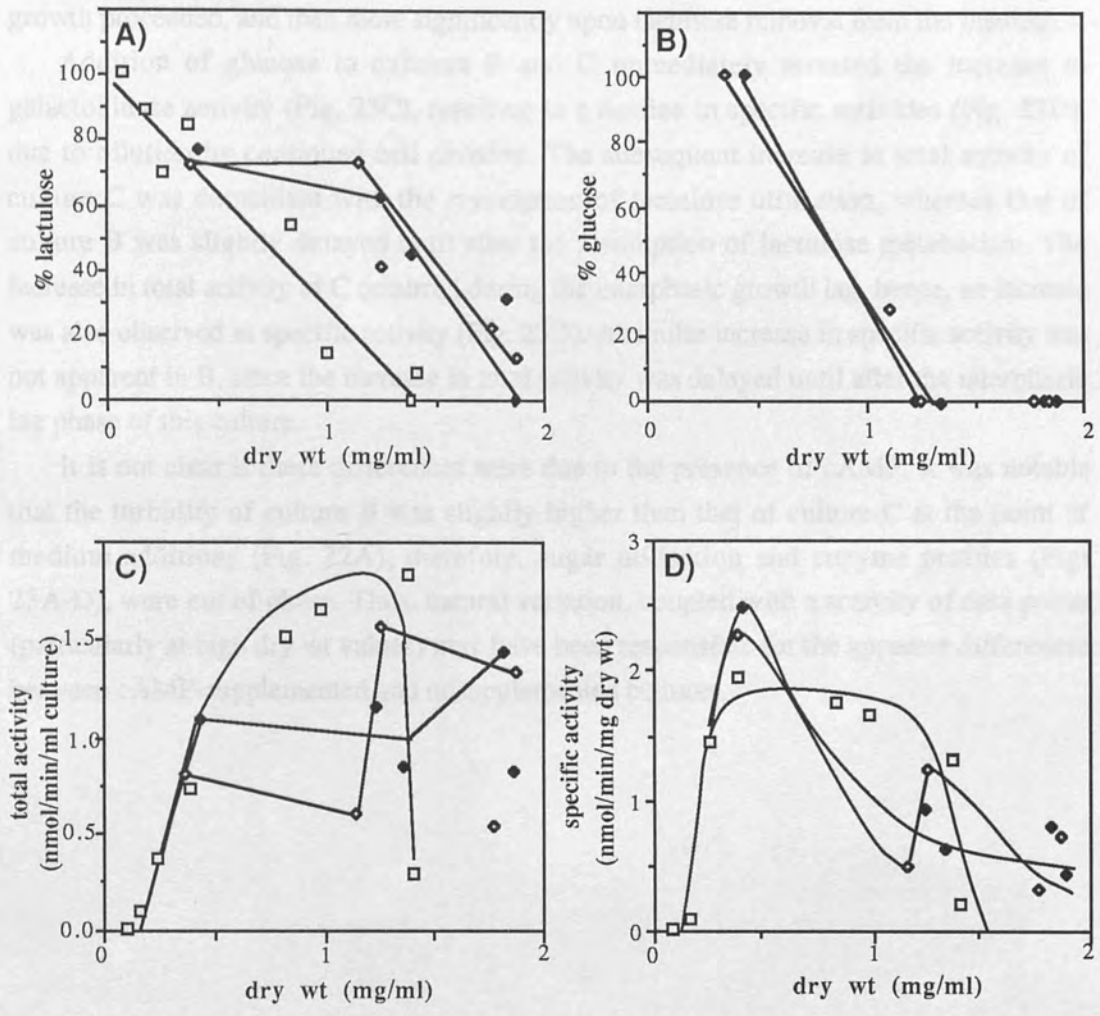
Figure 22: Effect of glucose on lactulose utilisation - growth and sugar utilisation data.



Growth media:

- basal medium + lactulose (5 mM) (culture A)
- basal medium + lactulose (5 mM), then glucose (5 mM) added at 255 min (culture B)
- basal medium + lactulose (5 mM), then glucose (5 mM) + cAMP (5 mM) added at 255 min (culture C)

Figure 23: Effect of glucose on galactokinase induction by lactulose.



Growth media:

- ▫ basal medium + lactulose (5 mM) (culture A)
- ◆ basal medium + lactulose (5 mM), then glucose (5 mM) added at 255 min (culture B)
- ◇ basal medium + lactulose (5 mM), then glucose (5 mM) + cAMP (5 mM) added at 255 min (culture C)

(A); the differential rate of synthesis was calculated as 3.32 nmol/min/mg dry wt from the linear portion of the curve. Once lactulose was exhausted from the medium, activity immediately declined (Fig. 23A), suggesting the operation of an inactivation mechanism; possibly enzyme degradation/inhibition. Specific activity reached a maximum value of 1.95 nmol/min/mg dry wt during early/mid exponential phase (Fig. 23D), declined slightly as growth proceeded, and then more significantly upon lactulose removal from the medium.

Addition of glucose to cultures B and C immediately arrested the increase in galactokinase activity (Fig. 23C), resulting in a decline in specific activities (Fig. 23D), due to dilution by continued cell division. The subsequent increase in total activity of culture C was coincident with the resumption of lactulose utilisation, whereas that of culture B was slightly delayed until after the resumption of lactulose metabolism. The increase in total activity of C occurred during the interphasic growth lag, hence, an increase was also observed in specific activity (Fig. 23D). A similar increase in specific activity was not apparent in B, since the increase in total activity was delayed until after the interphasic lag phase of this culture.

It is not clear if these differences were due to the presence of cAMP. It was notable that the turbidity of culture B was slightly higher than that of culture C at the point of medium additions (Fig. 22A), therefore, sugar utilisation and enzyme profiles (Figs 23A-D), were out of phase. Thus, natural variation, coupled with a scarcity of data points (particularly at high dry wt values) may have been responsible for the apparent differences between cAMP-supplemented and unsupplemented cultures.

RESULTS

CHAPTER 4: INVESTIGATION OF SUGAR TRANSPORT

4.1 Introduction.

Studies on sugar transport in bacteria are normally conducted with radiolabelled substrates, using rapid sampling techniques [159]. Rates of uptake are estimated by measurement of the initial accumulation of intracellular radioactivity. The use of such a procedure in this study was not feasible, due to the unavailability of radiolabelled lactulose.

Sugar transport can also be measured indirectly by a spectrophotometric technique [159-162]. When bacteria are presented with a hypertonic solution of impermeant molecules, they become plasmolysed due to the exit of water. This decrease in cell volume results in a concomitant increase in light scattering, because the turbidity of the cell suspension increases. In contrast, when bacteria are presented with a hypertonic solution of permeant molecules, there is an initial increase in turbidity (due to plasmolysis), which is closely followed by a decrease. The latter results from deplasmolysis of cells due to solute entry. The rate of this turbidity decrease is proportional to the rate of solute entry [160-162] and, in order to standardise results, is normally expressed as a function of the 'total shrinkage' [162]. This parameter corresponds to the difference in turbidities measured in osmotically equivalent solutions of a relatively impermeant substance (normally NaCl) and a readily permeant substance (normally glycerol).

Measurement of sugar transport by this osmotic procedure has been applied to whole cells of only Gram-negative bacteria. Early attempts to demonstrate optical effects (using NaCl and glucose) in a wide range of Gram-positive bacteria were unsuccessful [163], most likely due to the fact that such organisms are not easily plasmolysed [163,164]. Furthermore, salt-induced contraction of Gram-positives (*B. megaterium*, *Staph. aureus* and *Micrococcus lysodeikticus*), when measured by changes in the dextran-impermeable volume, cannot be considered primarily an osmotic response, but is caused by the electrostatic contraction of cell wall polymers [164,165]. However, osmotic swelling procedures have been used to study sugar transport in protoplasts of both Gram-negative (*E. coli*) [166] and Gram-positive (*Sarcina lutea*, *M. lysodeikticus*, *Str. faecalis*) bacteria [167,168]. A similar procedure has been used to measure the transport of weak acids/bases in protoplasts of *Cl. pasteurianum* [169].

Osmotic swelling would appear an appropriate technique to study lactulose transport in *Cl. perfringens*, in the absence of radiolabelled substrate. It was decided to conduct these studies on protoplasts, rather than whole cells, in order to remove any interference of the cell wall in interpretation of results and, more importantly, to remove any resistance to plasmolysis.

The first part of the results section is concerned with attempts to protoplast *Cl. perfringens* cells. The experimental procedures proved unsuccessful, therefore, a completely different approach was taken to investigate sugar transport. These studies are considered in the final section of the chapter.

4.2 Results.

4.2.1 Production of *Cl. perfringens* Protoplasts.

Bacteria are normally protoplasted by treatment with lysozyme (mucopeptide N-acetylmuramyl-hydrolase, EC 3.2.1.17), in the presence of an osmotic stabilising agent. The basic procedure adopted for protoplast formation was that of Chassy and Giuffrida (Materials and Methods, section 2.2.13), which employs PEG as the osmotic stabiliser [157]. In order to standardise incubations, cells were resuspended to yield an A_{612} of approx. 1 and the amount of lysozyme to be added was calculated as a function of the dry wt content of the resuspension [157]. Thus, amounts of lysozyme added to incubations are expressed both as a concentration (mg/ml) and as a function of the cell dry wt content (mg/mg dry wt). Cells were routinely cultured in basal medium supplemented with lactulose (30 mM).

The main aim of these experiments was to determine if high yields of *Cl. perfringens* protoplasts could be obtained for use in transport studies, therefore, the methods used were only semi-quantitative. Although incubation conditions were varied in an attempt to increase the protoplast yield, this study was by no means a comprehensive survey of all the possible factors that influence protoplast formation.

Before consideration of the results, it should be noted that the term 'population change' does not necessarily refer to the formation of true protoplasts (rounded sacs of protoplasm), but changes in cell morphology. Often, a variety of morphological changes were observed in any one sample, and these occurred in a specific sequence, as judged by the predominant changes evident in progressive incubation samples.

Rods firstly appeared feint and contained beaded areas of dark contrasting protoplasm. This beaded appearance may have been due to the formation of plasmolysis vacuoles [170], however, since similar changes were only rarely observed in control samples, this implies an initial action of lysozyme on the cell wall. Protoplasm then became concentrated towards one pole of the rod and, finally, the feint rod outline of the original cells completely disappeared, leaving ovate areas of dark contrasting protoplasm with tailed structures attached. This tailed appearance of protoplasts has been observed for many other Gram-positive bacteria (*B. licheniformis*, *B. cereus*, *B. subtilis*, *M. lysodeikticus* and *Staph. aureus*) [171-173] and has been attributed to mesosomal membranes which have remained attached to the protoplast membrane. Mesosomal tags are normally only observed in the presence of >10-20 mM Mg^{2+} [171,172] and, since this ion was not routinely included in incubation mixtures, it is unlikely that this phenomenon could account for the morphology of *Cl. perfringens* protoplasts. Rather, since the appearance of the appendages coincided with the disappearance of the rod outline of cells, it is more likely that they resulted from the collapse of the cell wall, or were remnants of cell wall material.

In a preliminary experiment, cells were harvested at early exponential phase and

resuspended to 0.57 mg dry wt/ml suspension. Cells were incubated in 10 mM Tris/HCl buffer, pH 8.0, containing PEG (12% (w/v)) and lysozyme concentrations of 0, 12.5, 125 or 250 μg/ml (0, 0.09, 0.88 and 1.75 mg/mg dry wt, respectively). After 30 min, definite changes were evident in the incubation containing 0.09 mg/mg dry wt. The cells appeared as rods, but approx. 20% of the population was reduced in size relative to the control incubation. This proportion increased to 50% after a further 30 min of incubation. No changes were observed in any of the incubations containing higher lysozyme concentrations.

A wider range of low lysozyme concentrations was next studied, with extended incubation times. Incubations contained mid exponential cells and lysozyme (0-50 μg/ml) (Table 16). Highest yields (30-40% population change) were obtained with 150-290 μg lysozyme/mg dry wt. These changes were apparent after 300 min in the incubations containing lysozyme at 220 and 290 μg/mg dry wt, whereas comparable changes were evident after only 100 min in the incubation containing 150 μg/mg dry wt. However, no further changes were observed in the latter incubation up to 300 min.

The optimal lysozyme concentration range (150-290 μg/mg dry wt or 25-50 μg/ml) determined for *Cl. perfringens* was similar to values reported in the literature for a variety of Gram-positive bacteria; especially when lysozyme concentrations are expressed as a function of the cell dry wt content of incubations. Examples include: *Str. faecalis* (50 μg/mg dry wt or 180 μg/ml) [167], *M. lysodeikticus* (50 μg/mg dry wt or 25 μg/ml) [172], *L. fermenti* (100 μg/mg dry wt or 250 μg/ml) [174], *B. cereus* and *B. megaterium* (500 μg/mg dry wt or 250 μg/ml) [172]. These bacteria were protoplasted using phosphate buffer/sucrose incubation systems, and protoplast formation was normally complete within 2 h. Although Chassy and Giuffrida routinely used a lysozyme concentration of 1.2 mg/mg dry wt in a Tris buffer/PEG system [157,175], detailed study with *Str. mutans* and *L. casei* revealed that the optimal concentration was 80 μg/mg dry wt [157].

In general, much higher lysozyme concentrations have been employed in studies with the clostridia. It is only possible to consider these values as a concentration, since the cell dry wt content of incubations was not given. Thus, the production of *Cl. pasteurianum* and *Cl. acetobutylicum* protoplasts is normally complete within 1 h, when cells are incubated with 0.2-1.0 mg lysozyme/ml in phosphate buffer/sucrose systems [169,176,177]. In the case of *Cl. perfringens* ATCC 3626B, 50% protoplast formation is obtained after 1 h when cells are incubated with 1 mg lysozyme/ml in a thioglycollate/Tris/sucrose system [175]. However, in the present study, no change was obtained in incubations containing 0.125 and 0.25 mg/ml lysozyme, after 1 h incubation, as reported above (preliminary experiment). When incubations were conducted for 80 min with even higher lysozyme concentrations of 0.6 and 1.2 mg/ml, there were no apparent changes in cell morphology, but cell clumping was observed in the incubation containing the highest lysozyme concentration. Other workers have reported that high lysozyme concentrations may be inhibitory for protoplast formation [157], and a similar situation appeared to exist for the strain of *Cl. perfringens* studied here.

Table 16: Formation of *Cl. perfringens* protoplasts in a Tris buffer system.

Lysozyme concentration		% Population change					
µg/ml buffer	µg/mg dry wt	30 min	60 min	70 min	100 min	160 min	300 min
0	0	0	0	ND	0	1	1
5	30	0	0	ND	1	5	ND
10	60	0	3	ND	4	3	7
15	90	0	5	ND	10	20	20
25	150	3	10	ND	40	40	30
38	220	2	ND	2	5	ND	40
50	290	2	ND	2	5	ND	30

Cells harvested from a mid-exponential phase, lactulose (30 mM)-grown culture were incubated with a range of lysozyme concentrations in 10 mM Tris HCl buffer pH 8.2, plus PEG (12% (w/v)) as stabiliser. Incubations contained 0.17 mg dry wt cells/ml buffer. Samples were removed at the indicated time point and examined for morphological change under phase contrast microscopy.

ND = not determined.

In an attempt to improve the yield of protoplasts, different incubation conditions were studied. Mid exponential cells were incubated in a phosphate buffer system (25 mM sodium phosphate, pH 7.0), with PEG (12% (w/v)) and a range of lysozyme concentrations (Table 17). The optimum lysozyme concentration appeared to be 90 μg/mg dry wt (13 μg/ml), which produced a 10% population change after 2 h incubation (Table 17). Therefore, substitution of Tris/HCl buffer, pH 8.0 with phosphate buffer, pH 7.0 was without any stimulatory effect. If anything, the Tris buffer system was more advantageous (40% change after 100 min with 150 μg/mg dry wt lysozyme) (Table 16).

Inclusion of Mg^{2+} ions in protoplasting media has been shown to improve protoplast yields of *Cl. acetobutylicum* [176] and *L. fermenti* [174]. Therefore, mid exponential cells were incubated in the PEG/Tris system with a fixed lysozyme concentration (37 μg/ml or 300 μg/mg dry wt) and a range of $MgCl_2$ concentrations. After 120 min, % population changes in incubations containing 0, 5, 10 and 20 mM $MgCl_2$ were 10, 4, 2 and 1%, respectively. Thus, Mg^{2+} ions exerted an inhibitory effect on protoplast formation. Similar findings have also been reported for oral streptococci and *L. casei* [157,178].

Mid exponential cells of the *Cl. perfringens* strain used in the present study exhibited greatly reduced susceptibility to lysozyme, relative to other clostridia [169,176,177], including *Cl. perfringens* ATCC 3626B [175]. Indeed, marked species and strain differences have been reported for other Gram-positive bacteria (lactobacilli, streptococci, propionibacteria, pediococci, actinomyces) [157]. In total, 5 different mechanisms have been proposed to account for the resistance of cell wall peptidoglycan to lysozyme [179,180]:

a) Presence of O-acetyl groups on amino sugar residues:
M. lysodeikticus, *B. megaterium*, *Str. faecalis*, *Staph aureus* and various lactobacilli.

b) Attachment of polymers such as teichoic acid or polysaccharide:
Staph. aureus, streptococci and lactobacilli.

c) Occurrence of free amino groups, probably in the the peptide portion:
M. lysodeikticus, *Corynebacterium tritia*, *Staph. aureus*, *Str. faecalis* and
B. megaterium.

d) High degree of peptide cross-linking:
Staph. aureus.

e) Occurrence of N-nonsubstituted glucosamine residues:
B. cereus, *B. megaterium* and *B. subtilis*.

The contribution of peptide cross-linking to lysozyme resistance can be reduced by supplementation of growth media with amino acids such as threonine, lysine and glycine [157,176,178]. Glycine has been shown to be stimulatory to the formation of protoplasts of *Cl. acetobutylicum* [176] and *Cl. perfringens* ATCC 3626B [175], even though these bacteria are relatively lysozyme-sensitive.

Glycine-supplementation of the basal medium was therefore investigated. Cultures of *Cl. perfringens* were grown to early exponential phase in basal medium supplemented with

Table 17: Formation of *Cl perfringens* protoplasts in a phosphate buffer system.

Lysozyme concentration		% Population change	
µg/ml buffer	µg/mg dry wt	60 min	120 min
0	0	0	1
1	10	1	5
3	20	3	5
13	90	4	10
25	180	2	3
125	890	1	1

Cells harvested from a mid-exponential phase, lactulose (30 mM) grown culture were incubated with a range of lysozyme concentrations in 25 mM potassium phosphate buffer pH 7.0, plus PEG (12% (w/v)) as stabiliser. Incubations contained 0.14 mg dry wt cells/ml. Samples were removed at the indicated time point and examined for morphological change under phase-contrast microscopy.

lactulose (30 mM) alone or lactulose (30 mM) plus glycine (0.4% (w/v)), and cells were resuspended to give 0.70 and 0.42 mg dry wt/ml, respectively. Samples were incubated with a fixed amount of lysozyme (230 μg/mg dry wt) in the PEG/Tris system. After 200 min, changes were apparent in approx. 75% of the glycine-supplemented cells and 80% of the unsupplemented cells.

Although glycine did not appear to have any stimulatory effect on protoplast formation, very high yields were obtained from cells cultured in the absence of glycine. This may have been related to the stage of culture growth at cell harvesting. Early exponential cells were also used in the preliminary experiment and relatively high yields (50% population change) were observed after only 1 h of incubation with 0.09 mg lysozyme/mg dry wt in the PEG/Tris system. Studies with other Gram-positives (*Str. faecalis*, *Str. mutans* and *L. casei*) have indicated an influence of growth phase on lysozyme-susceptibility, however, stationary phase cells of these bacteria are most sensitive [157,167].

The differences between early and mid exponential phase cells of *Cl. perfringens* may have been due to environmental effects on cell wall synthesis. Growth in medium supplemented with 30 mM lactulose was found to result in acidification of the medium to pH 6.7 in early exponential phase, and pH 5.7 in mid exponential phase. It is possible that these differences in medium pH may have effected cell wall synthesis, thereby resulting in concomitant differences in lysozyme-susceptibility with growth phase. Alternatively, studies with *B. megaterium* and *B. subtilis* have shown that culture pH can influence protoplast morphology [181-183]. Cells harvested from an acidic environment (pH 5) produce rod-shaped protoplasts, whereas cells harvested at pH 7 produce spherical protoplasts. These differences have been attributed to differences in the phospholipid composition of the protoplast membrane [181-183]. It is unlikely that the rod-shaped structures observed in incubations containing mid exponential phase cells of *Cl. perfringens* were in fact protoplasts, since there was no apparent change in phase contrast of these structures when compared with control cells.

It should be noted that changes (presence of tailed protoplasts) were occasionally observed in control incubations. These changes were minor (ca. 1%) (Tables 16 and 17), and only observed upon prolonged incubation. Control changes are most likely due to autoplast formation, which has been reported for *Cl. perfringens* strain 11268 CDR [184]. Overnight incubation of this strain at room temperature in brain heart infusion with glucose (1% (w/v)) and sucrose (0.4 M) resulted in 50-90% autoplast formation [184].

Although high yields of protoplasts were obtained with early exponential cells, rather long incubation times (200 min) were still required. This was undesirable, since it was intended to use the protoplasts in sugar uptake studies and prolonged incubation at 37 °C may have resulted in metabolic changes (due to protein turnover), or even damage to cell membranes. Therefore, it was decided to take a different approach to investigate sugar transport.

4.2.2 Whole Cell Studies: Assay of Sugar Utilisation.

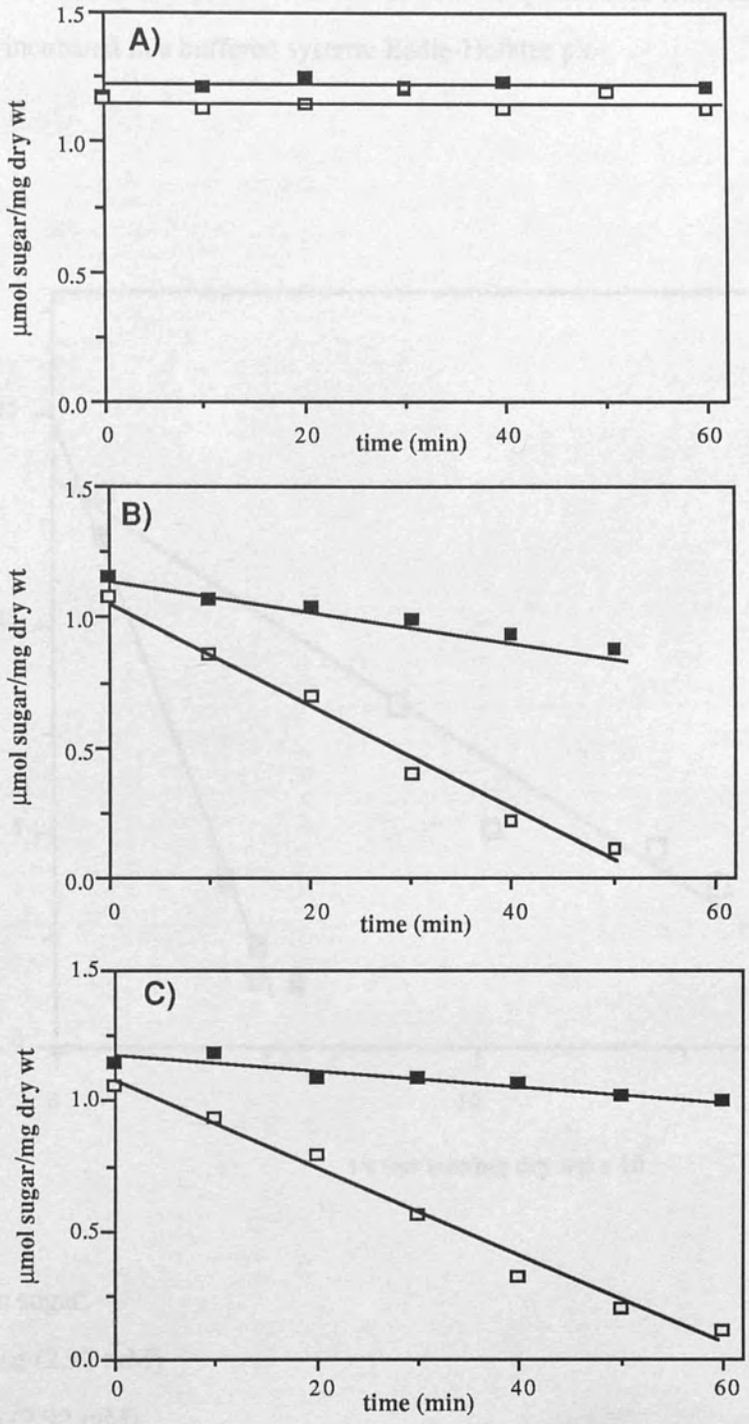
A buffered utilisation system was developed; consisting of a dense cell suspension (normally mid exponential cells), incubated with sugar, in sodium phosphate buffer pH 7.0 (50 mM), plus ascorbate (0.1% (w/v)) as reductant (Materials and Methods, section 2.2.12). Uptake was measured indirectly by assay of the sugar remaining in the medium, using colourimetric procedures. Medium sugar was expressed as a function of the cell dry wt content of incubations, since bacterial concentrations varied slightly between incubations. Various inhibitors, which interfere with the mechanisms of energy coupling, were added to incubations, in order to gain information regarding the mechanism(s) of sugar transport. Similar methodology has been employed to study lactose utilisation in lactic streptococci [94].

Typical utilisation patterns of lactulose (2.92 mM) or lactose (2.92 mM) by basal medium-grown, lactulose-grown and lactose-grown cells are depicted in Fig. 24. In these and subsequent experiments, sugars were added to growth media at 20 mM concentrations. Utilisation of lactulose or lactose by basal medium-grown cells was not detectable (Fig. 24A), whereas both sugars were used when incubations contained lactulose- or lactose-grown cells (Figs 24B and C, respectively). Identical findings were obtained when the experiment was repeated using cells harvested from another basal medium culture (data not shown). These findings suggest that the lactulose and lactose utilisation systems are inducible. Furthermore, induction of these systems only occurs when cells are cultured in nutrient-rich medium in the presence of β-galactosides.

When detectable, sugar utilisation was linear over the period studied (Figs 24B and C). The respective rates of lactulose and lactose utilisation were calculated as 5.2 and 20.1 nmol/min/mg dry wt for lactulose-grown cells, and 3.2 and 17.1 nmol/min/mg dry wt for lactose-grown cells. When the lactulose utilisation rate was expressed as a percentage of the lactose uptake rate, then similar values were obtained for both growth conditions; values were 25.9% for lactulose-grown cells, and 18.7% for lactose-grown cells. Thus, inclusion of either β-galactoside in the growth medium appears to result in the induction of similar metabolic systems for lactose or lactulose in *Cl. perfringens*.

In order to determine if both lactose and lactulose are used by a single utilisation system, kinetic studies were undertaken with lactulose-grown cells. Rates of lactose and lactulose utilisation were measured over a range of substrate concentrations (0.25, 0.33, 0.5, 1.0 and 10 mM sugar), and results were analysed by Eadie-Hofstee and double-reciprocal plots. Both procedures yielded similar kinetic parameters (Table 18), therefore, only the Eadie-Hofstee plot is shown (Fig. 25). Lactulose-grown cells possessed a greater affinity for lactose relative to lactulose, but, more importantly, the V_{max} values were almost identical. This indicates that both lactulose and lactose are substrates of the same utilisation system, produced in response to growth in lactulose-supplemented medium.

Figure 24: Time course of lactulose and lactose utilisation in a buffered incubation system.



Growth medium:

A) Basal medium

B) Basal medium + lactulose (20 mM)

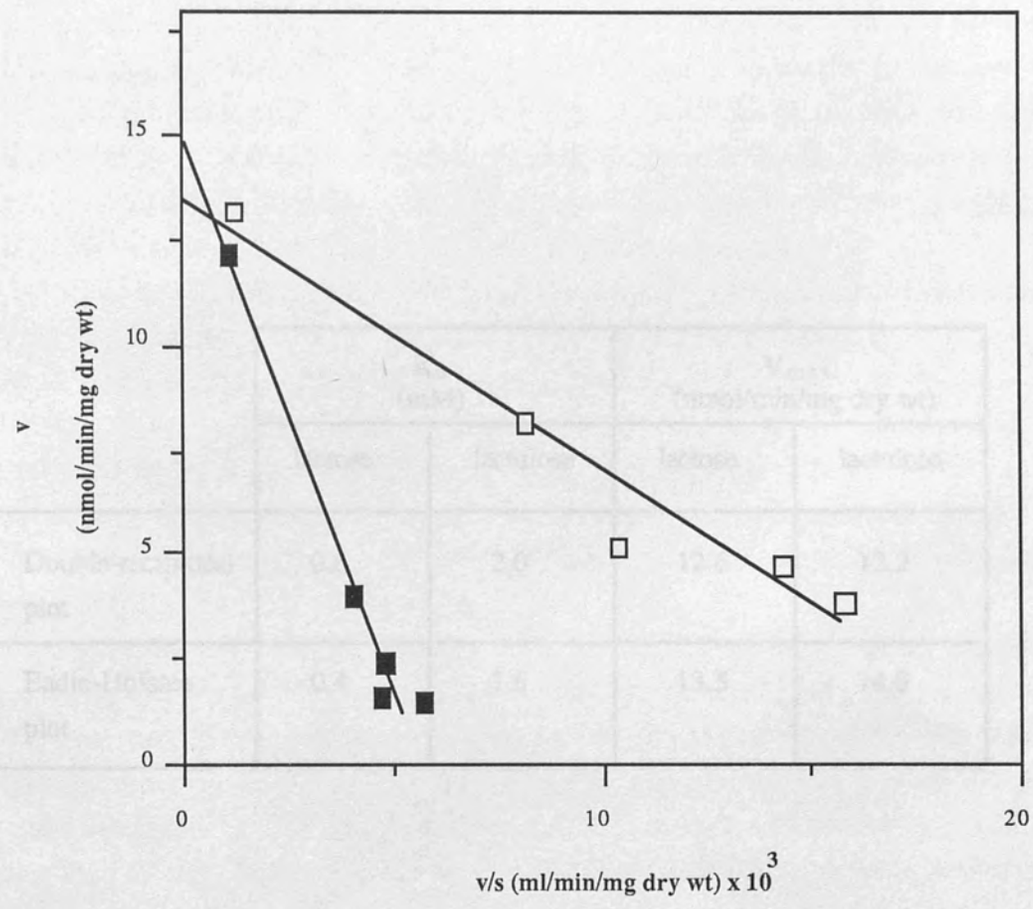
C) Basal medium + lactose (20 mM)

Incubation sugar:

■ - lactulose (2.92 mM)

□ - lactose (2.92 mM)

Figure 25: Kinetics of lactulose and lactose utilisation by lactulose-induced cells incubated in a buffered system: Eadie-Hofstee plot.



Incubation sugar:

■ lactulose (2.92 mM)

□ lactose (2.92 mM)

Table 18: Lactulose and lactose utilisation by lactulose (20 mM)-grown cells: kinetic constants determined in the buffered incubation system.

	K_m (mM)		V_{max} (nmol/min/mg dry wt)	
	lactose	lactulose	lactose	lactulose
Double-reciprocal plot	0.6	2.0	12.6	12.2
Eadie-Hofstee plot	0.4	1.6	13.5	14.8

A series of experiments were performed with sugar transport inhibitors which interfere with energy coupling mechanisms, in an attempt to gain information regarding the mechanism(s) of β-galactoside transport. The clostridia lack membrane-associated electron transport systems [40], and are dependent upon membrane-bound ATPase activity for the formation of a pmf [185,186]. The clostridial enzyme is inhibited by DCCD [185,186] and this property was exploited in the present study.

Addition of DCCD (50 μM) to incubations containing lactulose-grown cells plus lactulose (2.92 mM) (Fig. 26A), or lactose-grown cells plus lactose (2.92 mM) (Fig. 26B), resulted in inhibition of sugar utilisation. The DCCD had been dissolved in ethanol, and addition of identical amounts (0.5% (v/v)) of ethanol to control incubations was without any effect (Figs 26A and B). Degrees of inhibition of lactulose and lactose utilisation by DCCD were comparable; being 66.7% and 62.3%, respectively. Thus, similar mechanisms of energy coupling appeared to operate for lactulose transport by lactulose-induced cells, and lactose transport by lactose-induced cells.

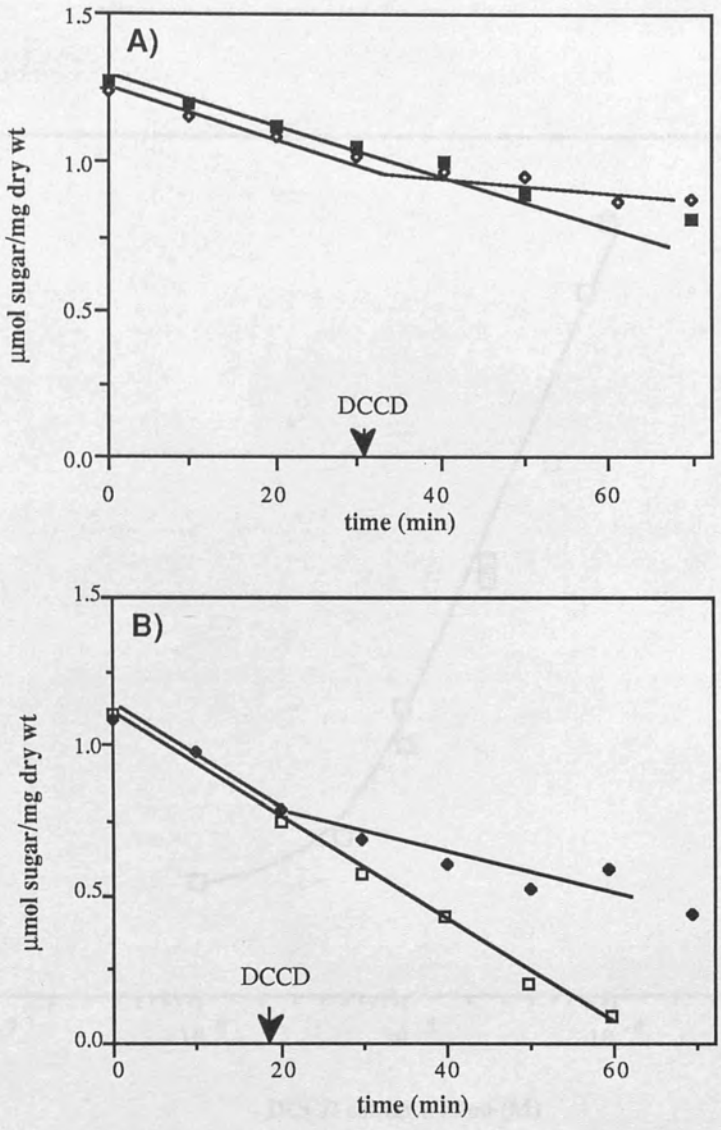
It was notable that inhibition of sugar utilisation was not complete in both incubations. This may be explained by the operation of additional transport system(s) which are not dependent upon the pmf (e.g. PTS systems), or incomplete inhibition of the membrane ATPase. Previous studies with membrane vesicles of *Cl. perfringens* have shown that ATPase activity is inhibited by a factor of 95% in response to 50 μM DCCD [186]. However, only 80% inhibition of the ATPase activity of *Cl. pasteurianum* is observed with DCCD concentrations as high as 100 μM [185]. It was therefore pertinent to study the effect of different DCCD concentrations.

Experiments were conducted with cells harvested from two different cultures of lactulose -grown cells. Incubations contained lactulose (2.92 mM), and a total of 8 different DCCD concentrations (0, 1, 5, 10, 25, 50, 75 and 100 μM) were studied. Utilisation was gradually inhibited as the DCCD concentration was increased, and this effect was non-linear (Fig. 27). At the highest DCCD concentration studied, utilisation was inhibited by 90%. These findings support the suggestion that incomplete inhibition of sugar transport by 50 μM inhibitor was due to the incomplete inhibition of ATPase activity.

The proton conductor CCCP dissipates the electrochemical proton gradient in *Cl. pasteurianum* [51] and the effect of this inhibitor on β-galactoside utilisation by *Cl. perfringens* was studied. Addition of CCCP (50 μM) completely abolished lactose and lactulose utilisation by both lactulose-induced (Fig. 28A) and lactose-induced (Fig. 28B) cells. Inhibition appeared to be instantaneous in all cases. These findings argue for the involvement of an electrochemical gradient of protons in the energisation of β-galactoside transport in both lactose- and lactulose-induced cells of *Cl. perfringens*. Furthermore, the results of the studies with DCCD suggest that the membrane-bound ATPase of this organism may play a major role in the generation of this gradient.

Finally, two experiments were conducted to study the effect of fluoride on lactulose utilisation by lactulose-induced cells. Fluoride prevents the formation of a PEP potential,

Figure 26: β-Galactoside utilisation in a buffered incubation system: effect of DCCD.



A) Lactulose utilisation by lactulose (20 mM)-induced cells:

■ control

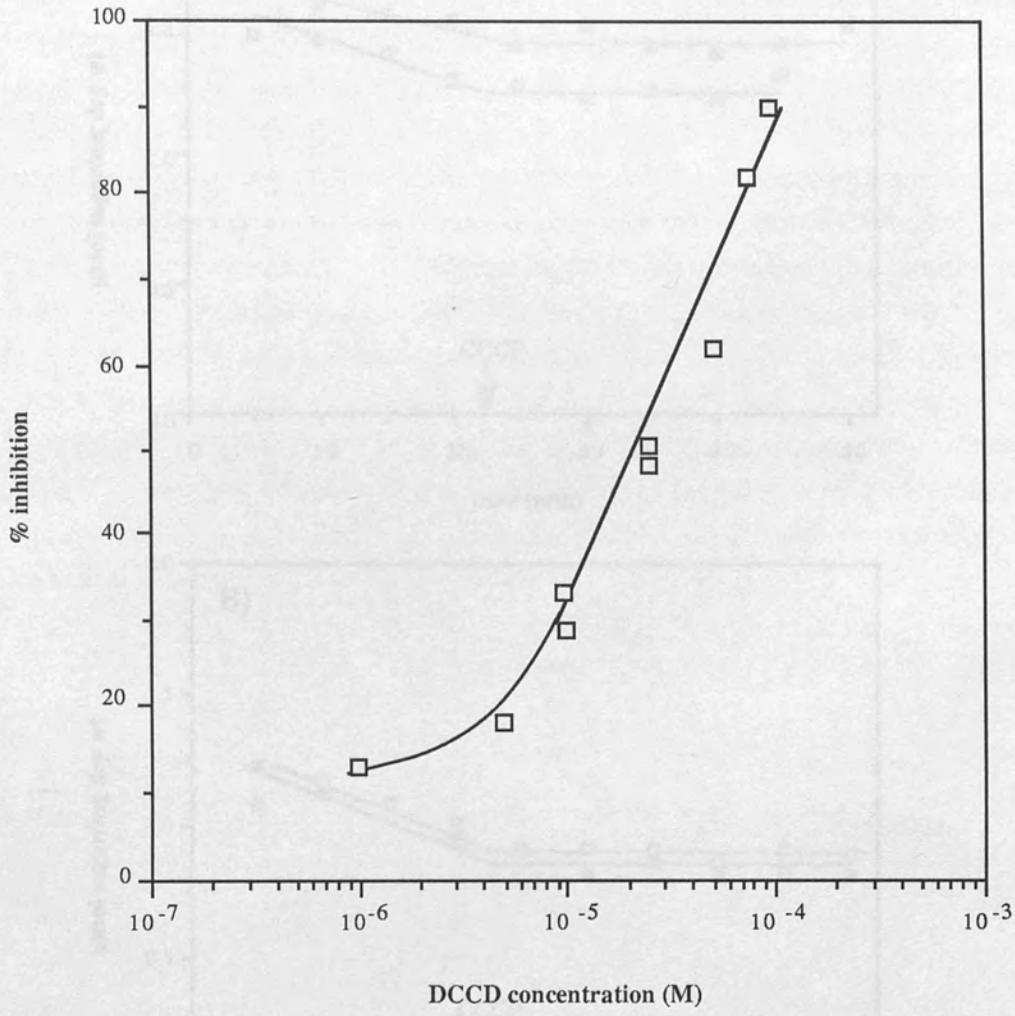
◆ + DCCD (50 µM)

B) Lactose utilisation by lactose (20 mM)-induced cells:

□ control

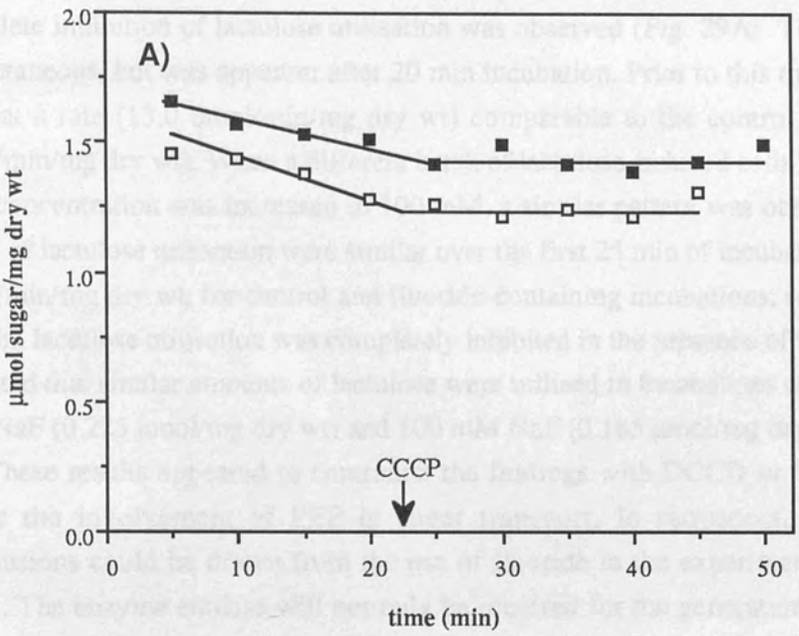
◆ + DCCD (50 µM)

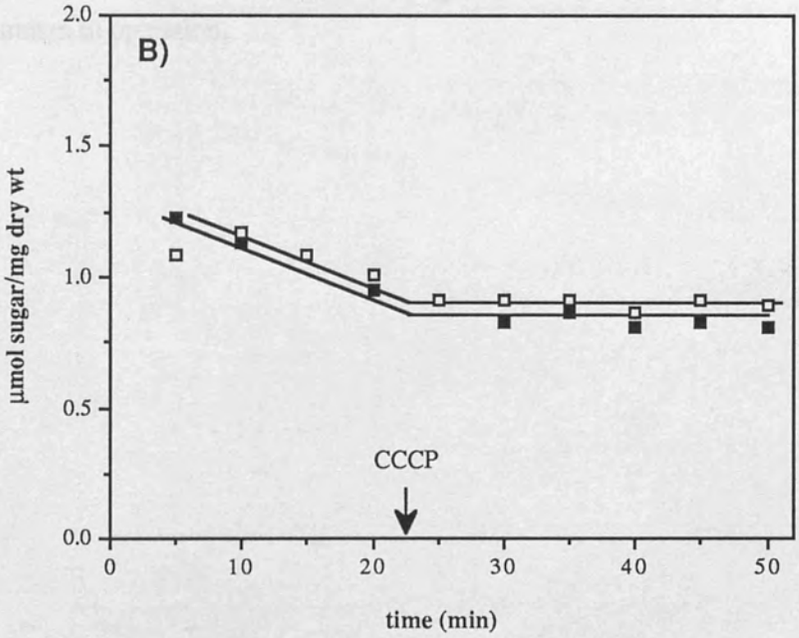
Figure 27: Effect of DCCD on lactulose utilisation by lactulose-induced cells in a buffered incubation system: dose-response curve.



Cells were induced for the lactulose utilisation system by growth in basal medium supplemented with lactulose (20 mM). Incubations contained 2.92 mM lactulose.

Figure 28: β-Galactoside utilisation in a buffered incubation system: effect of CCCP.





Growth conditions:

A) Basal medium + lactulose (20 mM)

B) Basal medium + lactose (20 mM)

Incubation sugar:

■ lactulose (2.92 mM)

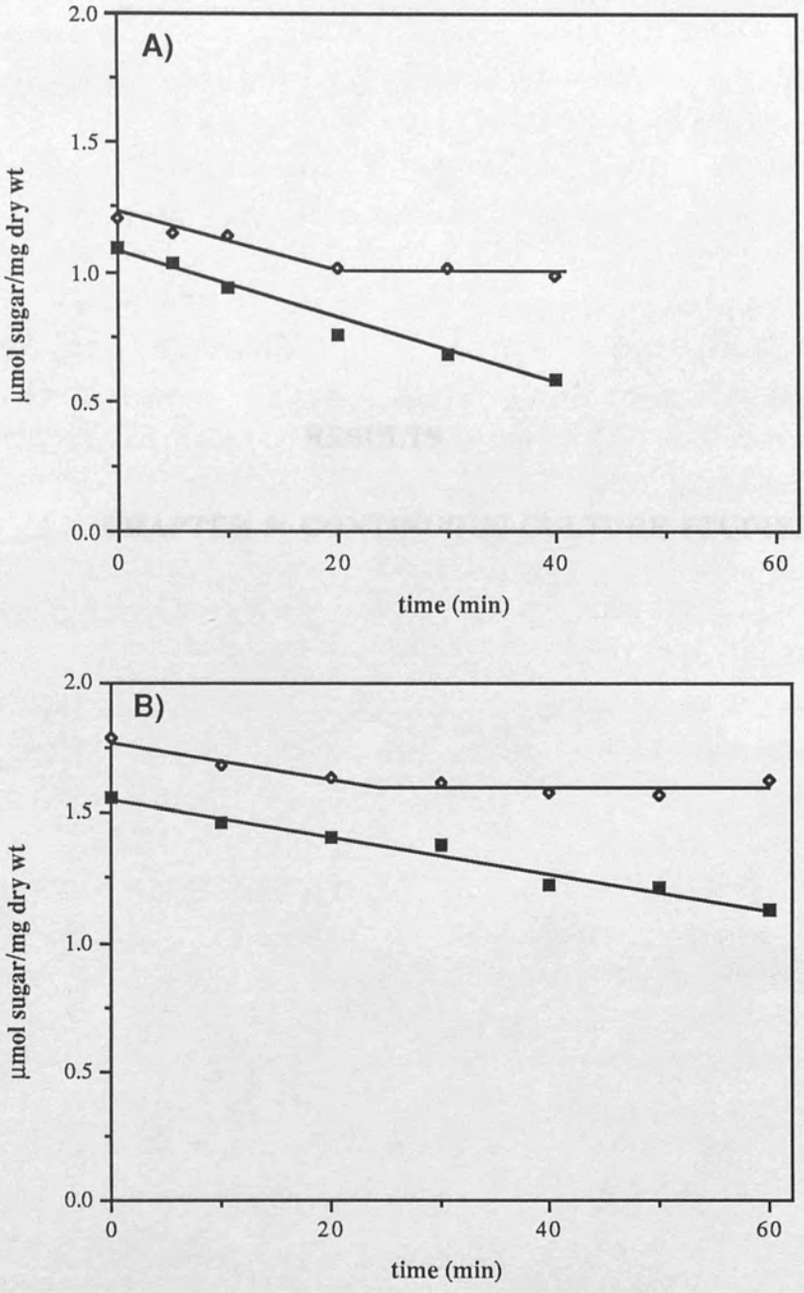
□ lactose (2.92 mM)

via the inhibition of the enzyme enolase (EC 4.2.1.11), and is often used in transport studies as an inhibitor of PEP-dependent systems.

When an incubation was conducted in the presence of 30 mM NaF (added at time 0), complete inhibition of lactulose utilisation was observed (Fig. 29A). This effect was not instantaneous, but was apparent after 20 min incubation. Prior to this time, lactulose was used at a rate (13.0 nmol/min/mg dry wt) comparable to the control incubation (13.1 nmol/min/mg dry wt). When a different batch of lactulose-induced cells was used, and the NaF concentration was increased to 100 mM, a similar pattern was obtained (Fig. 29B). Rates of lactulose utilisation were similar over the first 25 min of incubation at 7.4 and 6.7 nmol/min/mg dry wt, for control and fluoride-containing incubations, respectively. After 25 min, lactulose utilisation was completely inhibited in the presence of fluoride. It should be noted that similar amounts of lactulose were utilised in incubations containing both 30 mM NaF (0.225 μmol/mg dry wt) and 100 mM NaF (0.185 μmol/mg dry wt).

These results appeared to contradict the findings with DCCD or CCCP, since they imply the involvement of PEP in sugar transport. In retrospect, only ambiguous conclusions could be drawn from the use of fluoride in the experimental system under study. The enzyme enolase will not only be required for the generation of PEP, but also for the continued metabolism of the sugar under study, irrespective of the transport mechanism in operation.

Figure 29: β-Galactoside utilisation in a buffered incubation system: effect of fluoride.



Incubations contained lactulose-induced cells and lactulose (2.92 mM)

A) Control (■) and 30 mM fluoride (◆)

B) Control (■) and 100 mM fluoride (◆).

RESULTS

CHAPTER 5: CONTINUOUS CULTURE STUDIES

5.1 Introduction.

Batch cultures of bacteria normally exhibit characteristic growth curves consisting of lag, exponential and stationary phases. The lag phase corresponds to a period of metabolic adjustment, then cells increase in mass and divide, resulting in an exponential increase in culture density. Cells are in a constant state of physiological change during exponential growth, due to the continual removal of nutrients from the medium and excretion of metabolic end products. Growth eventually ceases, due to the depletion of essential nutrients and/or a build up of toxic metabolites, and the culture enters stationary phase.

Studies with batch culture systems are of limited value for extrapolation to natural systems, such as the large intestine. The exponential phase of batch culture growth is often characterised by high growth rates, since essential nutrients are normally present in excess, whereas growth in natural systems generally proceeds at low rates, since nutrients are present at low concentrations [187,188]. The use of an 'open' culture system, such as the chemostat, can permit closer approximation of these natural conditions.

Growth of organisms in continuous culture is controlled by limiting the supply of a single essential nutrient, all other nutrients being present in excess. The initial concentration of the growth limiting nutrient determines the bacterial cell density, whereas its rate of supply determines the growth rate. This allows growth rates to be varied independently of the initial substrate concentration [187]. A further important advantage of this type of culture sytem is that the environment can be rigorously controlled, thereby permitting the attainment of steady state growth conditions [187,188].

Continuous culture has certain limitations when attempting to model natural systems such as the large intestine [189,190]. Nutrients do not enter the colon in a steady stream, rather, there is pulsed entry of ileal fluid after a meal, followed by fluid absorption from the colon. Thus, changes in the effective culture volume also occur. In addition, growth does not proceed under steady state conditions; there may be intermittent periods of relatively rapid growth (after feeding), followed by long periods of slower growth. Finally, a further important consideration is that a mixed microbial flora is present *in vivo*. Thus, the growth of different bacterial groups may be limited by different factors, and this pattern may change with time.

It is clear that an accurate model of the large intestine may never be produced using *in vitro* culture techniques. Indeed, this was not a major aim of the present study. Rather, due to the limitation of time, only the effect of growth rate on lactulose metabolism was studied. These experiments were performed with a single organism, *Cl. perfringens*, under conditions of both C-limitation and environmental control. Although these conditions were by no means an accurate reflection of the *in vivo* situation, they were nevertheless a considerable improvement on batch culture conditions.

5.2 Results.

5.2.1 Determination of the Growth Limiting Substrate Concentration - Batch Growth.

Cells were cultured under batch conditions in basal medium supplemented with a range of lactulose concentrations (0-40 mM) in order to determine the growth limiting substrate concentration. Samples were periodically removed and both growth (turbidity) and sugar utilisation (t.l.c.) were determined. Turbidity readings were used to calculate culture dry wt from a calibration curve, and specific growth rates were determined from plots of log dry wt versus time (Materials and Methods, section 2.2.6.2).

A hyperbolic relationship was observed between growth rate and the medium lactulose concentration (Fig. 30A). Growth was limited by lactulose concentrations <10 mM, whereas at higher concentrations another component of the basal medium had presumably become growth limiting. This hyperbolic relationship suggested that growth rate was related to medium lactulose concentration according to the Monod equation [191]:

$$\mu = \mu_{max} .s / (K_s + s)$$

Where:

μ is the specific growth rate at limiting nutrient concentration s.

μ_{max} is the growth rate at saturating concentrations of limiting nutrient.

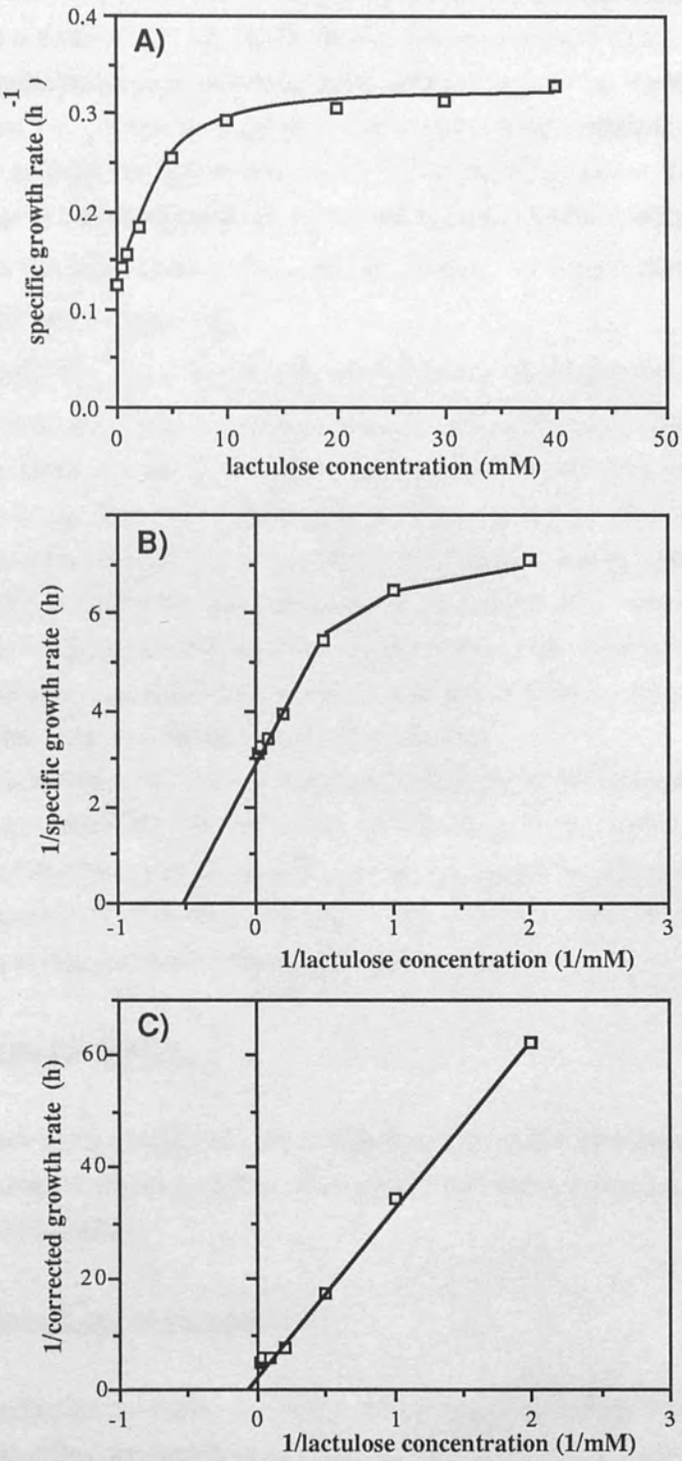
K_s is the saturation constant (the substrate concentration which supports a growth rate of $\mu_{max}/2$).

This equation is analagous to the Michaelis-Menten equation of enzyme kinetics, and when data were represented in the form of a double-reciprocal plot (Fig. 30B), a linear relationship was only observed at lactulose concentrations ≥2 mM. The linear portion of the curve was used to calculate values for μ_{max} and K_s of 0.341 h^{-1} and 1.85 mM, respectively.

The deviation from linearity at low lactulose concentrations was due to inordinately high growth rates (Fig. 30B). The basal medium was chemically undefined (Materials and Methods, section 2.2.1.3) and contained a complex mixture of potential carbon and energy sources in the form of proteose-peptone and yeast extract. It was possible, therefore, that non-linearity was due to the utilisation of these additional carbon and energy sources. Indeed, growth in basal medium alone was significant (μ = 0.126 h^{-1}; Fig. 30A).

The correction of all data for growth in basal medium alone appeared inappropriate, since the resulting double-reciprocal plot also exhibited non-linear behaviour (Fig. 30C). In this case, non-linearity was observed at lactulose concentrations ≥20 mM, and was due to inordinately low growth rates (Fig. 30C). Consideration of the linear portion of the curve permitted the calculation of a μ_{max} of 0.4 h^{-1} and a K_s of 10 mM.

Figure 30: Effect of medium lactulose concentration on the specific growth rate in batch culture.



Specific growth rates were determined from plots of log dry wt versus time.
Subtraction of the growth rate in basal medium alone gave the corrected growth rate.

The above findings can probably be best explained in terms of a repressive effect of lactulose on the utilisation of basal medium components. Repression may be weak at low lactulose concentrations, reaching maximum levels as the medium lactulose concentration is increased to a value of 2 mM. In the absence of data evaluating the utilisation of basal medium components at each lactulose concentration, it may be inappropriate to simply correct all data for growth in basal medium alone. On the other hand, it may also be erroneous to consider the linear portion of the uncorrected curve (Fig. 30B); although repression may be maximal at lactulose concentrations ≥2 mM, it may not necessarily be absolute. Thus, the true values for μ_{max} and K_s probably lie within the ranges 0.341-0.400 h^{-1} and 1.85-10 mM, respectively.

Growth yields (Y_{LL}) (Table 19) were determined at each lactulose concentration from plots of culture dry wt versus lactulose utilisation (data not shown). Values were expressed in molar terms (units = g dry wt/mol lactulose). Growth yields were fairly stable between 5-40 mM lactulose: mean ± SD values were 112 ± 11 g dry wt /mol lactulose (n = 5). At lower lactulose concentrations, yields were much higher, ranging from 213 to 435 g dry wt/mol substrate at lactulose concentrations of 2.0 and 0.5 mM, respectively. These data support the above suggestion that carbon and energy sources present in the basal medium may be used at low lactulose concentrations, and lactulose may repress the utilisation of these compounds in a concentration-dependent manner.

When yields were corrected for basal medium growth, values were constant at 74 ± 4 (mean ± SD) for lactulose concentrations of 2-40 mM (n=6) (Table 19). Slightly lower growth yields were obtained at 0.5 and 1.0 mM lactulose; values were 54 and 62 g/mol lactulose, respectively. However, correction for growth in basal medium alone may be inappropriate, as outlined above for the growth rate data.

5.2.2 Chemostat Studies.

All cultures were conducted with 5 mM lactulose as the limiting nutrient, at constant pH (7.0). In total, 4 separate culture runs were performed, covering a range of dilution rates from 0.030-0.436 h^{-1}.

5.2.2.1 Growth and sugar utilisation.

Culture turbidity increased gradually with dilution rate (Fig. 31A), whereas a more complex relationship existed between the residual medium lactulose concentration and dilution rate (Fig. 31B). The lactulose concentration was relatively high (3.5%) at the lowest dilution rate (0.030 h^{-1}) studied, and then declined as the dilution rate was increased to 0.120 h^{-1}. A steady increase in the residual medium lactulose concentration was observed with further increases in dilution rate.

The rate of lactulose utilisation in the chemostat was calculated using the following

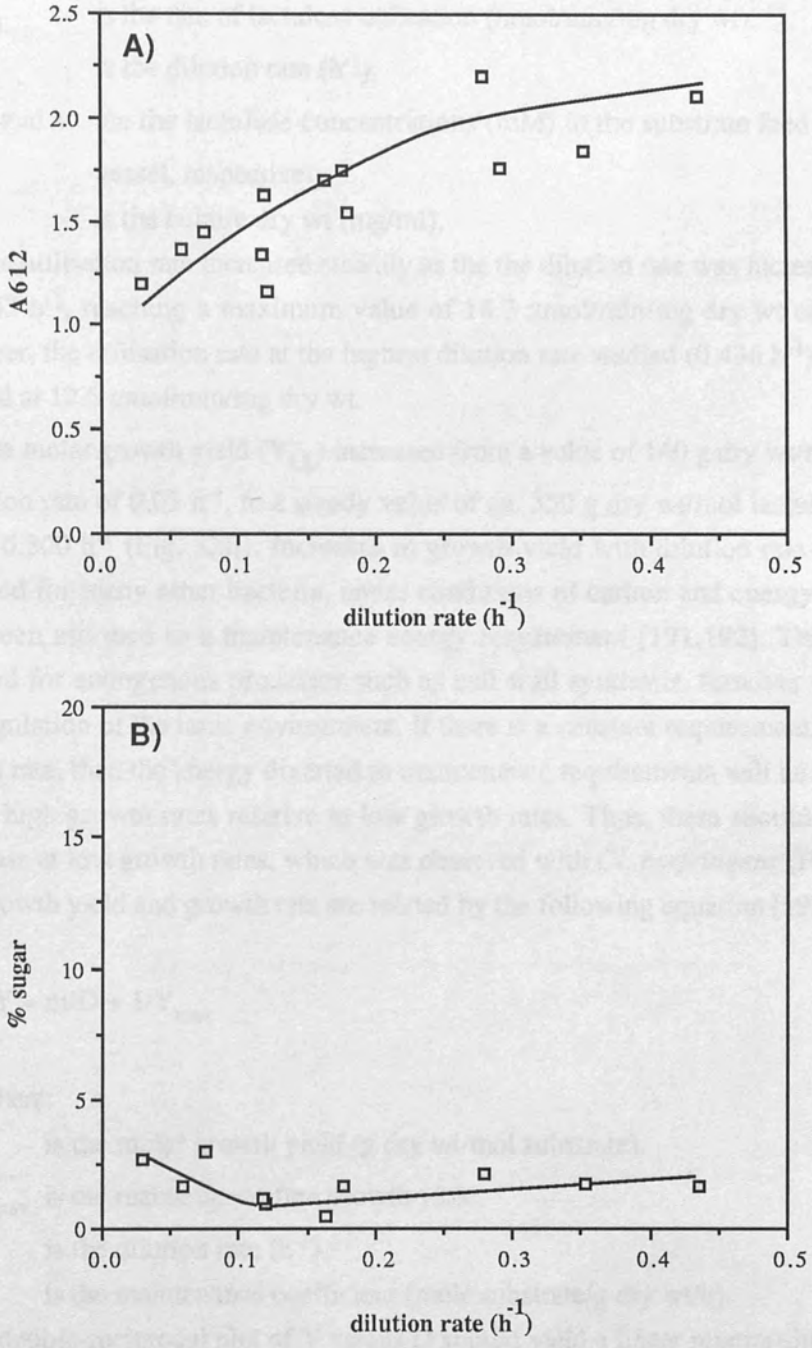
Table 19: Growth yields of *Cl. perfringens* cells grown in batch culture in basal medium supplemented with lactulose.

Lactulose concentration (mM)	Y_{LL} (g dry wt/mol lactulose)	Corrected Y_{LL} (g dry wt/mol lactulose)
0.5	435	54
1.0	301	62
2.0	213	75
5.0	130	80
10.0	116	77
20.0	108	74
30.0	105	71
40.0	102	68

Samples were removed throughout culture growth in basal medium supplemented with a range of lactulose concentrations. Yield coefficients were determined from plots of culture dry wt versus the residual medium lactulose concentration. Corrected yield coefficients were similarly calculated, except that culture dry wt was corrected for growth in basal medium alone.

Figure 31: Chemostat growth under conditions of lactulose-limitation:

effect of dilution rate on culture growth and residual medium lactulose.



The lactulose concentration in the substrate feed was 5 mM.

Lactulose was assayed by the cysteine-carbazole procedure.

equation:

$$q_{LL} = D\ (s_o - s) / 60\ x$$

Where:

q_{LL} is the rate of lactulose utilisation (nmol/min/mg dry wt).

D is the dilution rate (h^{-1}).

s_o and s are the lactulose concentrations (mM) in the substrate feed and the culture vessel, respectively.

x is the culture dry wt (mg/ml).

The utilisation rate increased steadily as the the dilution rate was increased from 0.030 to 0.353 h^{-1}, reaching a maximum value of 18.3 nmol/min/mg dry wt cells (Fig. 32A). However, the utilisation rate at the highest dilution rate studied (0.436 h^{-1}) was somewhat reduced at 12.6 nmol/min/mg dry wt.

The molar growth yield (Y_{LL}) increased from a value of 140 g dry wt/mol lactulose, at a dilution rate of 0.03 h^{-1}, to a steady value of ca. 350 g dry wt/mol lactulose, at dilution rates >0.300 h^{-1} (Fig. 32B). Increases in growth yield with dilution rate have also been observed for many other bacteria, under conditions of carbon and energy limitation, and have been ascribed to a maintenance energy requirement [191,192]. This is the energy required for endogenous processes such as cell wall synthesis, turnover of cell material and regulation of the ionic environment. If there is a constant requirement, irrespective of growth rate, then the energy diverted to maintenance requirements will be proportionately less at high growth rates relative to low growth rates. Thus, there should be a decreased cell mass at low growth rates, which was observed with *Cl. perfringens* (Fig. 31A).

Growth yield and growth rate are related by the following equation [192]:

$$1/Y = m/D + 1/Y_{max}$$

Where:

Y is the molar growth yield (g dry wt/mol substrate).

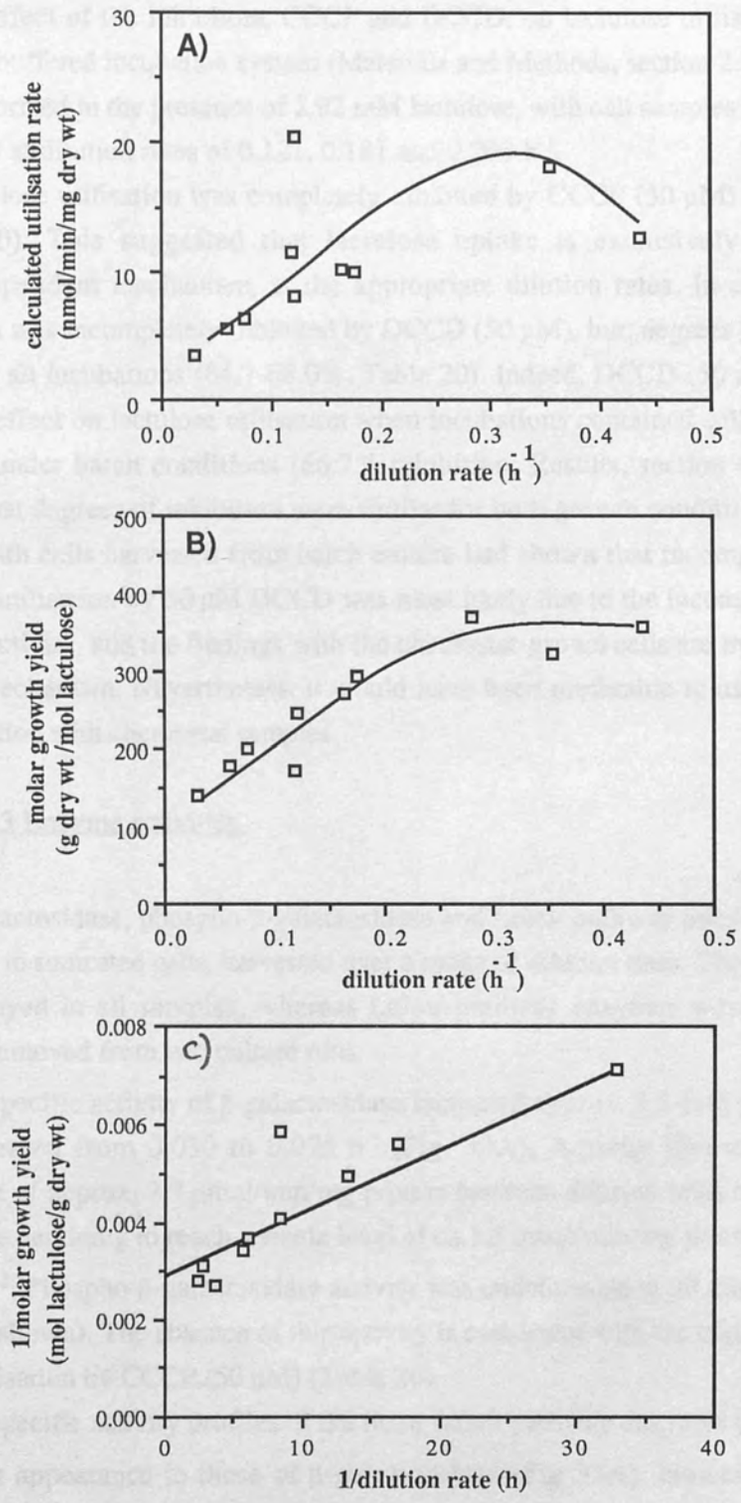
Y_{max} is the maintenance-free growth yield.

D is the dilution rate (h^{-1}).

m is the maintenance coefficient (mole substrate/g dry wt/h).

A double-reciprocal plot of Y versus D should yield a linear relationship, with slope m and intercept on the co-ordinate of $1/Y_{max}$, providing that there is no change in the fermentation product pattern with growth rate [192]. Indeed, when the data from *Cl. perfringens* were treated accordingly, the expected relationship was observed (Fig. 32C). The maintenance coefficient was calculated as 0.176 mmol/g dry wt/h, and Y_{max} as 388 g

Figure 32: Chemostat growth under conditions of lactulose-limitation: calculated utilisation rate and molar growth yield.



The lactulose concentration in the substrate feed was 5 mM.

dry wt/mol lactulose.

5.2.2.2 Sugar transport studies.

The effect of the inhibitors, CCCP and DCCD, on lactulose utilisation was studied using the buffered incubation system (Materials and Methods, section 2.2.12). Incubations were performed in the presence of 2.92 mM lactulose, with cell samples removed from the chemostat at dilution rates of 0.121, 0.181 and 0.293 h^{-1}.

Lactulose utilisation was completely inhibited by CCCP (50 μM) in all incubations (Table 20). This suggested that lactulose uptake is exclusively mediated via a proton-dependent mechanism, at the appropriate dilution rates. In contrast, lactulose utilisation was incompletely inhibited by DCCD (50 μM), but, degrees of inhibition were similar in all incubations (64.7-68.0%; Table 20). Indeed, DCCD (50 μM) resulted in an identical effect on lactulose utilisation when incubations contained cells which had been cultured under batch conditions (66.7% inhibition; Results, section 4.2.2), and it was notable that degrees of inhibition were similar for both growth conditions. More detailed studies with cells harvested from batch culture had shown that incomplete inhibition of lactulose utilisation by 50 μM DCCD was most likely due to the incomplete inhibition of ATPase activity, and the findings with the chemostat-grown cells are most likely due to a similar mechanism. Nevertheless, it would have been preferable to use a higher DCCD concentration with chemostat samples.

5.2.2.3 Enzyme activities.

β-Galactosidase, phospho-β-galactosidase and Leloir pathway enzyme activities were measured in sonicated cells, harvested over a range of dilution rates. The galactohydrolases were assayed in all samples, whereas Leloir pathway enzymes were only assayed in samples removed from two culture runs.

The specific activity of β-galactosidase increased approx. 2.5-fold as the dilution rate was increased from 0.030 to 0.075 h^{-1} (Fig. 33A). Activity then exhibited a broad maximum of approx. 2.7 μmol/min/mg protein between dilution rates of 0.090 and 0.180 h^{-1}, before declining to reach a stable level of ca.1.5 μmol/min/mg protein at dilution rates >0.293 h^{-1}. Phospho-β-galactosidase activity was undetectable at all dilution rates studied (data not shown). The absence of this activity is consistent with the complete inhibition of sugar utilisation by CCCP (50 μM) (Table 20).

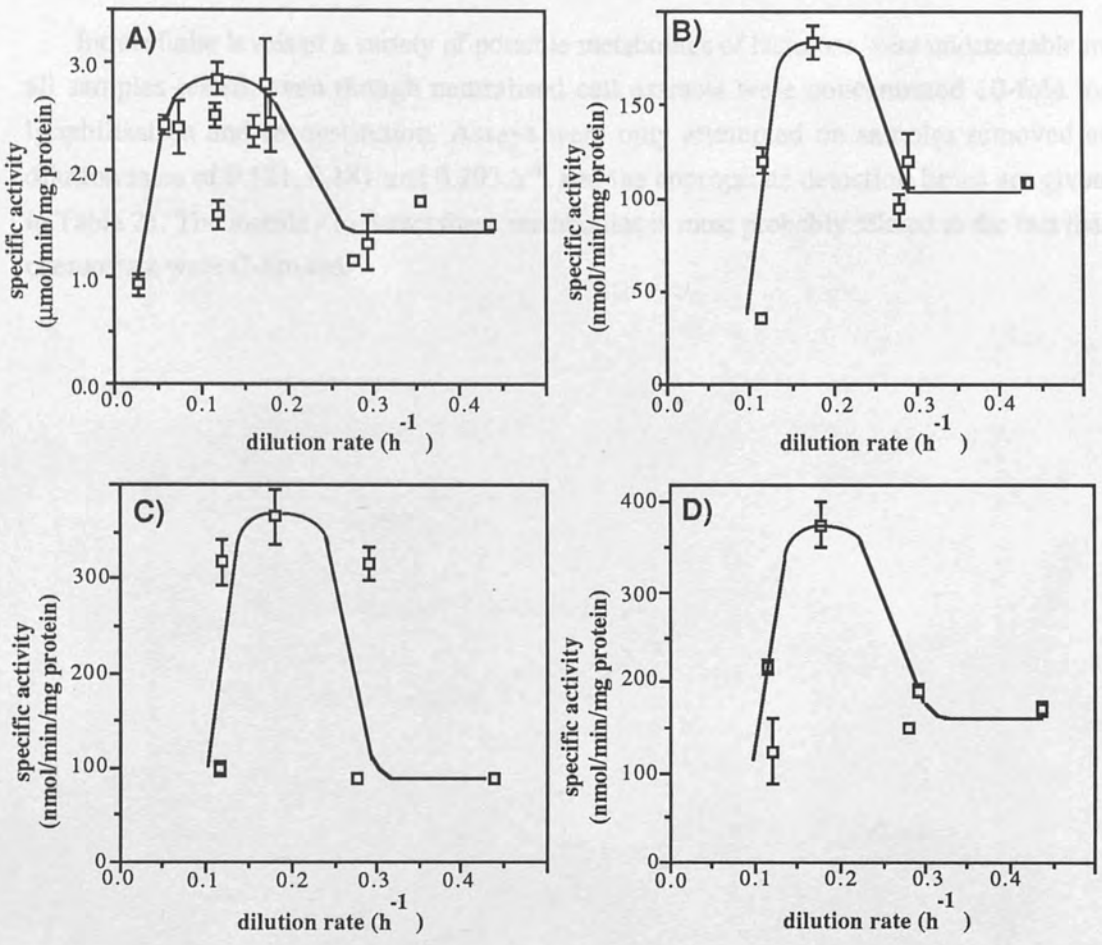
The specific activity profiles of the three Leloir pathway enzymes (Figs 33B-D) were similar in appearance to those of β-galactosidase (Fig 33A). However, maxima were shifted slightly towards the higher dilution rates. Thus, activities increased as the dilution rate was increased from 0.120 to 0.180 h^{-1}, exhibited broad peaks between dilution rates of 0.150 and 0.225 h^{-1}, and then declined to stable levels at dilution rates greater than

Table 20: Growth of *Cl. perfringens* in the chemostat under conditions of carbon (lactulose)-limitation: effect of inhibitors on *in vitro* utilisation rate.

Dilution rate (h^{-1})	Inhibitor	Concn. (mM)	Uptake rate (nmol/min/mg dry wt)	Inhibition (%)
0.121	CCCP	0	17.5	
		50	0.0	100.0
	DCCD	0	17.4	
		50	5.6	68.0
0.181	CCCP	0	25.7	
		50	0.0	100.0
	DCCD	0	25.0	
		50	8.8	64.7
0.293	CCCP	0	30.1	
		50	0.0	100.0
	DCCD	0	31.6	
		50	10.5	66.7

Cells were harvested from the chemostat at the indicated dilution rate and incubated with lactulose (2.92 mM) in a buffered utilisation system. Incubations were conducted in duplicate, and samples were removed at 10 min intervals. Transport inhibitors were added at 40 min and incubations were continued for a further 40 min. Lactulose remaining was determined using the resorcinol procedure.

Figure 33: Chemostat growth under conditions of lactulose-limitation: variation in β-galactosidase and Leloir pathway enzyme activity with dilution rate.



A) β-Galactosidase

B) Galactokinase

C) Galactose 1-phosphate uridylyl transferase

D) UDPgalactose 4-epimerase

The lactulose concentration in the substrate feed was 5 mM.

0.290 h^{-1}. Maximum levels were approx. 180 nmol/min/mg protein for galactokinase (Fig. 33B), 370 nmol/min/mg protein for galactose 1-phosphate uridylyl transferase (Fig. 33C) and 370 nmol/min/mg protein for UDPgalactose 4-epimerase (Fig. 33D).

5.2.2.4 Metabolite level determination.

Intracellular levels of a variety of possible metabolites of lactulose were undetectable in all samples tested, even though neutralised cell extracts were concentrated 10-fold by lyophilisation and reconstitution. Assays were only attempted on samples removed at dilution rates of 0.121, 0.181 and 0.293 h^{-1}, and the appropriate detection limits are given in Table 21. The inability to detect these metabolites is most probably related to the fact that chemostats were C-limited.

Table 21: Detection limits for metabolite assays using extracts prepared from cells cultured under conditions of carbon (lactulose)-limitation in the chemostat.

Metabolite	Detection limit (nmol/mg dry wt)
Galactose	0.2
Total galactose phosphate	0.2
Glucose 6-phosphate	0.1
Fructose/fructose 6-phosphate	0.1
Glucose 1-phosphate	0.1
Fructose 1,6-bisphosphate	0.4
Glyceraldehyde 3-phosphate	0.7
Dihydroxyacetone phosphate	0.9

CHAPTER 6: DISCUSSION

Previous studies of lactose metabolism in intestinal bacteria have revealed that the pathways of intermediary metabolism are in part determined by the mechanism of sugar transport (Introduction, section 1.6). Uptake by a PTS system results in the accumulation of intracellular lactose phosphate (phosphorylated at C-6 of the galactosyl moiety) and continued metabolism requires the participation of phospho-β-galactosidase, the tagatose 6-phosphate pathway and an ATP-dependent glucokinase. In contrast, transport by a non-PTS uptake system (normally an ion gradient-coupled system) results in the accumulation of unmodified substrate and further metabolism requires β-galactosidase, the Leloir pathway enzymes and an ATP-dependent glucokinase.

By analogy, possible routes of lactulose metabolism in *Cl. perfringens* are depicted in Fig. 34. It is assumed that phosphorylation of intracellular free fructose is mediated by an ATP-dependent fructokinase activity (or possibly mannofructokinase activity), specific for phosphorylation at C-6, based on the properties of other bacterial hexose kinases (Introduction, section 1.6.3). Although phosphorylation at C-1 of the galactosyl moiety during PTS transport seems unlikely (based on previous studies with lactose), phosphorylation of the fructosyl moiety at positions C-1 or C-6 during PTS transport cannot be entirely ruled out. However, the mechanism of hydrolysis of the intracellular phosphorylated disaccharide is unclear. Circumstantial evidence indicates that the fructosyl moiety of sucrose is phosphorylated at C-1 during transport by a PTS system in *Cl. pasteurianum*, since high levels of fructose 1-phosphate kinase are produced in response to growth in sucrose-containing medium [120]. If lactulose is transported by a similar mechanism in *Cl. perfringens*, then hydrolysis of the intracellular disaccharide may be mediated by an invertase-type activity, providing that it possesses sufficient broad substrate specificity with regard to the aglycone (galactosyl) moiety. Alternatively, β-galactosidase may function in this capacity, assuming that the enzyme could tolerate the introduction of a negatively charged substituent on the aglycone moiety of its substrate.

The results of this project will be discussed with reference to the possible metabolic schemes outlined in Fig. 34. It is pertinent to consider sugar transport first, since this step will ultimately determine the possible routes of intermediary metabolism.

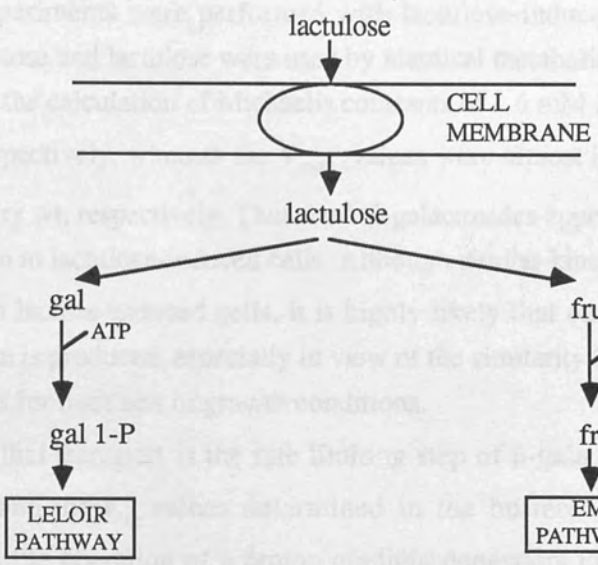
6.1 Sugar Transport.

A buffered incubation system was used to assay sugar transport in non-growing cells. It should be emphasised that this system did not permit the assay of the sugar transport step *per se*, rather, it provided a measure of the overall rate of sugar utilisation. Many studies have shown that the overall rate of sugar utilisation in bacteria is generally determined by the rate at which the carbohydrate enters the cell [193]. If a similar situation exists for lactulose utilisation in *Cl. perfringens*, then utilisation rates determined in the buffered system can be equated with net transport rates.

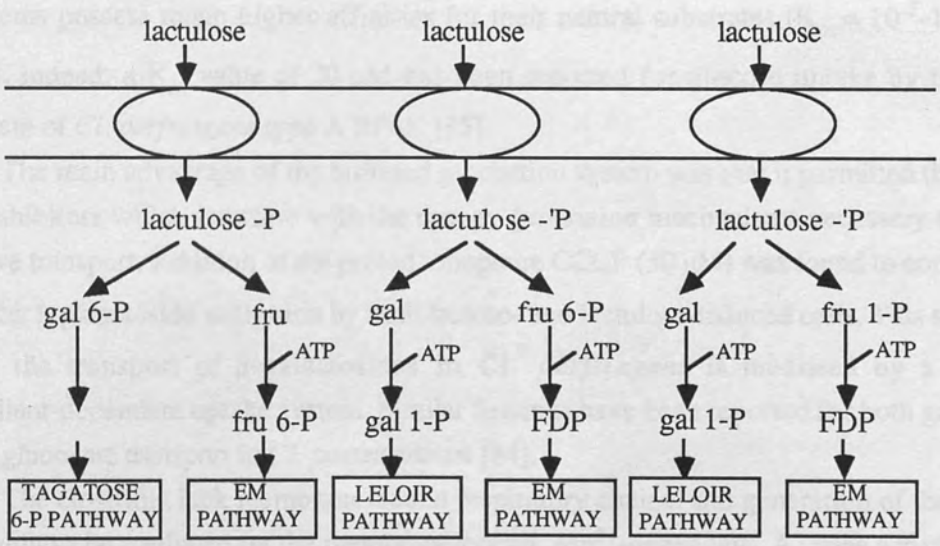
Utilisation of neither lactose nor lactulose was detectable when incubations contained

Figure 34: Possible pathways of lactulose metabolism.

A) Uptake by a non-PTS system.



B) Uptake by a PTS system.



P denotes a phosphate group

basal medium-grown cells, whereas both sugars were used when incubations contained lactose- or lactulose-grown cells. This suggests the induced synthesis of the respective utilisation systems. Lactose was used at a higher rate (4 to 5-fold) by both sets of cells, and absolute rates of utilisation were similar irrespective of the identity of the β-galactoside included in the growth medium. This suggests that lactose and lactulose are equally effective inducers of the β-galactoside uptake system(s) in *Cl. perfringens*.

Kinetic experiments were performed with lactulose-induced cells in an attempt to determine if lactose and lactulose were used by identical metabolic systems. Eadie-Hofstee plots permitted the calculation of Michaelis constants of 1.6 mM and 0.4 mM for lactulose and lactose, respectively, whereas the V_{max} values were almost identical at 14.8 and 13.5 nmol/min/mg dry wt, respectively. Thus, both β-galactosides appear to be metabolised by a common system in lactulose-induced cells. Although similar kinetic experiments were not performed with lactose-induced cells, it is highly likely that only a single β-galactoside utilisation sytem is produced, especially in view of the similarity between lactulose:lactose utilisation ratios for both sets of growth conditions.

Assuming that transport is the rate limiting step of β-galactoside utilisation in *Cl. perfringens*, then the K_m values determined in the buffered incubation system are consistent with the operation of a proton gradient-dependent uptake system. The value obtained for lactose in *Cl. perfringens* is very similar to that reported for lactose transport by proton-linked systems in both Gram-negatives (e.g. *E. coli*, K_m = 0.2 mM [43]) and Gram-positives (e.g. *Str. thermophilus*, K_m = 0.2-0.5 mM [194]). In general, PTS systems possess much higher affinities for their natural substrates ($K_m = 10^{-5}$-10^{-7} M) [76], indeed, a K_m value of 20 μM has been reported for glucose uptake by the PTS system of *Cl. perfringens* type A BP6K [85].

The main advantage of the buffered incubation system was that it permitted the study of inhibitors which interfere with the energy generation mechanisms necessary to drive active transport. Addition of the proton ionophore CCCP (50 μM) was found to completely inhibit β-galactoside utilisation by both lactose- and lactulose-induced cells. This suggests that the transport of β-galactosides in *Cl. perfringens* is mediated by a proton gradient-dependent uptake system. Similar findings have been reported for both galactose and gluconate transport in *Cl. pasteurianum* [84].

The clostridia lack membrane-bound respiratory chains, and generation of the pmf is thought to be mediated by the membrane-bound, Mg^{2+}-dependent, ATPase activity [40]. Studies with *Cl. perfringens* ATCC 3624 [186] and *Cl. pasteurianum* [51,185] have demonstrated that the main function of this enzyme is to provide a link between cellular metabolism and nutrient transport processes via the proton circulation. The ATPase activity of both these clostridia is sensitive to the inhibitor DCCD, which acts by binding to a site

located within the F_o-portion of the protein [177,185,186,195], in common with its mechanism of action in other prokaryotes [47]. The ATPase activity of *Cl. perfringens* ATCC 3624 has been shown to be inhibited 95% by 50 μM DCCD [186], therefore, a similar concentration of inhibitor was chosen for initial experiments in the present investigation.

It was surprising to find that utilisation of β-galactosides was inhibited by only 62-67% in the presence of 50 μM DCCD, especially since an absolute requirement for a proton gradient was suggested by analogous experiments with CCCP. Further study revealed that inhibition of β-galactoside utilisation was dependent upon the concentration of DCCD employed: the concentration required to produce 50% inhibition (I_{50} value) was determined as 24 μM, and a maximum observed inhibition of 90% was obtained with the highest concentration of inhibitor studied (100 μM). Exactly why transport activity in this strain of *Cl. perfringens* should possess reduced sensitivity to DCCD compared with the ATPase activity in *Cl. perfringens* ATCC 3624 is unclear, but strain differences may be important. On the other hand, the ATPase activity of *Cl. pasteurianum* is less sensitive to DCCD than that of *Cl. perfringens* ATCC 3624, since only 80% inhibition is observed in the presence of 100 μM DCCD [185]. Furthermore, it is possible to calculate I_{50} values of approx. 20-30 μM for inhibition of ATPase activity in *Cl. pasteurianum* using data reported in the literature [177,185]. These values are similar to that determined for inhibition of β-galactoside utilisation in *Cl. perfringens*. It is highly likely, therefore, that the concentration-dependent inhibition of sugar utilisation resulted from the concentration-dependent inhibition of the ATPase activity necessary to fuel active transport, rather than an effect on the transport system *per se*. Thus, ATPase activity appears to be necessary for the efficient utilisation of β-galactosides in *Cl. perfringens*.

Studies with *Cl. acetobutylicum* indicate that end product efflux may make an important contribution to the total pmf [52]. The ΔpH gradient of this organism is decreased by only 13% in the presence of 100 μM DCCD, and concentrations of inhibitor as high as 500 μM only produce a 62% reduction. In contrast, end product efflux appears to be unimportant in *Cl. pasteurianum* since 200 μM DCCD is sufficient to convert the internal alkaline ΔpH gradient into an internal acidic ΔpH gradient [51]. It is also unlikely that this process plays any significant role in the overall energy economy of *Cl. perfringens*, since 100 μM DCCD resulted in 90% inhibition of β-galactoside utilisation. Although higher concentrations of DCCD were not studied, it is evident from the dose response curve that inhibition had not reached its maximum value at 100 μM DCCD. However, direct measurement of the proton gradient response to DCCD is required, before drawing firm conclusions.

In retrospect, the use of fluoride as a probe of PTS-mediated uptake in the buffered incubation system was inappropriate. The site of action of this inhibitor is the glycolytic enzyme enolase, which is not only required to generate PEP necessary for PTS-mediated

uptake, but also for the continued utilisation of the sugars under study. Furthermore, inhibition of glycolysis at this step will reduce the net ATP yield to zero and, in the presence of a proton gradient-linked transport system substrate, will ultimately deplete cellular ATP levels via the action of the proton-translocating ATPase activity. In addition, inhibition of sugar utilisation may also result from the operation of feedback regulatory mechanisms, due to the build up of intracellular catabolites, since metabolism has been essentially blocked. It follows that inhibition of β-galactoside utilisation in *Cl. perfringens* does not necessarily rule out the operation of a proton gradient-linked uptake system, since fluoride-mediated inhibition may be expected regardless of the sugar transport mechanism in operation. Thus, it is concluded from the experiments with CCCP and DCCD that β-galactoside uptake in *Cl. perfringens* is mediated by a proton-dependent uptake system, analagous to the class of transport systems typified by β-galactoside uptake in *E. coli*.

Direct demonstration of proton movements in response to lactulose transport would have provided confirmatory evidence for the existence of such a transport mechanism. Such an approach has been used to demonstrate proton-dependent transport systems in *E. coli* [162,196], *Str. lactis* 7962 [197] and *Cl. pasteurianum* [84]. In brief, cells are incubated in lightly buffered medium, under conditions which result in inhibition of glycolysis and respiration. Alkalinisation of the medium is then measured upon addition of the sugar substrate of interest. An anion, which can readily penetrate the cell membrane (normally thiocyanate), is often included in incubations to prevent the build up of positive charge, since this may limit further proton influx.

6.2 Pathways of Intracellular Metabolism.

Transport of lactulose by a proton-dependent uptake system in *Cl. perfringens* precludes the operation of the PTS-dependent metabolic pathways depicted in Fig. 34B. Indeed, results obtained from the determination of both enzyme activities and metabolite levels are consistent with this proposal. Furthermore, the failure to detect monosaccharides in culture medium rules out any additional metabolic sequences involving extracellular cleavage of the disaccharide and subsequent uptake of the individual monosaccharides.

The presence of high levels of β-galactosidase specific activity, coupled with the failure to detect phospho-β-galactosidase activity in lactulose-grown cells of *Cl. perfringens*, confirms the absence of any PTS mechanism of sugar transport specific for phosphorylation of the galactosyl moiety of lactulose. In addition, levels of β-galactosidase specific activity (684 nmol/min/mg protein or 171 nmol/min/mg dry wt; determined using ONPG as substrate) are of a similar order of magnitude as those reported for a range of other Gram-positive bacteria which predominantly transport β-galactosides by non-PTS mechanisms. Examples include certain strains of lactic streptococci (approx. 200-800 nmol/min/mg dry wt)[95,96] and *B. megaterium* (245 nmol/min/mg protein) [198]. Levels of activity in Gram-negative bacteria are somewhat higher, examples include *E. coli* B

(6.6 μmol/min/mg dry wt) [199] and *K. aerogenes* (1.0 μmol/min/mg dry wt) [200].

Further support for the involvement of β-galactosidase in the metabolism of lactulose in intact cells came from the metabolite studies. High intracellular levels of the hydrolytic products, free fructose and galactose, were detected throughout growth in lactulose-containing medium, whereas galactose 6-phosphate was undetectable. The failure to detect this latter metabolite is consistent with the absence of both a PTS mechanism of lactulose transport (specific for phosphorylation of the galactosyl moiety) and phospho-β-galactosidase activity.

In view of these findings, the most likely routes of metabolism of intracellular free fructose and galactose are via fructokinase catalysed conversion to fructose 6-phosphate, and the Leloir pathway, respectively (Fig. 34B). The detection of fructokinase, the Leloir pathway enzymes, fructose 6-phosphate and certain intermediates of the Leloir pathway are in accordance with this view.

There are few other reports of fructokinase activity in the clostridia; probably related to the fact that fructose is generally transported by PTS systems in this group of bacteria [86,120]. Fructokinase activity has been reported in glucose-grown cells of *Cl. perfringens* type A BP6K, but levels were very low at 2 nmol/min/mg protein [89]. This strain was also found to possess high levels of glucokinase activity (59 nmol/min/mg protein) [89], even though it transports glucose by a PTS system [85], and it is not clear if these two activities are associated with a single enzyme of broad substrate specificity or two distinct proteins. In general, bacterial glucokinase possesses narrow substrate specificity (Introduction, section 1.6.3 and Table 8), as does the activity of *Cl. thermocellum* (no specificity for fructose) [119], therefore, the fructokinase and glucokinase activities in *Cl. perfringens* type A BP6K [89] are probably separate enzymes. If this is the case, then the high level of fructokinase activity reported in the present study may represent induced synthesis, in response to intracellular fructose generated as a result of lactulose hydrolysis.

The physiological role of the fructokinase activity of *K. aerogenes* [110] and oral streptococci [112] is considered to be the metabolism of fructosyl moieties of disaccharides such as sucrose. It is of interest, therefore, that levels of fructokinase activity in sucrose-grown cells of these organisms (280-980 nmol/min/mg protein in oral streptococci, and 46 nmol/min/mg protein in *K. aerogenes*) are similar to the maximum level (approx 400 nmol/min/mg protein or 56 nmol/min/mg dry wt) in exponential phase, lactulose-induced cells of *Cl. perfringens*. More importantly, fructokinase activity in *Cl. perfringens* was sufficient to account for the overall rate of lactulose utilisation (39.9 nmol/min/mg dry wt). Although the presence of intracellular fructose 6-phosphate in lactulose-grown cells is indicative of the involvement of fructokinase activity in cellular metabolism of the fructosyl moiety, it is not absolute proof, since this intermediate can also arise as a result of metabolism of the galactosyl moiety (Fig. 34B).

All three enzymes of the Leloir pathway were detected in lactulose-grown cells.

Maximum specific activities observed during exponential growth were 100.8 nmol/min/mg protein (17.9 nmol/min/mg dry wt) for galactokinase, 134 nmol/min/mg protein (26 nmol/min/mg dry wt) for galactose 1-phosphate uridylyl transferase and 653 nmol/min/mg protein (93 nmol/min/mg dry wt) for UDPgalactose 4-epimerase. Similar levels of kinase and transferase activities have been reported for lactose-induced cells of *E. coli* B (72 nmol/min/mg protein and 150 nmol/min/mg protein, respectively) and *K. aerogenes* (89 nmol/min/mg protein and 180 nmol/min/mg protein, respectively) [101]. These enteric bacteria transport lactose by proton- linked uptake systems and are thought to metabolise the galactosyl moiety exclusively via the Leloir pathway. In contrast, levels of epimerase activity in these two bacteria (14-32 nmol/min/mg protein) were much reduced relative to the values determined in *Cl. perfringens*. This may be related to the different assay procedures used; that used for *E. coli* and *K. aerogenes* was dependent upon galactose 1-phosphate uridylyl transferase activity present in cell extracts, and this may have resulted in an underestimation of activity.

Confirmation for the operation of the Leloir pathway in lactulose-grown cells of *Cl. perfringens* came from the metabolite studies. In the present study, it was not possible to directly determine galactose 1-phosphate as free galactose released upon acid hydrolysis, due to the presence of other acid labile galactose-containing material. However, the galactose phosphate species present was indirectly identified as galactose 1-phosphate, since galactose 6-phosphate was undetectable in acid hydrolysed cell extracts. More direct evidence came from the determination of glucose 1-phosphate and glucose 6-phosphate; the former metabolite is a product of the galactose 1-phosphate uridylyl transferase-catalysed reaction, whereas the latter metabolite is formed as a result of phosphoglucomutase-catalysed conversion of glucose 1-phosphate. Assay of intracellular UDPG and UDPgalactose was not attempted, since this would have required purified galactose 1-phosphate uridylyl transferase and UDPG dehydrogenase [201]. These enzymes are commercially available, but their use was financially prohibitive.

The operation of the Leloir pathway in the metabolism of galactose or galactose-containing saccharides has not been previously demonstrated in the clostridia. Indirect evidence suggests that intracellular phosphorylation of galactose is mediated by an ATP-dependent enzyme in *Cl. pasteurianum* [83]. This organism transports galactose by a proton-linked system [84], and the phosphorylation of this sugar has been measured in toluene-permeabilised, galactose-grown cells [83]. The phosphorylation rate in the presence of ATP (4.3 nmol/min/10^8 cells) is approx. 10-fold higher than that in the presence of PEP (0.425 nmol/min/10^8 cells) [83].

Although the maximum specific activities of galactokinase and galactose 1-phosphate uridylyl transferase determined in exponential cells of *Cl. perfringens* were of a similar order of magnitude as the specific rate of lactulose utilisation (39.9 nmol/min/mg dry wt), they were insufficient to account for the complete metabolism of the galactosyl moiety by the Leloir pathway. The reason for this discrepancy is unclear, but is most likely related to the assay conditions employed, rather than the existence of alternative catabolic pathways.

Enzyme assays were performed on cell extracts, and this may have resulted in the underestimation of cellular activities for several reasons:

a) The chemical composition of the assay system was much simpler than the intracellular environment. Possible activators may have been depleted during cell harvesting/preparation of cell extracts.

b) Cell extracts were prepared by sonication and, in order to compare enzyme activities with the specific rate of lactulose utilisation, specific activities were calculated as a function of the cell dry wt content rather than sonicant cell protein. For this comparison to be valid, enzyme release would have to be at, or near, completion, otherwise activities will be underestimated.

It should be noted that sonicated cells were routinely observed under the microscope to ensure >90% cell breakage and this tends to invalidate explanation b). More likely is the possibility that sonication resulted in enzyme damage, since long sonication times (normally 20 min) were required to ensure optimum cell disruption.

On the other hand, there is certain evidence in the literature which suggests that the phosphorylation of intracellular free hexoses may not be mediated exclusively by ATP-dependent hexose kinase activity. Several groups have suggested that the PTS systems of both Gram-positive and Gram-negative bacteria may be capable of catalysing PEP-dependent phosphorylation of intracellular hexoses [72,73,76].

Although the non-metabolisable sugar, TMG, is a substrate of the β-galactoside uptake system in *E. coli*, a mutant has been isolated which accumulates abnormally high levels of this galactoside; the majority of which is present in the phosphorylated form [202]. Time course experiments indicated that transport of TMG preceded phosphorylation, and experiments conducted with crude cell extracts demonstrated that phosphorylation required the particulate fraction, Mg^{2+} and PEP, but ATP was ineffective as a phosphoryl donor. It was suggested that intracellular phosphorylation was catalysed by a PTS system [202]. The identity of this system is unknown, however, the II^{Glc} system is known to possess limited substrate specificity for TMG, and it is possible that its affinity may be increased in the mutant [72].

Studies with membrane vesicles of *S. typhimurium* have indicated that the II^{Man} complex of this organism can catalyse intracellular phosphorylation [203]. When EI and HPr are present intravesicularly, both II^{Glc} and II^{Man} can catalyse PEP-dependent phosphorylation and uptake of their substrates. When the soluble components are present extravesicularly, the II^{Man} system can catalyse PEP-dependent phosphorylation (but not uptake), whereas the II^{Glc} system is unable to catalyse either reaction [203].

Strong evidence for PTS-mediated phosphorylation of intracellular sugar has come from studies with *Str. lactis* [118,204]. Starved cells of *Str. lactis* ML_3 maintain a high PEP potential (in the form of 2-phosphoglycerate, 3-phosphoglycerate and PEP; total concentration 40 mM) but contain negligible ATP levels [118]. Transport studies conducted with iodoacetate-treated, starved cells have shown that the ratio mole PEP

used/mole hexose accumulated is 1 in the case of various PTS monosaccharide substrates, whereas the value is 2 in the case of lactose. Lactose-accumulated cells also contained very high levels of glucose 6-phosphate, but low levels of free glucose, therefore, it was suggested that phosphorylation of the glucosyl moiety was catalysed by the mannose-PTS of this organism [118].

Additional support for this conclusion has come from a study with lactose analogues, including β-O-D-galactopyranosyl-(1,4')-2'-deoxy-D-glucopyranose (2'D-lactose) and β-O-D-galactopyranosyl-(1,4')-2'-deoxy-2'-fluoro-D-glucopyranose (2'F-lactose), which are high affinity substrates of the lactose PTS [204]. Transport of these analogues in starved cells of *Str. lactis* 133 resulted in phosphorylation of the galactosyl moiety at C-6, and the accumulated disaccharide-phosphates were substrates for phospho-β-galactosidase. A time course analysis of the intracellular hydrolytic products revealed that the aglycone moieties were present in both the free and phosphorylated (at C-6) forms, and the proportion of the latter increased with time. Phosphorylation of the aglycone was unaffected by inclusion of excess N-acetylglucosamine (substrate of the mannose PTS) in the extracellular medium and could not be simply attributed to efflux followed by re-entry via the mannose PTS. Cells defective in the mannose PTS were unable to phosphorylate the aglycone of 2'D-lactose but could phosphorylate that of 2'F-lactose, albeit at greatly reduced levels. Futher mutation of glucokinase activity completely abolished this residual phosphorylation. Thus, it was concluded that the intracellular phosphorylation of the glucosyl moiety of lactose may be mediated by both ATP-dependent and PEP-dependent activities [204].

The majority of the above studies were conducted with mutant cells or wild type cells under extreme physiological conditions (starvation), therefore, the relevance of such a mechanism in the mediation of lactulose metabolism in *Cl. perfringens* (under conditions of substrate excess) remains obscure. It is unlikely that PTS-mediated phosphorylation of intracellular galactose played a significant role in this organism, since the product of this reaction would be galactose 6-phosphate, which was undetectable. Although, fructokinase activity in *Cl. perfringens* was sufficient to account for the metabolism of intracellular free fructose, the PEP-dependent phosphorylation of this moiety cannot be entirely ruled out, since assay of fructose 1-phosphate (product of the clostridial fructose PTS system) was not performed. A spectrophotometric assay for this metabolite is available, which employs liver aldolase in conjunction with NAD^+ and glycerol 3-phosphate dehydrogenase [205]. Adaptation of this procedure for fluorimetric determination would have permitted more firm conclusions to be drawn regarding the role of PEP-dependent intracellular phosphorylation in the metabolism of lactulose.

The absence of galactose 6-phosphate also argues against the operation of any mechanism whereby the phosphorylation of the galactosyl moiety may be mediated via efflux, followed by re-uptake; catalysed by a PTS system. This is supported by the failure to detect extracellular hydrolytic products in the growth medium. This latter observation

may not be particularly significant since PTS systems generally have very low K_m values (0.1-10 μM), therefore, effluxed monosaccharides would not be expected to accumulate to a high level.

In general, levels of lactulose metabolites in *Cl. perfringens* were consistent with values reported in the literature for a range of bacteria cultured in the presence of a variety of sugars. In the present study, metabolite levels were expressed as a function of dry wt, since the intracellular (protoplast) volume was not measured. It should be noted that metabolite levels are more commonly expressed as a function of the intracellular volume, however, it is still possible to compare the data from *Cl. perfringens* with those from other bacteria, since the intracellular volume per unit dry wt is usually given.

Levels of galactose 1-phosphate in lactulose-grown cells of *Cl. perfringens* were stable, at a value of 2 nmol/mg dry wt, throughout the exponential phase of growth. Similar levels of this metabolite (range 3-8 nmol/mg dry wt) have been reported in galactose-grown cells of those lactic streptococci (*Str. lactis* ML_3, *Str. lactis* 7962, *Str. lactis* ML_8 and *Str. cremoris* AM_2) in which the Leloir pathway serves as a major route of metabolism [154]. In contrast, the metabolite is present at only low levels (0.1 nmol/mg dry wt) in those strains (*Str. cremoris* E_8 and *Str. cremoris* HP) which metabolise galactose predominantly via the tagatose 6-phosphate pathway [154]. Galactose 6-phosphate could not be detected (<0.2 nmol/mg dry wt) in lactulose-grown cells of *Cl. perfringens*, but this metabolite appears to be present at moderately high levels (3-4 nmol/mg dry wt) in all strains of lactic streptococci tested, irrespective of the major route of galactose metabolism [154].

Glucose 6-phosphate is also present at moderate levels (approx. 3 nmol/mg dry wt) in those lactic streptococci which predominantly metabolise galactose via the Leloir pathway [154]. Further study with *Str. lactis* ML_8 has shown that only slight variations in the level of this metabolite occur when cells are grown in the presence of different sugars [206]. Thus, levels of 1.7, 3.9 and 2.0 nmol/mg dry wt have been reported for galactose-, glucose- and lactose-grown cells, respectively [206]. Similar levels of glucose 6-phosphate are maintained in non-growing, glycolysing cells of *Str. lactis* ML_3 (1.0 nmol/mg dry wt) [207,208], *E. coli* (1.9 nmol/mg dry wt) [209] and *L. plantarum* (1.5 nmol/mg dry wt) [210]. The stable, exponential phase value of 0.9 nmol/mg dry wt in lactulose-grown cells of *Cl. perfringens* is consistent with these findings.

The levels of glucose 1-phosphate (0.3 nmol/mg dry wt) and fructose 6-phosphate (0.3 nmol/mg dry wt) in lactulose-grown, exponential phase cells of *Cl. perfringens* are also comparable with those of other bacteria. Examples include non-growing, glycolysing cells of *Str. lactis* ML_3 (fructose 6-phosphate = 0.1-0.2 nmol/mg dry wt) [207] and *L. plantarum* (glucose 1-phosphate = 0.4 nmol/mg dry wt, fructose 6-phosphate = 0.7 nmol/mg dry wt) [210]. No data are available regarding glucose 1-phosphate levels in the lactic streptococci.

Large differences were evident between the levels of FDP and triose phosphate determined in *Cl. perfringens* and those reported for other bacteria. Levels of FDP in glycolysing cells of lactic streptococci (10-14 nmol/mg dry wt) [207,208], *E. coli* (6.6 nmol/mg dry wt) [209] and *L. plantarum* (15.3 nmol/mg dry wt) [210], are much higher than those in lactulose-grown cells of *Cl. perfringens* (range 1.1-2.4 nmol/mg dry wt, during exponential growth). However, detailed studies with the lactic streptococci have shown that levels of this metabolite are dependent upon the identity of the sugar in the growth medium [154]. Galactose-, glucose- or lactose-grown cells of *Str. lactis* ML_3 and *Str. lactis* ML_8 contain FDP at approx. 8-15 nmol/mg dry wt . Although similar levels are also found in glucose- or galactose-grown cells of *Str. lactis* 7962, lactose-grown cells of this strain contain a level (2.9 nmol/mg dry wt) similar to that determined for *Cl. perfringens.*

The triose phosphates were only detectable in early exponential phase samples of lactulose-grown cells of *Cl. perfringens*; levels were 1.3 and 1.0 nmol/mg dry wt for GA3P and DHAP, respectively. These values are slightly higher than the respective levels of 0.47 and 0.45 nmol/mg dry wt in glycolysing cells of *E. coli* [209]. In the case of galactose-grown cells of lactic streptococci, levels of DHAP ranged from 2.1-4.5 nmol/mg dry wt, and those of GA3P ranged from 0.18-0.36 nmol/mg dry wt [154].

Strain-dependent differences in fermentation pattern in the lactic streptococci have been linked with differences in intracellular levels of FDP, since this metabolite serves as an activator of lactate dehydrogenase [154]. Thus, growth conditions which result in high levels of intracellular FDP are also associated with a predominantly homolactic fermentation of the medium sugar, whereas lactate is a quantitatively less important product in cases where the intracellular level of FDP is low. Furthermore, the triose phosphates serve as inhibitors of streptococcal pyruvate-formate lyase, and increased levels of these intermediates ensure a greater diversion of pyruvate to lactate [154]. Assuming that similar control mechanisms exist for *Cl. perfringens* and the lactic streptococci, then the presence of low levels of both FDP and triose phosphates in the former bacterium are consistent with the low amounts of lactate produced from lactulose [33,145].

FDP, along with other glycolytic intermediates, also serves as an activator of pyruvate kinase in the lactic streptococci [156,211]. By control of PEP levels, this mechanism serves to provide a link between PTS-mediated transport systems and subsequent metabolism [156,211].

Finally, intracellular levels of galactose 1-phosphate, fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate were relatively stable throughout exponential phase, whereas the levels of free galactose and fructose increased during early/mid exponential phase. This suggests that the phosphorylation of these two hexosyl moieties is the rate limiting step in their intracellular metabolism. The close correlation between galactose and fructose levels would further suggest that cellular activities of galactokinase and fructokinase are co-ordinately regulated, thereby ensuring the balanced metabolism of both

hexosyl moieties of lactulose. The exact mechanism of this regulation is unclear, it may involve controls at the level of enzyme synthesis and/or enzyme activity. Purification of galactokinase and fructokinase may permit a better understanding of the role of the latter mechanism.

6.3 Regulation of Sugar Metabolism.

In bacteria, the genes encoding sugar-specific catabolic enzyme systems are normally arranged in the form of polycistronic operons, thereby permitting the co-ordinate expression of the respective catabolic pathways. Regulation of operon expression occurs predominantly at the level of transcription, and controls exist at both the initiation and termination steps [212].

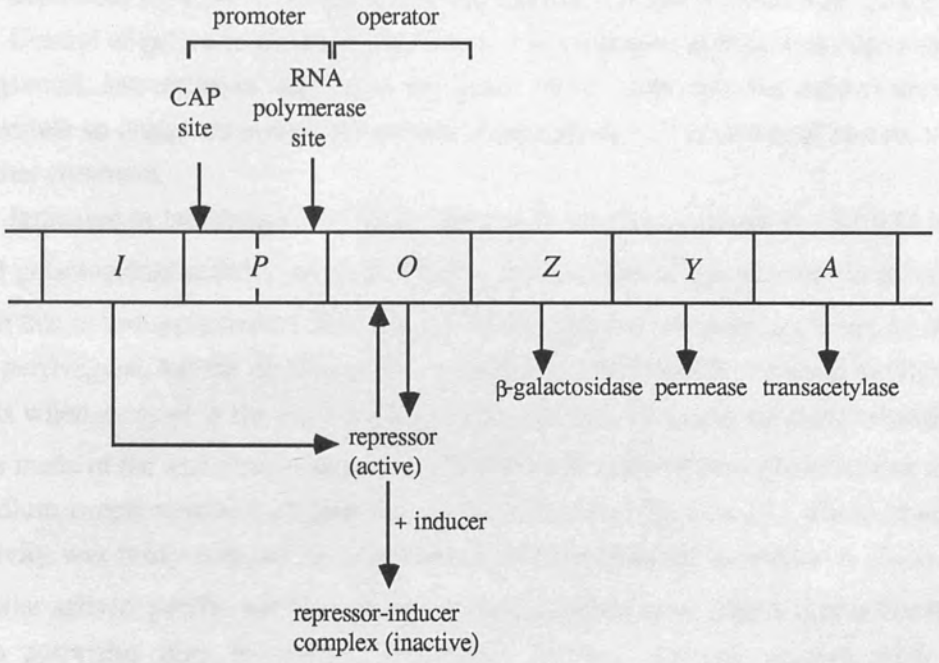
The most extensively studied system is the *lac* operon of *E. coli*, which contains 3 structural genes; coding for β-galactosidase (*lac Z*), the lactose carrier protein (*lac Y*) and thiogalactoside transacetylase (*lac A*), a regulatory gene (*lac I*) which encodes the *lac* repressor protein, and a control region consisting of the *lac* promoter and the *lac* operator (Fig. 35A) [102,121,213]. Transcription initiation is subject to both positive and negative control, mediated via protein-nucleic acid interactions, and the activities of these regulatory proteins are themselves modulated by low molecular weight effector molecules. Negative control is exerted by the *lac* repressor protein which binds to the operator region of the DNA and prevents the movement of RNA polymerase along its template. Binding of inducer to the repressor results in the dissociation of this protein from the DNA and permits transcription initiation to proceed. Full induction of the operon also requires the participation of the CAP protein, which is itself activated by cAMP binding (Introduction, section 1.7.1) [102,121,213].

The *gal* operon of *E. coli* encodes the genes for the Leloir pathway of this organism: galactokinase (*gal K*), galactose 1-phosphate uridylyl transferase (*gal T*) and UDPgalactose 4-epimerase (*gal E*). The organisation of the control region is more complex than that of the *lac* operon, and its regulation is poorly understood [102,213]. The *gal* repressor gene (*gal R*) is not closely linked to the operon, which is in itself not an unusual arrangement, but the operon possesses 2 operator regions, one of which is located within the structural gene for the epimerase (Fig. 35B) [214]. The operon also possesses two overlapping promoter sites (P_{G1} and P_{G2}) which control transcription initiation from two start sites (S_1 and S_2, respectively) separated by 5 base pairs [215]. Occupation of the CAP site is thought to exert both positive control on transcription initiation from the S_1 site and negative control on transcription initiation from the S_2 site [215]. Furthermore, binding of the *gal* repressor is only thought to inhibit initiation from the S_1 site [215].

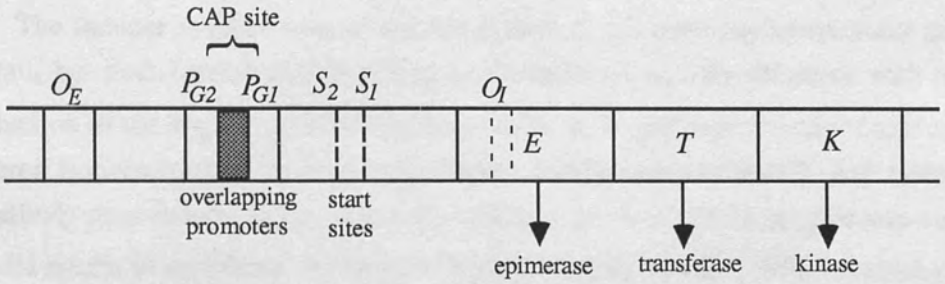
The cAMP-CAP complex also plays a significant role in the regulation of transcription termination of the *gal* operon of *E. coli*. In the presence of galactose, the intracellular level

Figure 35: The *lac* and *gal* operons of *E. coli*

A) The *lac* operon



B) The *gal* operon



O_E -external operator

O_I -internal operator

P_{G1} and P_{G2} are overlapping promoters which control transcription initiation from sites S_1 and S_2, respectively.

of cAMP is high and essentially equal amounts of the kinase, transferase and epimerase are produced. When the intracellular cAMP level is low, production of the epimerase remains high, whereas that of the promoter distal gene products declines [216,217]. This polar effect is thought to result from the premature termination of transcription at intercistronic sites, and requires the participation of a termination factor termed rho protein [102,217]. The cAMP-CAP complex is thought to decrease polarity by acting as an antiterminator at rho-dependent sites [217], but the actual mechanism is not fully understood [102].

Control of gene expression in the clostridia in particular, and Gram-positive organisms in general, has not been studied in any great detail. Although this subject area did not constitute an important part of the present investigation, several observations are worthy of further comment.

Inclusion of lactulose (20 mM) in the growth medium resulted in a 80-fold induction of β-galactosidase activity, as determined by comparison of the differential induction rate with that of unsupplemented cells. Lactulose also induced a β-galactoside uptake system in *Cl. perfringens*, but the activity of this system was not detectable in basal medium-grown cells when assayed in the buffered incubation system. Although no comprehensive study was made of the variation in β-galactoside transport activity throughout culture growth in medium supplemented with lactulose, consideration of the data as a whole revealed that activity was fairly constant in cells harvested from mid-late exponential phase. Since a similar activity profile was also observed for β-galactosidase, then it is possible that these two activities may be under co-ordinate control. Genetic studies with another Gram-positive organism, *Str. lactis* 7962 (transports lactose by a proton gradient-dependent system), have indicated that the structural genes encoding transport activity and β-galactosidase are closely linked [95].

The inducer requirements of the *lac* system in *Cl. perfringens* were not studied in detail, but both lactose and lactulose appeared to be equally effective with regard to induction of the β-galactoside utilisation system. In *E. coli*, the natural inducer of the *lac* operon is considered to be allolactose, rather than lactose *per se* [93], and galactose is a relatively poor inducer [218], whereas growth of *Str. lactis* 7962 in galactose-containing media results in significant induction of β-galactosidase activity [199]. In lactulose-grown cells of *Cl. perfringens*, a correlation was evident between the specific activity profile of β-galactosidase and the concentration profile of the unidentified pool of acid labile galactose (but not free galactose). Assuming that the identity of this compound is unmodified lactulose, it is possible that induction of the *lac* system in *Cl. perfringens* requires a β-galactoside, rather than galactose, however, more detailed study is needed.

Galactokinase and UDPgalactose 4-epimerase activities were not co-ordinately controlled in *Cl. perfringens*. The differential rate of galactokinase induction was increased 30-fold by inclusion of lactulose (20 mM) in the basal medium, whereas epimerase levels were high in cells cultured in basal medium alone, and lactulose-supplementation was

without effect on the differential rate of enzyme synthesis. Although galactose 1-phosphate uridylyl transferase activities were not measured in basal medium-grown cells, this enzyme and galactokinase appeared to be co-ordinately controlled, whereas the specific activity profile of the epimerase was distinct. As outlined above, the enzymes of the *gal* operon of *E. coli* are not co-ordinately regulated, therefore, these results do not necessarily preclude an operon arrangement for the Leloir pathway enzymes in *Cl. perfringens*. Constitutive levels of UDPgalactose 4-epimerase have also been reported for *K. aerogenes* and group N streptococci, but the enzyme is absent from *Staph. aureus* [101]. The finding for group N streptococci is perhaps surprising, since the enzyme plays only a minimal role in the metabolism of galactose or galactose-containing saccharides in certain species of this group of organisms [101]. Rather, the presence or absence of this enzyme is thought to correlate with the presence or absence of galactose in the cell wall [101]. It is possible, therefore, that the constitutive synthesis of the enzyme in *Cl. perfringens* is related to an absolute requirement for efficient cell wall synthesis. Indeed, this organism is known to contain galactose in its cell wall [219].

The physiological rationale for the natural polarity of the *gal* operon in *E. coli*, coupled with the existence of cAMP-dependent and cAMP-independent promoters, has been explained in terms of the dual role that the epimerase plays in catalysing both anabolic and catabolic reactions [128]. The cell wall precursor UDPgalactose will be required even when cAMP levels are low and, under such conditions, transcription will be controlled by the cAMP-independent promoter which is *gal* repressor-resistant. Polarity will also be high, thereby ensuring that superfluous levels of kinase and transferase activities are not produced. In contrast, in the presence of galactose, and when cAMP levels are high, transcription will initiate from the cAMP-dependent promoter and polarity will be low [128].

It is unlikely that such a mechanism can account for the constitutive expression of the epimerase in *Cl. perfringens*, since cAMP has been reported to be absent from this organism [145]. The distribution of this cyclic nucleotide among Gram-positive bacteria is not thought to be as widespread as that among Gram-negatives [128]. Its absence has been reported for a wide range of bacilli, including *B. subtilis* [123,220], *B. megaterium* [221], *B. brevis* [222], *B. licheniformis* [223], *B. cereus* [220], *B. pumilis* [220], and *L. plantarum* [224], whereas its presence has been reported for several species of lactic streptococci (but not *Str. lactis* 7962)[225], *Str. salivarius* [226], *M. lysodeikticus*, *Brevibacterium liquefaciens*, *Corynebacterium equi* and *Nocardia erythropolis* [220]. Control of gene expression in certain Gram-positive bacteria, especially bacilli, cannot therefore be explained in terms of the models for Gram-negatives. Whether an alternative positive regulatory mechanism exists, or additional factors are involved, is currently unknown. Indeed, the situation as regards *E. coli* is incompletely understood, and this has resulted in the hypothesis that additional regulatory factors, such as catabolite modulator factor (negative effector; Introduction, section 1.7.2), may be involved, at least under conditions of catabolite repression [102].

Study of the inducer specificity of galactokinase in *Cl. perfringens* revealed important differences between this organism and *E. coli*. Comparison of specific activities in cells grown in basal medium supplemented with a range of sugars (5 mM final concentration) suggested that lactulose, lactose and galactose were inducers: degrees of induction were calculated as 7-fold, 6-fold and 2-fold, respectively, relative to basal medium-grown cells. Essentially similar results were obtained when differential rates of induction were compared (no data available for lactose). The actual inducer of the *gal* operon is most likely galactose, and this is supported by the correlation between the concentration profile of intracellular galactose and the specific activity profiles of galactokinase and galactose 1-phosphate uridylyl transferase in lactulose (20 mM)-induced cells. On the other hand, exogenous galactose resulted in lower levels of induction of galactokinase than the β-galactosides tested, which is unexpected. This discrepancy may have been due to the experimental design, since the exogenous sugar concentrations (5 mM) employed were probably subsaturating for induction. Consistent with this view is the observation that 20 mM lactulose resulted in a 30-fold induction of galactokinase. Thus, the apparent difference in inducer efficiency may have resulted from different intracellular concentrations of free galactose, since β-galactosides and galactose are most likely transported by different systems.

Although the inducibility of fructokinase was not studied in detail, a similar correlation existed between the level of intracellular fructose and the specific activity profile of fructokinase, therefore, intracellular fructose may be the natural inducer of this enzyme. Since galactose and fructose are both products of the first intracellular reaction, and each may function as the inducer of their respective catabolic pathways, then the co-ordinate metabolism of these hexosyl moieties will be ensured.

Galactokinase activity in *Cl. perfringens* was not only subject to induction by galactose or galactose-containing disaccharides, but repression exerted by fructose or glucose, in the absence of any potential inducer. Repression was particularly significant (7-fold) in the case of glucose (5 mM). Similar repressive effects have been observed for certain other Gram-positive bacteria.

Sucrose is transported by a PTS mechanism in *Str. lactis* K_1, specific for phosphorylation of the glucosyl moiety [227]. Phosphorylation of the fructosyl moiety is mediated by an ATP-dependent mannofructokinase, and low levels of this enzyme (1.6-3.8 nmol/min/mg protein) are observed when cells are grown in the presence of glucose, fructose or lactose, whereas high levels (12.6 nmol/min/mg protein) are induced by growth in the presence of sucrose. Unexpectedly, maltose, ribose or galactose-supplementation of the growth medium was found to result in even higher levels of enzyme activity (14.2-20.6 nmol/min/mg protein). It was suggested that the PTS system may negatively control mannofructokinase expression, since, with the exception of sucrose, all the sugars which resulted in high levels of activity were either substrates of non-PTS uptake systems or only poor substrates of the PTS [227].

Similar repression/induction effects have been reported for the mannofructokinase of oral streptococci [112], and the lactose catabolic systems of lactic streptococci [118,228,229], oral streptococci [98] and *L. casei* [230]. Studies with glucose PTS mutants of *Str. lactis* ML_3 [142] and *L. casei* [230] have confirmed that the glucose PTS negatively regulates the expression of the lactose utilisation systems present in these organisms. Glucose-grown mutant cells metabolise glucose via a permease/glucokinase system and contain derepressed levels of lactose transport activity and phospho-β-galactosidase [142,230].

A similar mechanism may be responsible for the repressive effects of glucose and fructose on basal galactokinase expression in *Cl. perfringens*, since both sugars are PTS substrates in the clostridia [83,85,86,120]. Furthermore, glucose repressed galactokinase activity to a greater extent than did fructose, whereas no significant difference was observed between cells harvested from lactose- or lactulose-supplemented media. This implies the involvement of the glucose and fructose transport systems in repression, rather than a more direct intracellular effect. Further study, with a wider range of PTS and non-PTS sugars, and mutants defective in specific PTS systems, is required to clarify the role of such transport systems in regulation of operon expression in the clostridia.

Glucose not only regulated the low level of constitutive expression of galactokinase in *Cl. perfringens*, but also influenced the induced expression of this enzyme. Addition of glucose to growth media, along with an inducing sugar (lactulose or galactose), generally resulted in diauxie. In all cases, glucose appeared to inhibit the utilisation of the inducing sugar, and exhaustion of glucose from the medium relieved this inhibition. Inhibition of galactose utilisation was less severe than that of lactulose, indicating a differential sensitivity of the respective utilisation systems to glucose. The immediacy of the glucose effect on sugar utilisation, coupled with the fact that inhibition was observed in cells already induced for the *gal* operon (and presumably the β-galactoside or galactose transport sytems), suggests a direct inhibitory effect on the respective transport systems. This glucose effect is analogous, therefore, to the phenomenon of inducer exclusion (Introduction, section 1.7.3). The mechanism responsible may be similar to the allosteric regulatory model proposed for Gram-negatives (Introduction, section 1.7.3.1), since glucose is transported by a PTS sytem in *Cl. perfringens* [85], whereas lactulose and probably galactose are transported by non-PTS systems. In Gram-positives, PTS-mediated regulation of non-PTS uptake systems has not been studied in any great detail, preliminary evidence suggests that maltose uptake in *Staph. aureus* and glycerol uptake in *B. subtilis* are subject to inhibition by a factor III^{Glc} [138], whereas PTS-mediated regulation of glycerol uptake in *Staph. aureus* may be attributed to inhibition by intracellular sugar phosphate (Introduction, section 1.7.3.2) [140]. In theory, either mechanism could explain the effects of glucose on galactose or lactulose utilisation in *Cl. perfringens*. However, it is not known if the clostridia possess a soluble factor III^{Glc} molecule, which is a prerequisite for the allosteric mechanism. Thus, more detailed characterisation of the glucose PTS of

the clostridia is required. It should be noted that addition of glucose to cells already growing on galactose or lactulose did not result in measurable expulsion of the inducing sugar from the cell. This is not unexpected, since inducer expulsion is specific for PTS-mediated regulation of PTS uptake systems (Introduction, section 1.7.3.5) [231].

Although inhibition of galactokinase induction was evident during the periods of inducer exclusion, several observations suggest that this mechanism may not be the only glucose effect in *Cl. perfringens*. When glucose and galactose were added together to basal medium, prior to enzyme induction, analysis of the differential rate of enzyme induction revealed two components. The slowly increasing component was observed during the period of preferential glucose utilisation and, although inhibition of galactose was not complete, the rate was only similar to that for basal medium-grown cells. It follows that repression of catabolite gene expression, independent of inducer exclusion, may also play a role in the mediation of glucose effects in *Cl. perfringens*. Absolute proof for the involvement of such a mechanism in *Cl. perfringens* would require regulatory mutants, constitutive for galactokinase (Introduction, section 1.7), which are currently not available.

An important additional effect was observed when glucose was added to cells preinduced for galactokinase by growth in the presence of galactose or lactulose: a net increase in the differential rate of induction was converted to a net decrease. This effect was only slight in the case of cells preinduced with lactulose, but was more significant in the case of cells preinduced with galactose. The effect differed from the phenomenon of transient repression (Introduction, section 1.7) in that it persisted for as long as glucose was present in the medium.

The mechanism of this inhibitory effect is unclear. It may be due to activation of a degradation mechanism or direct inhibition of galactokinase activity. A similar decrease in galactokinase activity was observed upon exhaustion of lactulose (5 mM) from the control culture, but glucose-mediated inhibition of galactose utilisation in preinduced cells was incomplete, therefore, this effect occurred even though galactose was still being taken up, albeit at a greatly reduced rate. Purification of galactokinase, coupled with a study of its regulation by catabolites of lactulose and glucose, may help identify possible inhibitory compounds.

Similar effects have been reported in *Str. lactis* 7962 [206], *B. megaterium* [198] and *E. coli* [232]. Glucose-mediated inhibition of galactose metabolism in preinduced cells of *Str. lactis* 7962 has been attributed to a direct inhibitory effect on the enzymes of the Leloir pathway, since glucose does not directly inhibit galactose transport activity (as measured with thiomethyl β-D-galactoside) [206]. In the case of galactose-adapted cells of *B. megaterium*, glucose incompletely inhibits galactose transport (decreased by 50%), but completely inhibits β-galactosidase synthesis [198]. Furthermore, the rate of decay of β-galactosidase specific activity is much faster than can be accounted for simply by dilution due to cell division [198].

The possibility that glucose or a metabolite may directly inhibit the activity, rather than

the synthesis, of induced catabolic enzyme systems was first considered by McGinnis and Paigen (1969) [232]. Glucose or glucose 6-phosphate was found to inhibit the metabolism of galactose or lactose in preinduced cells of *E. coli*, and they termed this effect catabolite inhibition. However, they were only able to identify the actual site of inhibition as an early step in metabolism (transport or the first intracellular step) [232], therefore, catabolite inhibition in *E. coli* may be solely mediated via an inducer exclusion mechanism.

In the present study, no attempt was made to differentiate between glucose-mediated catabolite inhibition of the transport step and that of subsequent metabolic steps. This would have required the use of non-metabolisable analogues of the respective sugar transport systems and, therefore, a more detailed characterisation of the substrate specificity of these systems. However, inhibition/degradation of galactokinase was by no means sufficient to account for the degree of inhibition of sugar utilisation, therefore, inducer exclusion may be the more important glucose effect.

The effects of cAMP on glucose-mediated inhibition of lactulose utilisation and galactokinase activity were not absolutely clear. Differences were apparent in the galactokinase profiles of cAMP-supplemented and unsupplemented cultures, but these effects were observed after glucose exhaustion from the medium. The differences may have been due to natural variation between the two cultures, as discussed in detail elsewhere (Results, section 3.2.3.2). More significantly, cAMP had no effect on the severe inhibition of both galactokinase synthesis/activity and lactulose utilisation. However, since the main glucose effect in this organism is most likely inducer exclusion, then this finding is not totally unexpected because cAMP does not play any significant role in the mediation of this regulatory effect.

In conclusion, at least two, and possibly three, distinct mechanisms have been identified whereby *Cl. perfringens* can regulate sugar utilisation: induction/repression, inducer exclusion and catabolite inhibition. All of these mechanisms may play an important role in nature, where the organism has to adapt to an ever changing environment. Thus, under conditions of sugar starvation, inducible catabolic operons may be constitutively expressed at low levels to ensure the immediate utilisation of any suitable sugar. If an easily metabolisable sugar becomes available, and if this also a substrate of a PTS system, then the basal synthesis of inducible catabolic systems may be repressed, thereby minimising wasteful expenditure of energy. However, in the case of galactokinase at least, repression is never complete, but may be dependent upon the degree of saturation of that PTS system. In contrast, if a less readily metabolisable sugar substrate, such a disaccharide substrate of a non-PTS system, should become available in the absence of any PTS sugar, then induction ensures that only the synthesis of the relevant catabolic system is increased.

It is more likely that several sugar substrates (both PTS and non-PTS) may become available simultaneously. Under such conditions, PTS-mediated inhibition of non-PTS uptake systems will ensure the preferential utilisation of the more readily metabolised PTS sugars. Repression and inhibition probably also play important roles under such

conditions, especially if the PTS sugars are present at high levels. Finally, catabolite inhibition may be important in ensuring the preferential utilisation of PTS sugars, should these sugars become available when the organism is already metabolising non-PTS sugar(s). Under such conditions, the level of expression of the induced catabolic systems may be high and inducer exclusion (by an allosteric mechanism) may be insufficient to titrate out non-PTS transport proteins.

6.4 Continuous Culture Studies.

The growth yield coefficients (Y_{LL}) from both batch culture (range 102-435 g dry wt/mol) and continuous culture (range 140-350 g dry wt/mol) studies were very high, since values of approx. 20 g dry wt/mol glucose fermented (40 g dry wt/mol disaccharide) are normally obtained when bacteria are grown under anaerobic conditions [232]. High values have also been reported for a range of anaerobic bacteria (including other strains of *Cl. perfringens*) grown in both batch (BC) and continuous culture (CC). Examples include: *Cl. perfringens* strains ATCC 3624 (Y_{glc} = 40 g dry wt/mol in BC) [234] and 13124 (Y_{glc} = 64 g dry wt/mol in BC or 43 g dry wt/mol in CC) [235], lactic streptococci (Y_{glc} ≤40 g dry wt/mol in BC or 43.8 g dry wt/mol in CC) [233,236], *Bact. fragilis* (Y_{glc} ≤105 g dry wt/mol in BC or 120 g dry wt/mol in CC) [237], *Bact. thetaiotamicron* (Y_{glc} = 76 g dry wt/mol in CC) [190] and rumen bacteria such as: *Selemonas ruminatium*, *Bact. ruminocola*, *Megasphera elsdenii* (Y_{glc} ≤99 g dry wt/mol) [238,239] and *Bact. amylophilus* ($Y_{maltose}$ = 160 g/mol) [240]. Indeed, it has been suggested that high yield coefficients may be the general rule for rumen bacteria and other obligate anaerobes [241]. This has been tentatively linked with the operation of anaerobic electron transfer-dependent phosphorylation reactions (many of the above bacteria possess functional electron transfer components), and end product efflux in symport with protons may be a contributory factor [241].

The present study indicated that end product efflux does not play a major role in energy generation in at least one strain of *Cl. perfringens*, and other evidence suggests that membrane-linked electron transport chains are absent from the clostridia [233,242]. These organisms can generate ATP by substrate level phosphorylation reactions involving acylate kinases (acetate kinase and butyrate kinase), and such reactions may contribute to the high yields. However, assuming a Y_{ATP} value of 10.5 g dry wt/mol [233], and an ATP yield of approx 3.3 mol/mol hexose fermented (as reported for *Cl. pasteurianum*) [242], then a Y_{hexose} value of approx. 35 g dry wt/mol hexose (or 70 g dry wt/mol disaccharide) can be calculated. This value is similar to the Y_{glc} values reported for *Cl. perfringens* ATCC 3624 in batch culture [234] and strain ATCC 13124 in continuous culture [235], but much less

than that reported for *Cl. perfringens* ATCC 13124 in batch culture [235], and the *Cl. perfringens* strain studied in the present investigation (both batch and continuous cultures).

The above discrepancies may have been due to the complex nature of the basal medium. In the present study, this contained yeast extract and proteose peptone supplements, whereas in the study with strain ATCC 13124, it contained a proteose peptone supplement [235]. A pre-requisite for the determination of accurate yields is that the substrate under study must be the sole source of energy for growth [233,241]. It is feasible, therefore, that components of the growth medium may have served as additional energy sources. Indeed, batch growth in basal medium alone was significant. Although the correction of all data for basal growth resulted in more acceptable values, it is likely that the true yield may have been underestimated. No attempt was made to identify the component(s) of the basal medium or quantitate their utilisation, and it does not necessarily follow that the additional energy source(s) will be used to the same degree in the absence or presence of lactulose.

In the case of yields determined in continuous culture, other studies with oral streptococci in glucose-limited chemostats have shown that complex media (containing tryptose, yeast extract and casamino acids) results in higher growth yields (range 25-41 g dry wt/mol glucose) than defined media (range 15-25 g dry wt/mol glucose) [243]. Unfortunately, no chemostat experiments were conducted with *Cl. perfringens* cells growing in basal medium alone, therefore, it is not possible to correct for basal growth. Although the increase in yield coefficient with increasing dilution rate implies that the utilisation of the unknown energy source increases with growth rate, this relationship may also be explained in terms of a maintenance requirement [192], in which case the unknown compound may be used to a similar degree at all growth rates. However, in the absence of basal growth data, it is impossible to draw any valid conclusions regarding a maintenance energy requirement in this organism. More useful information may have been obtained from both the batch and continuous culture studies if a minimal or chemically defined medium had been used. On the other hand, measurement of growth yields in *Cl. perfringens* was not a major objective of this study, rather, investigation of the metabolic pathways was the major aim, and this may have been compromised by poor growth.

Problems were also encountered with regard to the accurate measurement of specific growth rates in batch culture, due to the utilisation of components of the basal medium. Double-reciprocal plots of specific growth rate versus lactulose concentration deviated from linearity at low lactulose concentrations, and correction for basal medium growth appeared inappropriate. Consideration of the uncorrected data, from the linear portion of the curve (lactulose concentration $\geq$2 mM), permitted the determination of a half saturatic constant for growth of 1.85 mM, whereas corrected data yielded a value of 10 mM. The former value is consistent with the half saturation constant for lactulose utilisation determined in the buffered incubation system. The maximum specific growth rates determined from both uncorrected (0.341 h^{-1}) and corrected data (0.400 h^{-1}) were clearly erroneous, since samples were removed from the chemostat at dilution rates of 0.353 h^{-1}

and 0.436 h^{-1}. The increase in medium sugar at the latter dilution rate suggests that the culture is approaching washout, but no decrease in culture turbidity was apparent, which is normally associated with this phenomenon [191]. The discrepancy may have been due to the fact that the batch culture experiments were conducted in unstirred flasks incubated in the gas jar, rather than in the chemostat vessel. The different culture conditions present in the chemostat, namely; agitation, direct gas transfer and, most importantly, pH control, are probably responsible for the higher than expected growth rates.

The lactulose utilisation rate in the chemostat increased linearly with dilution rate up to approx. 0.3 h^{-1}, and then more slowly as the dilution rate was increased further. The observed maximum specific utilisation rate was 18.3 nmol/min/mg dry wt, which was approx. half that (39.9 nmol/min/mg dry wt) obtained in batch culture with 20 mM lactulose. When cells were harvested at different dilution rates and incubated in a buffered system, the resulting utilisation rates were approx. 2-fold higher than those determined in the chemostat, even though incubations were conducted with only 2.92 mM lactulose. The reasons for this are unclear; a component of the basal medium may be inhibitory, or different control mechanisms may operate in the two systems. However, utilisation rates determined in the buffered system showed a similar trend to those calculated in the chemostat, indicating that increases in expression of the lactulose utilisation system occur in response to increases in growth rate.

Inhibition of lactulose utilisation by CCCP (50 μM) was complete in all incubations, suggesting that a proton gradient is required for sugar utilisation over the range of dilution rates studied. In contrast, inhibition of utilisation by DCCD (50 μM) only amounted to 65-68% in all incubations. Similar degrees of inhibition (62-67%) were obtained when batch-grown cells were incubated with an identical concentration of this inhibitor. By analogy, incomplete inhibition of β-galactoside utilisation in chemostat-grown cells was most likely due to incomplete inhibition of the proton-translocating ATPase activity, rather than the operation of alternative (PTS) uptake systems.

The absence of phospho-β-galactosidase and the presence of β-galactosidase in chemostat-grown cells is in agreement with the above findings. This would suggest that *Cl. perfringens* does not possess the ability to produce a high affinity system (e.g. PTS) for β-galactosides under conditions of carbon limitation, even at slow growth rates. The production of high affinity sugar systems in response to carbon limitation has been observed for *K. aerogenes* [188], *Ps. aeruginosa* [244] and *Str. mutans* [245]. However, the inability to produce high affinity pathways is not necessarily uncommon behaviour, for example, *E. coli* and *K. aerogenes* appear to transport lactose by a proton-dependent system irrespective of lactose availability [246].

Bacteria generally respond to carbon limitation by derepressing the synthesis of their catabolic enzyme systems, while at the same time restricting the synthesis of their anabolic systems to match the growth rate requirements [188,247]. Unfortunately, no chemostat experiments were performed with an excess of lactulose, therefore, only tentative

conclusions can be drawn regarding the operation of a similar phenomenon in *Cl. perfringens*. Nevertheless, high activities of enzymes, relative to the utilisation rate, were observed at all dilution rates. This is perhaps best illustrated by consideration of the galactokinase data, since this enzyme gave the lowest measurable specific activity out of all the enzymes assayed. Low levels of specific activity were observed at dilution rates of 0.118 h^{-1} and 0.300 h^{-1}, with higher levels of activity present at the intermediate dilution rates. Recalculation of these specific activities as a function of dry wt gave values of 12.82 and 28.04 nmol/min/mg dry wt, at D = 0.118 and 0.300 h^{-1}, respectively, which were approx. 1.5-fold higher than the corresponding rates of lactulose (and hence galactose) utilisation (approx. 8 and 16 nmol/min/mg dry wt, respectively). Specific activities at intermediate dilution rates reached a maximum of 45 nmol/min/mg dry wt, which was approx. 4.5-fold higher than the corresponding rate of lactulose utilisation. Although these results by no means indicate a massive derepression of enzyme synthesis, studies with batch cultures indicate that galactokinase activity may have been underestimated in the assay system; the differential rate of galactokinase induction in batch cultures of lactulose (20 mM)-grown cell was approx. 2.5-fold lower than the specific rate of lactulose utilisation. These culture conditions supported a specific growth rate of approx. 0.3 h^{-1} and, at the appropriate dilution rate in the chemostat, enzyme activity was approx. 4.5-fold higher than the calculated utilisation rate. However, large differences exist between the environmental conditions in the two culture systems, therefore, such comparisons are not really justified.

A total of 5 different patterns of variation in enzyme activity with dilution rate have been commonly observed when bacteria are grown in the chemostat [246,247]:

a) A constant level irrespective of dilution rate.

b) An increase in activity with an increase in dilution rate.

c) A decrease in activity with an increase in dilution rate.

d) Activity passes through a maximum value at an intermediate dilution rate.

e) Activity passes through a minimum at an intermediate dilution rate.

Responses a) and e) are only infrequently observed, whereas response b) is normally characteristic of enzymes involved in biosynthesis or associated with respiratory chains. Responses c) and d) are invariably characteristic of catabolic enzymes. Indeed, all of the catabolic enzymes assayed in *Cl. perfringens* exhibited response d).

Maxima in catabolic enzyme activities, under conditions of carbon limitation, have generally been interpreted in terms of a balance between induction and catabolite repression [246,247]. The residual concentration of the growth limiting carbon source in the medium should increase as the growth rate is increased [246] and, since induction will be submaximal at low growth rates, increasing the dilution rate should result in a progressive saturation of inducing sites (specific activity increases). However, the effect of an inducer will also be dependent upon the metabolic state of the cell, with respect to the level of catabolite repressor compounds. Thus, an increase in the steady state concentration of the limiting substrate will also result in increased catabolite pools, therefore, catabolite

repression will also increase with dilution rate. A point may be reached whereby repressing compounds are produced at a sufficiently high rate so that repression dominates over induction. Consequently, specific activities may be expected to decline at high dilution rates.

Evidence to support this interpretation has come from studies employing regulatory mutants or non-metabolisable analogues of inducing compounds [246,247]. In a classical study, Clarke and coworkers (1968) studied the expression of the inducible enzyme amidase in acetamide (inducer)-limited cultures of *Ps. aeruginosa*, at various dilution rates [248]. In wild type cells, a sharp peak in activity was observed at D = 0.30-0.35 h^{-1}. Addition of succinate (repressing compound) to such cultures resulted in a more severe decrease in activity at the higher dilution rates, whereas the decline in activity was less severe in mutants with decreased sensitivity to catabolite repression by succinate. In fully constitutive mutants, the maximum in activity was observed at the lowest dilution rate studied, and this declined as the dilution rate was increased.

Several studies suggest that a more complex regulatory pattern may be responsible for the maximum in β-galactosidase activity observed in carbon-limited cultures of enteric bacteria [246]. When *E. coli* B6 is grown in a lactose-limited chemostat, a broad peak in activity is observed, with a maximum in activity at D = 0.7 h^{-1} [249]. The expected decrease in activity is observed when a constitutive mutant is grown under C-limitation with a mixture of glucose and lactose, but a maximum in activity (at D = 0.8 h^{-1}) is observed on lactose alone [249]. Sharp maxima in activity have also been observed for constitutive β-galactosidase activities in *E. coli* ML308, grown under succinate- or glycerol-limited conditions, and *E. coli* CA8.000c, grown under glycerol- or glucose-limited conditions [250]. Furthermore, a catabolite-insensitive mutant of strain CA8.000c had an almost constant level of β-galactosidase at D >0.3 h^{-1}, but activity declined at lower dilution rates, as for the constitutive parent strain [250]. Although addition of cAMP could relieve catabolite repression in glycerol-limited *E. coli* ML308 at high growth rates (D = 0.4 h^{-1}), it was ineffective at low values (D = 0.12 h^{-1}), where activity was submaximal. The decline in activity at low dilution rates in constitutive strains was attributed to a restricted supply of components necessary for enzyme synthesis, rather than a decrease in intracellular cAMP levels [250].

Assuming that catabolite repression is exclusively mediated by changes in cAMP levels, then one should expect a decrease in the level of this nucleotide with increasing dilution rate. Studies with *E. coli* K-12 indicate that the level of cAMP is fairly constant at all dilution rates (0.05-0.40 h^{-1}) when glucose is limiting, whereas the expected decline in levels occurs when succinate is limiting [251]. In contrast, another group has reported a decrease in cAMP with increasing dilution rate for *E. coli* 451-B variant under conditions of glucose-, glycerol-, or lactose-limitation [252]. Similar changes in cGMP concentration were also observed, and it was suggested that different enzyme systems may be subject to regulation by different small molecules (including cAMP and cGMP) [252]. Furthermore,

each system may not necessarily respond in the same way to changes in the levels of these compounds [252]. Hence, the possibility arises that the decreases in constitutive β-galactosidase synthesis at low dilution rate may result from the operation of cAMP-independent repression, rather than limitation of compounds necessary for its synthesis.

Returning to the maxima in catabolic enzyme activity in *Cl. perfringens*. It is feasible that this pattern may have resulted from the operation of differential induction/repression effects at the various dilution rates. The actual mechanisms of repression are unidentified, but direct involvement of PTS systems seems unlikely, since such mechanisms were found to be inoperative in the metabolism of lactulose. Repression is most likely mediated by metabolites of lactulose, and components of the basal medium may also play a role. The actual mechanism involved must be cAMP-independent, due to the reported absence of cAMP from this strain [145], and it is unclear if catabolite inhibition is also involved. A more detailed characterisation of regulatory mechanisms would have been aided by the availability of regulatory mutants or non-metabolisable analogues of the β-galactoside utilisation sytem. Studies of a range of nutrient limitations may have given some clue to the chemical identity of the repressing compound(s). Such studies with *Ps. aeruginosa* have shown that the molecules responsible for catabolite repression are most likely compounds of C, N and P, since the activities of repressible enzymes decreased as the dilution rate was increased when any one of these three nutrients was growth limiting [247]. Improvement of the detection limits of the metabolite assays (possibly by increased concentration of extracts) may have permitted more firm conclusions to be drawn regarding the involvement of metabolites in induction and catabolite repression/inhibition.

According to theory, the residual concentration of growth limiting substrate should increase as the dilution rate is increased [191,247]. Residual lactulose concentrations were low (≤3%) at all dilution rates studied and, although the theoretical relationship was observed at dilution rates >0.1 h^{-1}, the residual lactulose concentration was relatively high at the lowest dilution rates studied. It is possible, therefore, that a component of the basal medium may have been limiting growth at very low dilution rates. If this is the case, then the increase in enzyme activities at low dilution rates may have resulted from derepression, as lactulose becomes limiting, rather than an increase in induction. Hence, enzyme activity may simply decline as the dilution rate is increased under lactulose-limiting conditions. In view of these findings, use of a lower medium lactulose concentration would have been preferable.

It is of interest that the levels of all three Leloir pathway enzymes varied in parallel as the dilution rate was increased, whereas UDPgalactose 4-epimerase appeared to be independently regulated in batch culture. This may have been due to the differences in environmental conditions in the two culture systems. In batch culture, the levels of galactokinase and galactose 1-phosphate uridylyl transferase levelled off as the culture approached stationary phase, whereas that of epimerase continued to increase. This latter

enzyme is required for cell wall synthesis, in addition to lactulose metabolism, and it is possible that the adverse culture conditions (pH of approx. 5) resulted in changes in cell wall synthesis. It would be pertinent, therefore, to study the expression of this enzyme at different pH values in the chemostat.

It should be emphasised that the major aim of the continuous culture studies was not to produce an accurate model of the large intestine, due to the constraints of time. This would have required a much more complex culture system, and significant advances have been made in this subject area recently [189,253-255]. However, some of the above findings may be pertinent to the *in vivo* situation.

Under conditions of C-limitation, at low dilution rates, *Cl. perfringens* appears to derepress the synthesis of induced catabolic enzymes. Increased enzyme synthesis at low growth rates would at first appear a wasteful response, however, the physiological rationale for such a response is clear [247]. No evidence was obtained for the production of high affinity (PTS) uptake systems at low dilution rates, therefore, the only way to increase the organism's metabolic effectiveness at low lactulose concentrations is to increase the levels of the appropriate catabolic systems [247].

The mean generation time in the human colon has been estimated as approx. 40 h [190], but it is unlikely that growth and metabolism occur at steady rate. More likely, these processes will be most rapid immediately following the pulsed entry of ileal fluid after feeding [189]. Furthermore, growth rates may be expected to vary along the length of the colon, being most rapid proximal to the ileocaecal valve [190]. In the case of *Cl. perfringens*, β-galactosidase activity was studied over a range of dilution rates equivalent to mean generation times of 1.6-23.1 h. These results are only pertinent, therefore, to those areas of the colon where growth proceeds at rapid rates. Clearly, further study is required at lower dilution rates, preferably using lower medium lactulose concentrations.

Although carbon sources are considered to be the growth limiting nutrient *in vivo* under normal conditions [256], this may not necessarily be the case immediately following the administration of 20-30 g of oral lactulose. In addition, other sugars will also be present *in vivo* (Introduction, section 1.3.2), and it is not clear if these may exert repressive effects on the utilisation of lactulose, or vice versa. Thus, studies are required with both single and mixed sugar substrates, under conditions of carbon limitation and carbon excess.

An important consideration is the pH of the culture medium. Lactulose metabolism was studied at a fixed pH of 7, whereas oral administration is known to result in a marked acidification of the colonic contents (Introduction, section 1.1 and Table 1). Studies with a faecal incubation system indicate that acid is inhibitory to lactulose metabolism [25], and others have shown changes in the fermentation product pattern with decreasing pH [33]. This latter finding suggests that the contribution of different bacterial groups to lactulose breakdown may change as a direct consequence of metabolism [33]. However, since butyric acid is produced in large amounts, irrespective of culture pH, the clostridia may be relatively refractory to changes in pH [33]. This could have been tested using the

chemostat, by simply measuring utilisation rates at various pH values and fixed dilution rates.

Finally, a mixed flora is present *in vivo* and, although *Cl. perfringens* appears to be an active metaboliser of the sugar under laboratory conditions, it is unknown if this bacterium would be able to compete effectively with other organisms present in the colon. It is likely that those bacteria which transport the sugar by high affinity systems will be more effective in scavenging the sugar, and bacterial numbers need also to be taken into account. The clostridia constitute only a minor proportion of the total flora, therefore, more detailed studies are required with representatives of more numerically important bacterial groups, such as the bifidobacteria, eubacteria and bacteroides (see Table 4).

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