The Metabolism of Lactulose by Intestinal Bacteria

by

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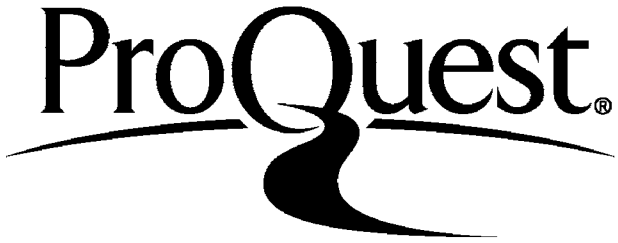

ABSTRACT

About 60 strains of intestinal bacteria were cultured in lactulose-containing media to quantitate both sugar fermentation and non-gaseous end-products. Certain species of clostridia (especially C.perfringens), lactobacilli and bacteroides utilised the disaccharide extensively, while other organisms were unable to metabolise this substrate. β-Galactosidase activity did not parallel growth on lactulose in all cases. The major fermentation products were acetic, lactic and butyric acids. Hydrogen and carbon dioxide were the only gases qualitatively detected. Clostridial strains exhibited a butyric type fermentation, and most lactobacilli were homofermentative. Fermentation products were estimated for selected species throughout their growth cycles. The products of lactulose fermentation by mixed bacterial cultures varied with incubation conditions such as pH, but correlated well with those produced by pure cultures.

Studies on lactulose transport by C.perfringens indicated methodological limitations in assaying (^{14}C) lactose uptake and in the use of NADH-based procedures. o-Nitrophenyl β-D-galactopyranoside uptake by lactulose grown whole cells and an absence of phospho-β-D-galactosidase suggested an active transport of the disaccharide. The inducible β-galactosidase was partially purified and characterised; fructose 1,6-bisphosphate inhibited enzyme activity by 26%, and lactulose or lactose hydrolysis required K^+ ions. Galactokinase was inducible in galactose, lactulose or lactose grown cells. Fructose 1-phosphate kinase (FIPK) and fructose 6-phosphate kinase were detected in fructose grown cell-free extracts; FIPK was partially purified five-fold by affinity chromatography.

The glucose effect was observed in C.perfringens grown on lactulose, and could not be eleviated by external cyclic AMP or dibutyryl cAMP. Assays for the cyclic nucleotide in lactulose grown cells and extracellular fluid were in the negative. This inhibition was not observed during growth on a mixture of lactulose with fructose, and co-utilisation of lactulose with galactose and lactose respectively was apparent.

CONTENTS

To my family and friends

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Abbreviations

The recommended abbreviations described in Instructions to Authors, Biochem. J. (1986) 233, 1 - 24, are used.

CHAPTER 1: INTRODUCTION

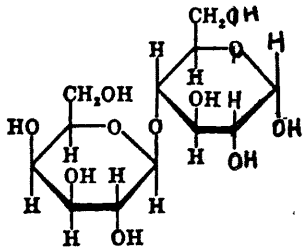
1. PREFACE

Lactulose (4-o-β-D-galactopyranosyl-α-D-fructofuranose; Fig. 1.1) is a synthetic disaccharide used clinically in treating the comagenic disorder portal-systemic encephalopathy (PSE) as well as other minor syndromes (Conn and Lieberthal, 1979; Weber, 1981.) Remarkably, these applications arise from the microbial degradation of the disaccharide in the gut, as the human intestinal lactases (EC 3.2.1.2.3) do not hydrolyse ingested lactulose (Dahlquist and Gryboski, 1965) and the sugar is poorly absorbed in the small intestine (Maxton et al., 1986; Noone et al., 1986). Despite its widespread use, little is known of the biochemical nature of microbial dissimilation of the disaccharide. Consequently, the aim of the present study was to examine specific intestinal bacteria for their ability to utilise the disaccharide, quantitatively assess fermentation products, and investigate biochemical aspects of lactulose uptake, metabolism and regulation in a selected species.

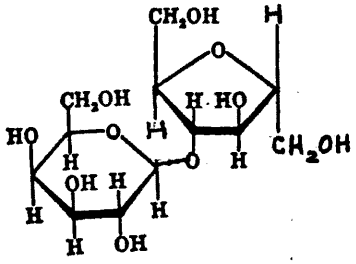
1.1 CHEMISTRY OF LACTULOSE

Lactulose does not occur naturally; although absent in raw milk, it has been detected in milk products subjected to heat treatment (Adachi and Naganishi, 1958; Ersserand Mitchell, 1984.

The laboratory synthesis of the sugar was first described by Montgomery and Hudson (1930), making use of the Lobry de Bruyn-Alberda van Elkenstein rearrangement of aldoses to ketoses in alkaline solution at 35°C for 36h using lactose as precursor. In subsequent work, replacing calcium hydroxide with other alkaline agents minimised side-products, and modified procedures to separate the new ketose from the mixture of aldoses



α-Lactose: 4-O-β-D-galactopyranosyl-β-D-glucopyranose



α-Lactulose: 4-O-β-D-galactopyranosyl-β-D-fructofuranose

Fig 1.1 Structural formulae of lactose and its keto-analogue lactulose (from Mendez and Olano, 1979)

and decomposition products involved ion-exchange resin columns or direct acetylation (review, Adachi and Patton, 1961). Alternative methods for obtaining lactulose were much less efficient.

Montgomery and Hudson (1930) reported the structure of lactulose as 4-0-β-D-galactopyranosyl-D-fructose, with the crystalline sugar in an α-form. Optical rotatory studies of the disaccharide in a buffered solution found crystalline lactulose to be a furanose (Isbell and Pigman, 1938). More recently, n.m.r. conformation analyses of freshly dissolved crystals of lactulose in deuterium-oxide and methyl-sulphoxide-d_6 indicated the predominant isomer to possess a β-D-fructofuranose moiety (Mendez and Olano, 1979). However, in the latter report, a different n.m.r. procedure resolved the β-D-fructopyranose as the major form. Although this discrepancy has not been further resolved cited literature usually describes lactulose as a furanose in its fructose constituent (review, Mendez and Olano, 1979).

A number of properties of lactulose have been described. It is considerably sweeter than its corresponding aldose, a physiological quality thought to result from several structural criteria (Lee and Birch, 1976). Lactulose gives the Seliwanoff test and Dische-Borefreund test for ketoses and reduces Fehling's solution on heating (Adachi and Patton, 1961). The white transparent crystals have a m.p. between 168.5° and 169.0°C, (Oosten, 1967a), and are very soluble in water, with a solubility of 76.4 ± 1.4% ($^{w}/_{v}$) at 30°C which increases to over 86% ($^{w}/_{v}$) at 90°C(Oosten, 1967b).

Separation of lactulose from lactose or other sugars can be achieved by a number of chromatographic procedures, by

using paper chromatography in isopropanol/water as the mobile phase (Bernhard et al., 1965; Oosten, 1967b), by t.l.c. (see Section 2.2.4), g.l.c. (Müller et al., 1969), and by anion-exchange chromatography (Ersser and Mitchel, 1984). Spectrophotometric methods to quantitate lactulose involve the cysteine-carbazole-sulphuric acid reaction (5 - 100 µg lactulose; Adachi, 1965a), or for larger amounts of sugar (2 - 8 mg), a methylamine-sodium hydroxide reagent (Adachi, 1965b). A simple procedure described by Vachek (1971) is a reliable quantitative assay for 0 - 8 mg lactulose, based on heating the aqueous solution with dilute sulphuric acid and measuring the stable coloured product at 400 nm (Section 2.2.4).

1.2 CLINICAL USES OF LACTULOSE

1.2.1 The treatment of portal-systemic encephalopathy

Portal-systemic encephalopathy (PSE) is a condition of cerebral and neuromuscular dysfunction caused by a combination of cirrhosis, portal hypertension and/or portal-systemic shunting, where a significant volume of portal blood bypasses the liver (Sherlock, 1977; Conn and Lieberthal, 1979). PSE aetiology, clinical symptoms and diagnosis have also been reviewed in the latter reports.

1.2.1.1 Pathogenesis of PSE

The pathogenesis of PSE is thought to be due to gut-derived toxins remaining in the systemic circulation due to inadequate liver function or because of the spontaneous portal-systemic collateral circulation (Muting et al., 1973; Fischer et al., 1976; Vince and Burridge, 1980). A complication arises as some of these toxins are metabolites also released from impaired hepatic or

muscle-cell function (Sherlock, 1977). Putative toxins of gut origin are numerous: ammonia (Conn, 1978), short-chain fatty acids (Chen et al., 1970), false neurotransmitters (Fisher and Baldessarini, 1971), amino acids (Fischer, 1979), and bacterial metabolites of methionine (Zieve et al., 1974). However, PSE therapy is generally aimed at controlling systemic nitrogen levels (Bircher et al., 1971a; Sherlock, 1977).

Ammonia remains the most extensively investigated nitrogenous metabolite. Early studies in the 1950's showed that oral doses of urea, dietary protein, ammonium salts or intestinal haemmorhage caused mental disturbances in cirrhotic patients (review, Conn and Lieberthal, 1979). Several sources of ammonia are available in vivo. Bacterial deamination of amino acids yield fatty acids and ammonia (Sabbaj et al., 1970), and metabolism of residual dietary protein produces ammonia and amines (Phear and Ruebner, 1956). Autolysis of bacterial protoplasm and shed mucosal cells are another source (Wrong, 1978). Large amounts of ammonia were generated from these sources in a virtually urea-free in vitro faecal homogenate system (Vince et al., 1976). Urea is an important substrate as bacterial ureases release ammonia for absorption along the entire length of the intestine (Evans et al., 1966; Wrong, 1971). Diffusion of urea from the systemic circulation is mainly in the small intestine (Fordran et al., 1965; Brown et al., 1975). Since hepatic biosynthesis of urea is the main detoxification pathway for blood ammonia (Visek, 1972), hepatic dysfunction in PSE is deleterious. The main ammoniagenic reaction, the deamination of glutamine, contributes host 'cellular' ammonia (Conn and Lieberthal, 1979).

However, although several studies have reported an acceptable correlation between blood ammonia and grade of PSE (White et al., 1955; Elkington et al., 1969) this is by no means always the case (Dastur, 1961; Zeegan et al., 1970; Sherlock, 1977), possibly due to venous blood ammonia assays being less reliable than arterial measurements, or to misdiagnosis of PSE (Conn and Lieberthal, 1979).

Several mechanisms have been postulated to explain the potential cerebrotoxicity of ammonia: a) a direct inhibitory effect on the respiratory chain; b) a decrease in available NADH; c) accumulation of the inhibitory neurotransmitter γ-aminobutyric acid; d) and others (review, Summerskill, 1971). Experimental evidence though, is inconclusive.

1.2.1.2 Clinical trials

It was suggested that as lactulose increased colonic lactobacilli (Petuely, 1957), which lack urease and other ammonia-genic enzymes, it would prove useful in the treatment of PSE (Ingelfinger, 1964). This postulate was subsequently confirmed by Bircher et al., (1966) by the successful treatment of 2 PSE patients with lactulose syrup. Over the last 20 years, a success rate of 75% has been reported in the clinical use of lactulose in PSE therapy. (Conn and Lieberthal, 1979; also see Rossi-Fanelli et al., 1982; Desai, 1983) Appropriate medication for the underlying liver disease or other causes and any concomitant disorders is also necessary. Clinical side-effects, abdominal distension, cramp or flatulence can be controlled by adjusting the lactulose dose (Carlin, 1973). Other side-effects have been reported, hyper-natraemia

(Nanji &Lauener,1984), and severe lactic acidosis in one patient (Mann et al., 1985).

To assess drug efficacy, measurements of faecal frequency, weight and bacteriology, as well as estimations of arterial and venous blood ammonia, and a number of psychometric tests are employed (Zeegan et al., 1970; Conn, 1977; McClain et al., 1984). Three types of clinical trials have been conducted. Firstly, an empirical approach administering lactulose orally or by enema in controlled doses (Lande and Clot, 1968; Cassi et al., 1972). Secondly, a comparative experimental design where PSE patients receive lactulose or neomycin respectively or in conjunction during alternative periods. For example, Fessel and Conn (1973) studied 24 patients, and 20 out of 24 responded to lactulose and 23 out of 24 to neomycin, although lactulose treatment improved mental state faster (2.2 -vs- 3.7 days; $p < 0.01$). In chronic PSE however, a continuous administration of a broad spectrum antibiotic such as neomycin may result in Staphlococcal enterocolitis, or malabsorption of sugars and fats (Elkington, 1970). Other comparative studies have contrasted lactulose with sorbitol, which also has a laxative effect but is ineffective in PSE (e.g. Bircher et al., 1966).

The third type of clinical trial is the most important, i.e. the double-blind controlled study (Elkington et al., 1969; Atterbury et al., 1976; Conn et al., 1977). In the investigation by Conn et al., (1977) for example, neomycin plus sorbitol and lactulose plus placebo tablets were administered to patients in a cross-over manner, preceded and followed by control periods. Both neomycin/sorbitol and lactulose/placebo were effective in the

majority of patients (83% and 90% respectively), and each tested parameter of PSE improved significantly.

1.2.1.3 Mode of lactulose action

Generally, the metabolism of lactulose by intestinal bacteria is thought to explain its efficacy in PSE therapy; more recently, a lactulose effect independent of microbial degradation has been suggested, but as yet not explained (Soeters et al., 1984; van Leeuwen et al., 1984a, b). Several hypotheses on the mode of lactulose actions following bacterial fermentation have been examined: promoting urease-negative bacteria and therefore altering composition of gut flora to a less toxicogenic form (see e.g. Bircher et al., 1966; Bircher et al., 1971a); a purgative effect which rapidly removes the offending toxin (Elkington, 1970); acidification of the gut to low pH which ionises ammonia thereby preventing its absorption (Agostini et al., 1972); and finally, the substrate effect of lactulose, where growth on a good carbon source reduces luminal NH_3 levels as NH_3 is used for bacterial nitrogen (Vince et al., 1978). However, anomalies exist in the experimental verification of these proposals.

The original hypothesis (Ingelfinger, 1964; Bircher et al., 1966) that bacterial growth or lactulose would produce a more non-ammoniagenic balance in the flora has received little experimental support. For instance, while Bircher et al., (1970) noted a significant lactulose-dependent increase in anaerobic lactobacilli, and an acceptable decrease in bacteroides numbers, no corresponding reduction in urease activity was shown. Furthermore, Conn (1978) has pointed out that bacterial quantitation in

the latter study may be an artifact as specimens were processed up to 48h after sampling. A similar pattern of change in lactobacilli and bacteroides occurred 4 - 20 days after clinical improvement of PSE (Bircher et al., 1969; 1971a). An increase in faecal lactobacilli, but with bacteroides or other groups remaining constant correlated in one study with clinical improvement (Vince et al., 1974) but not in another (Zeegan et al., 1970). Converseley, Elkington et al., (1969) noted negligible change in stool bacterial composition following successful lactulose therapy in PSE. The absence of consistent results would suggest that gross changes in specific bacterial populations are either not important or occur in a magnitude which is physiologically effective but methodolically not feasible to quantitate (Hill, 1981). Assaying bacterial enzyme activities may therefore be more pertinent following lactulose administration.

The purgative effect of lactulose, proposed to decrease the retention time of nitrogenous toxins, is mainly due to its fermentation products of organic acids, which impart an osmotic pull of water into the colon (Conn and Lieberthal, 1969). Intake of lactulose as a hypertonic syrup reinforces this effect. A calculation that 20 g of lactulose increases the colon volume by about 600 ml after 1 to 3h, implies a significant effect if a normal colon volume of 200 - 600 ml is accepted (Bircher, 1972). However, in dose-response experiments with normal subjects, 73 - 146 mmol lactulose caused faecal output of water to exceed 400 ml only after 48h (Saunders and Higgins, 1981). This cathartic effect alone may not be important, as control substances such as sorbitol (Bircher et al., 1966; Elkington et al., 1969) and magnesium sulphate (Zeegan et al.,

1970) which have a purely laxative action produced no clinical improvement.

Bacterial breakdown of lactulose to fermentation acids should also lower intestinal pH. Chronic ingestion of lactulose has been shown to increase caecal lactic and acetic acids and total volatile fatty acids (Florent et al., 1984). Clinical trials confirmed that lactulose therapy significantly reduced faecal pH from a mean control of 7.0 to 5.4 (Elkington et al., 1969; Zeegan et al., 1970; Bircher et al., 1971a). Depending on the intestinal site however, a variable pH effect was produced by lactulose (Bown et al., 1974). These authors used a novel pH-sensitive radiotelemetric device to measure luminal pH. Lactulose produced the largest median pH decrease to 4.8 (if control, 6.0) in the proximal colon, lower pH values throughout the intestinal tract, but values approaching neutrality in the distal colon and rectum. Sodium sulphate also produced a consistent faecal acidification (Agostini et al., 1972; Bown et al., 1974), but has no beneficial effect in PSE. This may mean that a pH effect is most important in the ileo-caecal region. Gastrointestinal pH measurements are influenced by absorption of organic acids by colonic mucosa (Dawson et al., 1964) and the buffering of colonic contents by mucosal secretion of bicarbonate (Bown et al., 1974). Overall, the evidence suggests that lactulose significantly decreases intestinal pH. As to how acidification of the gut reverses pathogenesis of PSE, three explanations have been reported. Firstly, Bennett and Eley (1976) suggested a laxative effect due to fermentation acids, since perfusion of isolated guinea-pig intestine with lactic acid (pH 4.5 - 5.0) or HCl (pH 4.3 - 4.8) caused peristaltic stimulation of gut circular muscle.

However, as discussed previously, a purely laxative action of lactulose appears unacceptable (p.10). The second proposal concerns the phenomenon of 'ammonia-trapping' by acid gut environment. The dissociation constant (pK_a) for NH_4^+ is 8.9 at 37°C, and only the NH_3 form is able to permeate cell membranes by passive diffusion, whilst NH_4^+ translocation is energy dependent (Conn and Lieberthal, 1979). Below a faecal pH of 6.2, the movement of NH_3 from colonic lumen into blood has been shown to be reversed in colon perfusion studies in dogs (Bircher et al., 1971b). Experimental isolation of the colon in chronic PSE patients showed that by varying the pH of the perfusate, diffusion of NH_3 into the blood depended on intraluminal pH (Castell and Moore, 1968). Consequently, Agostini et al., (1972) argued that ammonia concentration of acid stools should rise significantly as ammonia was 'trapped' and also continually produced by bacteria. However, in their report, in vivo faecal dialysis measurements indicated that lactulose did not increase faecal ammonia levels. In contrast, faecal ammonia concentration was notably elevated in patients treated with lactulose (Hedger and Ing, 1977). Such an increase though, is a small fraction of the large colonic ammonia pool (Conn and Lieberthal, 1979). Therefore, retention of ammonia by acidic gut contents is probably a minor component of the overall lactulose effects. A third postulate linked intestinal acidification with inhibition of bacteria producing ammonia from urea or other nitrogenous substrates. Evidence from in vitro studies indicated that Gram-negative anaerobes, clostridia, enterobacteria, and Bacillus spp. formed less ammonia at pH 5.0 than at pH 7.0 (Vince and Burridge, 1980). This proposal

may, in part, explain lactulose efficacy. Lactulose has also been shown to cause a 25% reduction in urea production rate (Weber et al., 1982).

Finally, the substrate effect of lactulose may well be the key mechanism in explaining its mode of action. In the presence of fermentable carbohydrate, ammonia is thought to be incorporated into bacterial cells, whereas growth solely on nitrogenous substrates releases ammonia (Mason, 1974; Vince et al., 1978; Vince and Burridge, 1980). In rats fed egg albumin, certain carbohydrates (raw potato, yam, cellulose) resulted in a significant increase in total faecal nitrogen; lactulose too produced a notable increase, but less so than the other carbohydrate preparations (Mason, 1974). In an in vitro faecal homogenate incubated with lactulose, or other fermentable substrates (glucose, mannitol, sorbitol), ammonia concentration was significantly reduced during fermentation (Vince et al., 1978). By contrast, acidification to pH $\leq$ 5.0 with HCl or a lactic-acetic acid mixture reduced ammonia generation appreciably, but without lowering existing ammonia content. This observation may also explain the previous inability (Agostini et al., 1972) to detect sufficiently elevated faecal ammonia levels. With four isolates of Bacteroides, one of C.perfringens, and two isolates of S.faecium, less ammonia production was estimated from alanine, methionine or histidine after growth in the presence of either lactose or lactulose (Vince and Burridge, 1980).

Finally, although experimental evidence from a group of co-authors indicates that lactulose reduces in vitro mucosal cell metabolism of glutamine independently of bacterial action, producing ammonia and amino acids, the effect has not been rationalised (Soeters et al., 1984; van Leeuwen et al., 1984a, b). Enterocyte

preparations, from conventional and germ-free rats, generated equal amounts of ammonia: lactulose (10%; 29 mM) reduced ammonia levels by about 20%, whereas neomycin had a variable effect (Soeters et al., 1984). Sections of gut tissue incubated with glutamine generated more ammonia, alanine and glutamate in the small intestine than large intestine medium; lactulose (25%; 73 mM) decreased ammonia production in the small bowel preparation to less than 40% of control, but had no effect on the large intestine sections (van Leeuwen et al., 1984b). Although whole tissue preparations were washed in ice cold saline and Krebs Ringer buffer, no estimate of epithelial microbes was made. In laboratory rodents, the attachment of microbes to epithelia is a secure one, and the microbes are difficult to wash from the surface (Savage, 1978). Biopsy tissue from the human large intestine, after six saline washes, is thought to be associated with bacteria (Hartley et al., 1979). Portal-arterial differences in rats given lactulose and neomycin respectively by stomach tube for seven days showed a significant amount of ammonia to be generated in the small intestine, although ammonia levels were 25% higher in the colon, but the blood flow rate of small:large bowel of 3:1 indicates that the small bowel is a larger producer; lactulose and neomycin markedly reduced net production of alanine and ammonia in the small bowel, but the presence of diarrhoea seems to be important for the action of lactulose in the colon, since in the lactulose without diarrhoea group, a significant increase in ammonia production was noted (van Leeuwen et al., 1984a). A selective colonisation of germ-free rats with anaerobes increased portal ammonia levels, whereas introduction of a population of aerobes did not alter ammonia levels (Soeters et al., 1984). However, in

the absence of bacterial metabolism, it is difficult to envisage a direct effect of lactulose or neomycin, especially as lactulose is poorly absorbed by the gut epithelia (Menzies et al., 1974; Maxton et al., 1986), and is not metabolised systemically (Evered and Sadoogh-Abasian, 1979). The lactulose effect in the studies of Soeters et al., (1984) and van Leeuwen et al., (1984a, b) was not investigated with control substances, and metabolism of the disaccharide not quantitated. Furthermore, the in vivo experiments did not permit a precise estimation of NH_3 production by bacteria or by intermediary metabolism.

Therefore, evidence reviewed in this section largely suggests that degradation of lactulose by bacteria is implicated in reducing gut ammonia production. The role of a non-bacterial mediated lactulose effect awaits further clarification.

1.2.2 Other uses of lactulose

Two diagnostic applications of lactulose have been reported: (1) as a marker to assess intestinal permeability, which increases in disease due to a structurally damaged mucosa (Menzies, 1974; Maxton et al., 1986; Noone et al., 1986) and, (2) in assaying small bowel transit time, where expired hydrogen is quantitated (Bond and Levitt, 1974; Flatz & Lie 1982). This has been found proportional to amount of non-absorbed sugar fermented.

In other clinical applications, lactulose is effective in treating mild to chronic constipation (Wesselius-de Casparis et al., 1968; Brocklehurst et al., 1983), and has been used in controlling antibiotic-resistant shigellosis (Levine and Hornick,

1975; Hansson et al., 1981). An effective role for lactulose in reducing endotoxinaemia in hepatitis patients has also been reported (Magliulo et al., 1979; van Vugt et al., 1983).

1.3 GASTROINTESTINAL BACTERIA

Although intestinal bacteria are regarded as the major component of the gut microbiota, it should be recognised that protozoa and yeasts are frequently isolated (Clark and Bauchop, 1977). A brief look at the non-bacterial microbes shows the following types (from Roseman, 1962). The eumyces, or true fungi, are mainly the yeasts, such as Candida albicans (for a review, see Shepherd et al., 1985) or Can. tropicalis, and to a lesser extent include species of Saccharomyces and Torulopsis (e.g. T.glabrata), more doubtfully indigenous. Protozoa belong to two classes, mastigophora or flagellates, and amoebas are mostly enteric (such as Entamoeba coli and Endolimax nana) except Entamoeba gingivalis. Quantitative data on relative proportions of each type appears lacking. This discussion however, looks at the numerically predominating bacteria, in particular at the types of intestinal bacterial, their relative importance, and ecological factors affecting bacterial growth and metabolism.

Bacterial colonisation of the gut begins at birth, following contamination from air and the diet. Ecological succession produces a relatively constant, indigenous population termed the autochthonous flora (Savage, 1977), to which additions of transient microbes, the allochthonous forms, occurs regularly. Excepting pathogenic infection from an external source, the gut flora is

regarded as a stable, self-regulating system (Drasar and Hill, 1974).

1.3.1 Factors controlling bacterial colonisation

Physico-chemical factors regulating bacterial survival include temperature, pH, and viscosity (see Clarke, 1977). The role of pH in bacterial cell homeostasis has been reviewed by Booth (1986). Gases in the intestine, apart from N_2 and O_2, are of microbial origin, specifically CO_2 and H_2 (Levitt, 1969), CH_4 (Calloway, 1969), and H_2S, also thought to be an unresolved factor in repression of E.coli populations (Savage, 1977). Diffusion of O_2 from the blood and tissues of the gut may account for a greater number of aerobic organisms adjacent to the bowel mucosa (Wrong et al., 1981). During succession, O_2 utilisation by aerobic bacteria creates a sufficiently low oxidation-reduction potention (E_h) to permit growth of obligate anaerobes (Savage, 1978; Hoogkamp-Korstanje et al., 1979). The E_h within the large intestine is strongly reducing, normally about -200mV (Wrong et al., 1981). Mechanisms of oxygen toxicity to obligate anaerobes have been reviewed by McCord et al., (1971) and Morris (1975). Intestinal motility is a crucial factor. It has a clearing action and determines flow-rate, as the rate of peristalsis decreases with luminal contents descending along the intestine (Drasar and Hill, 1974). Surgical strangulation of the small intestine in dogs produced a marked increment in bacterial numbers (Cohn and Bornside, 1965).

Manipulating bacterial substrate requirements by dietary alterations has produced equivocal changes in microflora composition. Comparisons of geographically and culturally distinct populations indicate significant differences in several genera (Aries et al., 1969; Finegold et al., 1977). Contrasting experimentally controlled high protein, carbohydrate or fat diets with normal intake in specific populations of subjects produced only minor changes in individual bacterial genera or species (e.g. Speck et al., 1970; Drasar et al., 1976; Hentges et al., 1977), possibly due to the short study span of several weeks (Hill and Drasar, 1975). Only with a soluble and rigorously defined diet of various sugars and oligosaccharides has a significant decrease in all or specific genera been found (Hill, 1981). Assaying changes in bacterial enzyme levels may be more useful: total faecal β-glucuronidase levels reflected the effect of diet on the flora within 4 weeks (Wynder and Reddy, 1974). In vivo bacterial multiplication must proceed at a rate sufficient to avoid being diluted out, varying between 0.5 and 6 divisions a day in different mammals (Drasar and Hill, 1974).

A number of intestinal secretions may be bacteriocidal but the extent of their effect in situ is unknown. Aseptic saliva was shown to inhibit the growth of several cocci strains (Zeldow, 1961), and significant colonisation by bacteria in patients with gastric achlorhydria indicated the germicidal effect of gastric acid (Drasar et al., 1969). Bile acids show variable effects: conjugated and unconjugated forms have an inhibitory effect on some bacteria but stimulate growth in others (Binder et al., 1975).

Lysozyme in the intestinal mucosa is also important (Clarke, 1977). Several effects have been suggested for immunoglobulins, specifically IgA: inhibition of bacterial motility and adherence to mucosal surfaces, and others (Drasar and Hill, 1974). Phagocytosis in scavenging contaminating microbes at epithelial surfaces has also been implicated (Savage, 1977).

Bacterial interactions provide another category of factors controlling microbial adaptation. Bacterial metabolites can be strongly antimicrobial: a) organic fermentation acids were inhibitory to coliforms and shigella under low E_h conditions (Lee and Gemmell, 1971; Levine and Hornick, 1975); b) several enterobacteria produce antibiotic bacteriocins or colicine (Tagg et al., 1976), although their role in emergence of dominant strains is unclear (Cooke et al., 1972).

1.3.2 Types of intestinal bacteria

Diller et al., (1970) suggested the following definitions to distinguish between anaerobes and aerobes: anaerobes do not possess cytochrome oxidase and oxygenases (the physiology of obligate anaerobiosis has been reviewed by Morris, 1975), with facultative anaerobes having an oxygenase-independent metabolism, obtaining their energy both by oxygen-dependent (cytochrome oxidase) and by oxygen-independent (pyridine nucleotide-regenerating dehydrogenases or reductases) redox processes. As for obligate aerobes, these organisms always depend upon cytochrome oxidases for their energy metabolism and usually upon oxygenases for their anabolism.

The relative tolerance of many anaerobes to experimental exposure to air has led to an extensive listing of intestinal species (Holdeman and Moore, 1972; Drasar and Hill, 1974; Drasar

and Barrow, 1985; Finegold et al., 1986). Anaerobic methodology has also been reviewed by Holdeman and Moore (1972), and Drasar and Hill (1974); it may be noted here that the anaerobic jar procedure is both commonly used and effective. Of the actual bacteria present in the gut, it is quite likely that all the major genera have been characterised to date; these are listed together with main species, in Table 1.3.1. General references for Sections 1.3.2.1 and 1.3.2.2, below, are Buchanan and Gibbons (1974) and Cowan (1975).

1.3.2.1 Gram-negative bacteria

Of the anaerobic rods, the family Bacteroidaceae comprises the genera Bacteroides, Fusobacterium and Leptotrichia. Many species are pleiomorphic, and all members are non-sporing obligate anaerobes. Bacteroides include between 20 - 30 different species, notably B.fragilis, B.vulgatus and B.uniformis (Moore et al., 1981; Salyers, 1984) many of which grow well in a defined medium containing only glucose, ammonia, sulphide, hemin, vitamin B_{12}, CO_2 and minerals in sufficient concentrations (Bryant, 1974).

The family Veillonellaceae are anaerobic cocci, with three genera of varying size (diameter 0.3 - 2.5 μm): Veillonella, Acidoaminococcus and Megasphaera. Carbohydrates, including glucose, are not fermented by Veillonella strains; lactic acid is metabolised to acetic and propionic acids, CO_2 and H_2.

Of the 12 constituent genera of the family Enterobacteriaceae, which are facultative anaerobic rods, Escherichia, Enterobacter, Klebsiella and Proteus occur frequently in the intestine, some species being pathogenic (Drasar and Hill, 1974); Klebsiella aerogenes fixes nitrogen in the intestine (Bergerson and Hipsley, 1970). Most

Table 1.3.1 Bacterial groups and selected species isolated in the intestinal tract (from Buchanan and Gibbons, 1974; Drasar and Hill, 1974).

Families and genera represented	Prominent Species	Other species isolated from the intestine
Pseudomonadaceae		Ps. aeruginosa
Pseudomonas		Ps. faecalis
Enterobacteriaceae		
Escherichia	E.coli	
Enterobacter		Ent.(Aerobacter) aerogenes
Klebsiella		K. aerogenes
Proteus		P. mirabilis
Bacteroidaceae		
Bacteroides	B.fragilis	B. oralis
	B.vulgatus	B. thetaiotaomicron
	B.uniformis	B. coagulans
Fusobacterium		F. nucleatum
		F. fusiforme
Leptotrichia		Lep. buccalis
Veillonellaceae		
Veillonella		V. parvula
		V. alcalescens
Acidoaminococcus		Ac. fermentans
Neisseriaceae		
Neisseria		N. catarrhalis
Propionibacteriaceae		
Eubacterium	Eu. aerofaciens	Eu. rectale
		Eu. lentum
Propionobacterium		Prop. acnes
Lactobacilliaceae		
Lactobacillus	L. acidophilus	L. brevis, L. fermentum
		L. casei, L. leichmanis
Streptococcus	S. faecalis	S. salivarius
		S. viridans
		S. intermedius
Bifidobacterium	Bifid. bifidum	Bifid. breve, Bifid. longum, Bifid. infantis
Peptococcaceae		
Peptococcus		Pt. asaccharolyticus
Ruminococcus	R. bromii	
Peptostreptococcus		Pst. intermedius
		Pst. productus
Bacillaceae		
Clostridium	C.perfringens	C.butyricum, C.difficile,
	C.paraputrificum	C.cadaveris,
Bacillus		Bc.subtilis

enterobacteria are urease-positive, and attack sugars fermentatively, with gas as an end-product.

Neisseria strains, family Neisseriaceae, are facultatively anaerobic cocci, usually occurring in pairs; although frequent in the mouth, they are a minor component of the gut flora.

1.3.2.2 Gram-positive bacteria

Important genera of the non-sporing rods are Eubacterium, Bifidobacterium, Lactobacillus and Propionibacterium. The eubacteria and bifidobacteria are obligate anaerobes, whereas the Lactobacillus genus comprises some strains which are obligately anaerobic and others which are facultatively so.

The genus Eubacterium (family Propionibacteriaceae) is numerically one of the most important in the colonic flora (Drasar and Hill, 1974), with Eu. aerofaciens being the fourth most frequent organism isolated in faecal samples (Moore and Holdeman, 1974). Eu. rectale strains ferment cellubiose, mellibiose and raffinose to produce large quantities of H_2; Eu. aerofaciens also produces formic acid and H_2 from sugar and peptone degradation.

Bifidobacteria (family Lactobacillaceae) dissimilate glucose to mainly lactic and acetic acids; gas, propionic and butyric acids are not produced. Of about 16 species, Bifid.bifidum is important. Strictly anaerobic lactobacilli require care in distinguishing them from bifidobacteria. Lactobacillus species are strongly saccharoclastic, yielding predominantly lactic acid as a fermentation end-product. Some heterofermentative strains produce gas from glucose, and ammonia from arginine under suitable conditions. Table 1.3.1 lists frequently isolated lactobacilli.

Streptococci are Gram-positive spheres which usually occur in pairs/chains, typically non-motile and attack carbohydrates fermentatively (see Kandler, 1983); lactic acid is the predominant end-product of fermentation. Classification of the strains is based on haemolytic activity; e.g. β -haemolysis is a clear-colourless zone produced by S.faecalis.

Four genera of anaerobic cocci comprise the family Peptococcaceae: Peptococcus, Peptostreptococcus, Ruminococcus and Sarcina. Ruminococcus is considered the most important genus and in fact the species R.bromii was not detected in the faecal microflora until its description by Moore et al., (1972). Peptostreptococcus isolates are non-proteolytic but ferment esculin, raffinose and other sugars (Willis, 1977). Pst.productus is numerically important in the gut (Moore and Holdeman, 1974).

Of the two genera of the family Bacillaceae, Clostridium and Bacillus, the former is important in the gut. Clostridia are anaerobic but several species are relatively aerotolerant. Some species are spore-formers, and appear distended during sporulation. Characteristically, strong proteolytic (e.g. C.botulinum) or saccarolytic (e.g. C.perfringens, C.butyricum) or both (e.g. C.tetani) are present. Since enumeration of clostridia, especially vegetative forms, is difficult due to the lack of a suitably selective or diagnostic medium, the clostridia may be far more numerous in the colonic flora than is realised generally (Hill and Drasar, 1975).

1.3.3 Bacterial distribution in the digestive tract

The gastrointestinal tract is a single, continuous organ, but with distinct functional regions: the buccal cavity, oesophagus, stomach, small intestine and the large intestine comprising the caecum, colon and rectum (Wrong et al., 1981). The term 'colon' is often used to refer to the large intestine as a whole. A number of habitats are available to bacteria: the gut lumen, surface mucus of epithelial cells, and immobilisation between gut cells, such as in the crypts of Lieberkühn (O'Grady and Vince, 1971). In enumerating bacterial distribution along the gut, an obvious problem is technical inaccessibility, giving rise to inconsistencies in comparing different habitats and regions. The mucosal flora may be more important than the luminal flora in, for example, urea metabolism (Hill and Drasar, 1975), and in possessing a higher proportion of aerobic organisms (Hill, 1981). The comparative enumeration in this section is generally based on data of lumen flora. An attempt to overcome the accessibility problem by studies of ileostomy and colostomy effluents (Finegold et al., 1970) and by various intubation procedures (Drasar et al., 1969) led to an uncertainty as to how closely this sampling method resembles the normal microbiota (Wrong et al., 1981). The microbial flora of the distal ileum in these patients (Finegold et al., 1970; Vince et al., 1973) is x100> than the normal distal ileal flora (Gorbach et al., 1967).

The overall numerical distribution of bacteria in the gastrointestinal tract is shown in Table 1.3.2. It is apparent that there are degrees of qualitative and quantitative differences between different intestinal regions, such as the preponderance of streptococci

Table 1.3.2 Enumeration of selected bacterial groups in the intestinal tract (from Drasar and Hill, 1974)

Region	Number of Subjects	$\log_{10}$ bacteria ml^{-1} intestinal content. Mean (Range)					
		Entero-bacteriaceae	Bacteriodes	Streptococci	Lactobacillus	Clostridia	Gram +, non-sporing anaerobes
Jejenum	20	D	D	2.4 (N-3.9)	2.4 (N-3.9)	No data[a]	D
Ileum	6	D (N-6)	N	N	D (N-3.7)	No data	D
Terminal ileum	7	3.3 (n-6)	5.7 (3.4-8)	3.4 (3.1-4.1)	D (N-8)	N	5.7 (4.1-8)
Caecum	3	6.2 (5.6-7.4)	7.9 (6.4-9.1)	2.6 (N-3.6)	D (N-2.8)	No data	5.2 (2.3-7.6)
Faeces	25[b]	6 (4-9)	10.5 (10-11.5)	5 (2-8)	4 (2-7)	3 (N-6)	10.5 (9-11)
	31[b]	6.6 (N-10.1)	9.8 (8-11.4)	4.9 (N-9.4)	3.5 (N-10)	3.3 (N-9.6)	5.6 (N-10.8)

[a] No data in references quoted by Drasar and Hill (1974)

[b] Values are $\log_{10}$ bacteria/g faeces. Mean (Range); N = Not detected; D = Less than 100 bacteria

and lactobacilli in the jejenum. Furthermore, biotypes of bacteroides differ in saliva and faecal samples (Vince, 1971). There is a marked increase in numbers of most bacterial groups, especially enterobacteria and bacteroides when comparing numbers in the jejenum with those in faecal samples (Table 1.3.2). Details of regional distribution of bacteria are discussed below from selected reports.

Samples of saliva from 132 fasting subjects (Drasar et al., 1969) were found to contain streptococci (10^6/g saliva), neisseria (10^5/g), veillonella (10^4/g) and fusobacteria (10^4/g). S.salivarius and viridans streptococci were prominent, and bacteroides (10^3/g) and bifidobacteria (10^2/g) present in all samples. Lactobacilli (10^2/g) isolates appeared in over 90% of samples, yeasts in 26%, enterobacteria in 26%, and staphlococci in 11%. Comparable bacterial counts have also been reported by Handelman and Mills (1965). The mixture of bacteria in the saliva passes into the stomach, mainly with dietary intake (Drasar and Hill, 1974).

In fasting subjects, a pH of 3 or less ensures the absence of viable organisms; food and saliva neutralise gastric acid, enabling bacteria to survive, with counts of up to 10^5 organisms/ml made after a meal (Drasar et al., 1969). These included streptococci, enterobacteria, bacteroides and bifidobacteria. These bacteria are considered transients, although lactobacilli and yeasts may be autochthonous, colonising the epithelial surface of the gastric mucosa (Savage, 1977). Gastric contents enter the small intestine continuously, the pyloric sphincter releasing about 6 - 9 ml min^{-1} at pH 5 - 7 (Luckey, 1974).

The fasting and post-prandial pattern of bacterial species in the duodenum and upper jejenum is generally the same as found in the stomach, but from a slightly higher percentage of subjects (Vince, 1971).

The lower small intestine, especially the terminal ileum, contains a larger and more permanent flora than the proximal regions. The stasis at the ileocaecal valve is an important factor in allowing the concentration and multiplication of bacteria in the distal and terminal ileum (Draser et al., 1969). In ileoproctostomy aspirates, a total of 10^7 organisms/ml have been reported (Dickman et al., 1975), comparing well with a range of 10^5 - 10^7/ml enumerated by Gorbach et al., (1967). Although lactobacilli (10^5/ml) and streptococci (10^5/ml) numbers appear prominent (Dickman et al., 1975), bacteroides and enterobacteria, especially E.coli (10^6/ml), have been isolated more consistently (Drasar and Hill, 1974). Clostridial presence (0 - 10^7/ml) varies, detected in 7 - 75% of subjects (Vince, 1971).

The bacterial composition of the caecum has been thought to be similar to that in faecal material (Tabaqchili, 1974), but Hill (1981) suggested that there is no evidence to show this. However, Table 1.3.2 shows a qualitative similarity; this may only be on the genus level, with important variation in types of species present. The caecum and right colon are probably the most important regions of the large intestine for bacterial metabolism, as intestinal contents are relatively fluid and permit a more ready interaction between bacterial enzymes and their substrates (Hill, 1981). The increased stagnation of luminal contents in the caecum and the remainder of the large bowel results in a rate of passage

which does not exceed the doubling times of bacteria (Savage, 1977).

Non-sporing anaerobic bacteria overwhelmingly predominate in human faecal flora, accounting for more than 99% of the total organisms (Drasar et al., 1969). Moore et al., (1981) found the major species to be the bacteroides representatives: B. uniformis, B. vulgatus, B. fragilis; and three Eubacterium strains (Eu. rectale, Eu. aerofaciens, Eu. lentum), and Bifid. adoloscentis. The remainder were mainly E.coli, S.viridans, S.salivarius and lactobacilli. Clostridial enumeration varies, from being a minor component to a significant one in the faecal flora (Hill and Drasar, 1975).

1.3.4 Bacterial metabolism: a selective look at carbohydrate dissimilation

Microbial activities in the gut are notably diverse, ranging from nitrogen fixation to the metabolism of bile acids (Wrong et al., 1981). Carbohydrate metabolism is discussed here to identify the substrate available in vivo; the biochemistry of fermentation under these conditions is an intriguing area of work. In addition, an evaluation of these nutrients is of use in testing 'lactulose-analogues' for their substrate effect in altering bacterial metabolism (discussed in Section 1.2.1.3).

A range of dietary and endogenous carbohydrates are putative substrates for bacterial metabolism (Prins, 1977; Cummings, 1982). Complex polysaccharides from the diet are mainly plant cell-wall associated (cellulose, hemicellulose, xylans, pectic polyuronides), although plant storage polysaccharides, such as starch and inulin, may also be present. Polysaccharides of endogenous origin are the laevans and dextrans synthesised by oral bacteria,

and mainly salivary mucins and epithelial mucopolysaccharides from the host. Key structural linkages of these polysaccharides are listed in Table 1.3.3. These substrates do not usually occur as isolated compounds, but rather as components of a complex matrix. Plant cell walls, for example, consist of an intricate network of polysaccharides, protein, and lignin, held together by covalent as well as non covalent bonds (Salyers, 1979).

Oligosaccharides may occur freely in dietary vegetables and mature leguminous seeds (e.g. stachyose, verbascose fermented in the lower gut; Cristofaro et al., 1973), or are formed extracellularly as intermediates or reaction products in the breakdown of polysaccharides by bacteria, but do not accumulate due to rapid fermentation (Prins, 1977). Disaccharides and monosaccharides may be similarly produced; they also occur as plant glycosides (Drasar and Hill, 1974).

Fibre as a convenient description of a group of polysaccharides has received considerable clinical interest, being implicated in the prevention of diseases of major importance, such as diverticular disease, lowering serum cholesterol, and possibly in reducing the risk of colon cancer (Cummings, 1977; Cummings and Branch, 1982). Sources of fibre used in clincal studies include wheat bran, vegetable/fruit fibre, plant gums such as guar, and mucilaginous substances from psyllium seeds and isphaghula (Mitchell and Eastwood, 1977). Defining fibre has presented a number of difficulties, largely due to differences in fractionnation methodology. The major fraction of fibre was described by Cummings (1981) as non-starch polysaccharide (NSP). Further, NSP was divided into cellulose and non-cellulose polysaccharide (NCP). Lignin is not a

Table 1.3.3 Source composition, and bacterial utilization of some types of polysaccharide that are components of plant cell walls or mucins (from Salyers, 1979)

Polysaccharide	Composition	Source	Bacterial species able to utilise the polysaccharide
Cellulose	Linear β(1,4) glucan	Plant cell walls	Bacteroides sp.
Xylan	β(1,4) linked xylose back-bone; arabinose side chains	Plant cell walls	B.ovatus, Bacteroides eggerthii, Bacteroides fragilis subspecies a, Bacteroides vulgatus, Bifidobacterium adolescentis, Bifidobacterium infantis
Noncellulosic glucans (laminarin, lichenan)	Linear β(1,3) or mixed β(1,3), β(1,4) glucans	Plant cell walls	B.thetaiotaomicron, B.uniformis, Bacteroides distasonis, Bacteroides "T4-1"
Pectins	Linear α(1,4) linked polymers containing galacturonic acid (and esters)	Plant cell walls	B.thetaiotaomicron, B.ovatus, Bacteroides "3452 A", B.fragilis subspecies a, B.vulgatus, Eubacterium eligens
Galactomannans (guar gum, locust bean gum)	β(1,4) linked mannose backbone with galactose side chains	Plant gums; food additives	B.ovatus, B.uniformis, Ruminococcus albus
Arabinogalactans	β(1,4) linked galactose backbone, arabinose side chains	Plant cell walls	B.thetaiotaomicron, B.ovatus, B. "3452 A", B.uniformis, B.vulgatus, B. "T4-1", Bifidobacterium longum
Mucopolysaccharides (chondroitin sulphate heparin, hyaluronic acid)	Linear polymers containing hexosamine and uronic acid, sulphate esters	Sloughed epithelial cells, dietary meat	B.thetaiotaomicron, B.ovatus, B "3542 A", B.fragilis subspecies a.
Mucin glycoprotein	Polysaccharide (sialic acid, L-fucose, hexose, hexosamine), protein	Swallowed saliva goblet cell mucin	A few strains of Bacteroides, Ruminococcus torques. Bifidobacterium bifidum

carbohydrate, and should be considered separately in any definition. A typical fibre, bran, consists of: cellulose 21%; pentosan 20 - 26%; starch 7.5 - 9%; sugar 5%; protein 11 - 15%; fat 5 - 10%; ash 5 - 9%; and water 14% (Cummings, 1981).

Evidence for polysaccharide breakdown by intestinal bacteria comes from two types of study, from quantitating faecal recovery of ingested fibre, and from in vitro work with purified substrates. Effluent from ileostomy patients fed bran NCP indicated 80 - 100% recovery, indicating little metabolism in the small bowel (Sandberg et al., 1981). In contrast, only 3 - 5 g/day of fibre remains in the stool (Stephen and Cummings, 1980), considering an average intake between 16 - 28 g/day (Cummings, 1973). The physico-chemical properties of fibre, such as branching, methoxylation and gelling affect degradation (Salyers, 1979) as well as the lignin and silica content (Eastwood et al., 1977). Fibre from faecal samples has been shown by scanning electron microscopy to have bacteria associated with it, surrounded by areas of cell-wall breakdown (Williams et al., 1978). This extracellular hydrolysis, as prementioned, releases oligo-, di-, and monosaccharides for subsequent fermentation (Clarke, 1977; Eastwood et al., 1980). Polysaccharide breakdown probably involves the sequential action of several bacteria (Robertson and Stanley, 1982).

Surveys of 25 major species of colonic bacteria have shown that many strains are capable of fermenting NCP of plant cell wall and mucin polysaccharides in vitro (Salyers et al., 1977a, b, c; Vercellotti et al., 1977; Salyers et al., 1978). Details of these studies are shown in Table 1.3.3. Bacteroides is notable as the dominant saccharolytic genus, and 3 species, B.ovatus, B.thetaiotaomicron

and B.uniformis ferment a range of polysaccharides of varying chemical composition. Starch, pig colon mucin and various cell-wall polymer fermentation by B.fragilis has also been reported by Clarke (1977); oligosaccharide side chains of hog gastric mucin are degraded by cell-bound and extracellular glycosidases in anaerobic faecal cultures (Hoskins and Boulding, 1981).

Inclusion of the mucopolysaccharide chondroitin sulphate in the growth medium of B.ovatus increased chondroitin lyase activity within 30 min; glucose repressed activity of this periplasmic enzyme but not completely so (Salyers, 1979). B.thetaiotaomicron digests chondroitin sulphate when covalently bound to proteoglycan (Kuritza and Salyers, 1983) or when supplied in a free state (Linn et al., 1983). Laminarin, β-(1→3)-glucan, degrading enzymes of at least two bacteroides species may also be periplasmic, yielding glucose, laminarabiose, and laminatriose (Salyers, 1979). It is also hydrolysed by E.coli and S.faecalis, but not by C.perfringens (Fomunyam et al., 1984). Xylan, a branched partially insoluble polysaccharide also appears to be degraded by cell-associated enzymes to yield xylose (Salyers, 1979). A significant find was the detection of a cellulolytic bacteroides species (10^8/g) in one of five human faecal samples (Betian et al., 1977; Bryant, 1978).

An endo-β-galactosidase hydrolysing internal β-galactosidic linkages of oligosaccharides from B.fragilis (Scudder et al., 1983) and a cytoplasmic exo-enzyme attacking (1 → 6) linked α-D-glucans from B.oralis (Takahishi et al., 1985) are notable polysaccharide degrading enzymes. Glucans, extracellular bacterial polysaccharides, are considered important matrix substances in dental plaque. The exo-β-D-galactosidase and acetyl-α-D-galactosaminidase from

culture fluids of C.perfringens hydrolysed a number of oligosaccharide-linked chromogenic substrates (DiCioccio et al., 1980).

Oligosaccharides mainly undergo extracellular hydrolysis to permit bacterial uptake of di- or monosaccharide reaction products. Organisms able to use only those substrates, such as soluble sugars, that disappear rapidly from the gut either by fermentation or absorption, are at a disadvantage unless they are preferentially retained in the gut by sequestration or are able to store reserve polysaccharide (Clarke, 1977). Biochemical versatility in such cases confers a survival advantage, e.g. the ability of B.fragilis to use starch and various polymers in addition to soluble sugars.

Several glycosidases, α- and β-glucosidases, α- and β-galactosidases, and β-glucuronidase have been detected in induced strains of most of the major genera (Hawksworth et al., 1971; Holdin, 1986). B.fragilis possesses several glycoside hydrolases which can cleave low-molecular weight polysaccharides and glycosyl moieties of glycoproteins or glycolipids into monosaccharides; six glycoside hydrolases, α-glucosidase, α-galactosidase, β-galactosidase, β-N-acetylglucosaminidase and α-L-fucosidase have been purified (Berg et al., 1980).

The final phase of carbohydrate fermentation to end-products (pathways are discussed in Section 1.4.3) is important in vivo for a number of reasons (Clarke, 1977; Cummings, 1981; Wolin, 1981). For example, isobutyric and 2-methylbutyric acids are required for growth of Ruminococcus albus, and growth on lactate of Veillonella alcalescens and Vibrio succinogenes (Clarke, 1977). The chemistry of rumen carbohydrate fermentation has been quantitated, and a similar equation proposed for fermentation in the human colon

based on known volatile fatty acid (VFA) concentrations and a number of assumptions (Cummings, 1981; Wolin, 1981):

$$34.5\ C_6H_{10}O_5 \rightarrow 48\ CH_3COOH + 11\ CH_3CH_2COOH +$$
Acetic acid Propionic acid
$$5\ CH_3(CH_2)_2COOH + 23.75\ CH_4 +$$
Butyric acid
$$34.25\ CO_2 + 10.5\ H_2O$$

Three important products of fermentation suggested are VFA (acetic, propionic, and butyric acids), gas (CO_2, H_2, CH_4) and energy. Alcohols appear absent or unimportant. Methanobrevibacter smithii, which uses H_2 to reduce CO_2 to CH_4, is responsible for almost all the CH_4 produced in the intestine (Miller et al., 1984).

1.4 BACTERIAL SUGAR METABOLISM

The purpose of this discussion is to present an overview of major developments in: a) the transport of sugars by bacteria, b) hexose fermentation pathways in anaerobic bacteria, which may be implicated in lactulose utilisation for cellular carbon and energy, and c) regulatory mechanisms that facilitate priority selection of available substrates.

1.4.1 Sugar Transport by Bacteria

Translocation of externally available solutes across the cytoplasmic membrane is a pre-requisite of bacterial growth. This uptake may be energy-dependent, via the active transport and group translocation modes, or energy-independent, mediated as passive or facilitated diffusion. Sugar uptake in bacteria is in the main energy-dependent and involves specific inducible proteins. In active transport uptake occurs across an electrochemical gradient and solutes appear in the cell in chemically unchanged forms whereas group translocation covalently modifies the solute during transport.

The group translocation system found most commonly in bacteria is the phosphoenolpyruvate-phosphotransferase system (PEP:PTS) which phosphorylates sugars and concentrates sugar phosphates intracellularly.

1.4.1.1 Active Transport

The analyses of molecular mechanisms involved in active transport seek to describe substrate binding, translocation and release. Two important questions are: (1), the nature of the solute-specific carrier (the number and location of proteins involved), and (2), the mode of energy coupling. Most studies have centred upon two specific active transport systems, the lactose (lac) permease of E.coli and the histidine (his) permease of Salmonella typhimurium. The overall features of active transport are probably best described with reference to the former system. However, the his permease of Sal.typhimurium, comprising four proteins, is referred to as a binding-protein-dependent system (see Section 1.4.1.1.2) and differs from the E.coli lac carrier, which is largely thought to be a one component system.

1.4.1.1.1 The lactose permease of E.coli

Fox and Kennedy (1965) isolated an inducible membrane bound protein (M protein) in E.coli cells in a partially pure form; this protein possessed similar binding kinetics to that of the β-galactoside permease. The M protein was then characterised as a single polypeptide chain with a molecular weight of 30,000, considerably hydrophobic and containing one -SH group which is involved in the inhibition of transport by -SH reagents (Fox and Kennedy, 1965; Jones and Kennedy, 1969). The lac operon has been extensively studied; it consists of a control region (lac I) and

three structural genes (lac Z, lac Y and lac A), coding for β-galactosidase, β-galactoside permease and thiogalactoside transacetylase respectively (see e.g. Miller and Reznikoff, 1978; Glass, 1982). Following the cloning of the lac Y gene (Teather et al., 1978), the DNA sequence of the Y-gene was determined and used to predict the lactose permease monomer to consist of 417 amino acid residues, with a molecular weight of 46504 (Büchel et al., 1980). SDS-Polyacrylamide gel electrophoresis under appropriate sieving conditions indeed determined this value as approximately 46000 (Beyreuther et al., 1980). Using carrier-overproducing strains, the active lactose carrier has been purified to near homogeneity, and the molecular weight confirmed as 46500 (Overath and Wright, 1982). Reconstitution of the solubilised lac permease in proteoliposomes to a functional state has been reported in wild and mutant strains of E.coli (Seto-Young et al., 1984). It is apparent from kinetic experiments on proteoliposome reconsituted with lac carrier protein that the product of the lac Y gene is solely responsible for β-galactoside transport (Kaback, 1983). Extraction of the lac permease in the nonionic detergent dodecyl-O-β-maltoside found the protein to exist in a monomeric state (Wright et al., 1983). However, kinetic studies and radiation inactivation analysis are consistent with the idea that upon energisation, a major structural alteration is induced, e.g. dimerization (Kaback, 1983). More recently, purification of the lac permease in another nonionic detergent, dodecyl octaethylene glycol monoether, in which the protein is more stable, and analytical ultracentrifugation and gel filtration experiments showed that in this detergent the lactose permease existed mainly as a dimer (Houssin et al., 1985). Similarly,conflicting reports on the oligomeric state

of the protein within the membrane have recently appeared (Kaback, 1986). Characterisation of the protein by circular dichroism measurements has disclosed a high degree of α-helicity, suggesting this form of secondary structure (Foster et al., 1983) spanning the inner membrane (Seckler , 1986). Monoclonal antibodies against purified lac carrier protein have been prepared and characterised; one of these antibodies markedly inhibits transport without altering the ability of the carrier to bind substrate (Carrasco et al., 1982). More specific antibodies directed against a particular number of amino acid residues indicated that the COOH terminus is localised on the cytoplasmic face of the membrane, and hydrophilic regions are buried in the interior of the protein (Seckler 1986). The in vitro biosynthesis of the lac Y gene product has been found to be identical to the lactose permease isolated from cytoplasmic membranes as determined by apparent molecular weight and N-terminal amino acid sequence, and does not undergo post-tranlational modification (Ehring et al., 1980). Alteration of the Cys 148 residue, essential for its sulphydryl group at or near the active site, to a glycine residue by oligonucleotide-directed, site-specific mutagenesis resulted in cells bearing the mutated lac Y exhibiting initial rates of lactose transport that were about one fourth of wild-type cells (see Kaback, 1986). Mutagenesis of other residues and resultant effects on lac permease function were also reviewed by Kaback (1986).

Since the early studies of sugar uptake, a fundamental question has been the source and mechanism of energy coupling for active transport. This is now well known, following the experimental verification of Mitchell's chemiosmatic hypothesis (Mitchell 1969, 1970). This proposal starts by recognising that an important means

of energy storage in bacterial cells is ionic gradients across the cytoplasmic membrane. These act as the medium for both conservation of energy and its transmission from energy-producing to energy-consuming membrane reactions. Ion gradients normally observed are those of H^+, Na^+ and K^+, although anion transport systems are also known (Rosen, 1986). The membrane components which couple the translocation of these ions to a chemical reaction (e.g. terminal oxidation or ATP hydrolysis, such as the Na^+-stimulated ATPase or K^+-ATPases from S.faecalis; Rosen, 1986) or to photochemical events (e.g. in bacteriohodopsin) are termed primary energy transducers, or pumps (review, Lanyi, 1979). Others, which couple the translocation of inorganic ions or electroneutral metabolites to one another (e.g. the proton-driven lactose carrier) are termed secondary energy transducers. In the latter class, three types can be defined uniport, symport and antiport, to denote processes catalysed by appropriate porters which result in either single translocation across the membrane, coupled translocation in the same direction, or coupled translocation in the opposite direction, respectively (Lanyi, 1979). For example, Maloney et al., (1984) have described an anion/anion antiporter system in S.lactis strain 7962 for phosphate/hexose 6-phosphate antiport. Subsequent work (see Rosen, 1986) indicated that the exchanger was energy-independent.

The energy stored as ionic gradient across the cytoplasmic membrane amounts to the electrochemical potential difference ($\Delta\tilde{\mu}$), which has osmotic and charge components. For protons ($\Delta\tilde{\mu}_{H+}$), the term proton-motive force (Δp) is substituted (e.g. review, Booth, 1980). Indeed the "common currency of energy exchange, particularly in the bacterial cell, is not ATP, but $\Delta\tilde{\mu}_{H+}$" (Kaback, 1983).

As the plasma membrane is impermeable to H^+, a Δp (in mV) is set up by the combined electrical potential difference ($\Delta \psi$) and the pH gradient (ΔpH):

$$\Delta p = \Delta \psi - 2.3 \frac{RT}{F} \Delta pH$$

where the value of 2.3 RT/F at 25°C is approximately 60 mV (Booth, 1980).

That the lac permease is a Δp-dependent system and more specifically a H^+: solute symporter has been substantiated experimentally. Using E.coli constitutive for lac permease, and with an anaerobic energy depleted cell suspension, addition of lactose caused alkalization of the medium (West, 1970). West and Mitchell (1973) then showed that stoichiometry of the lactose transport system is one proton per lactose. More recent observations (Ahmed and Booth, 1981, a, b) suggest that galactoside: H^+ stoichiometry is variable, arising from inability of experimental data to demonstrate thermodynamic equilibrium between lactose accumulation and the proton-motive force. The non-equilibrium has been ascribed, in part, to lactose efflux without protons, also termed leakage (Therisol et al., 1982). However, Wright et al., (1986) have attributed this deviation of stoichiometry to the difficulty in performing these measurements; stoichiometries near unity have been reported for other sugar: H^+ cotransporters, including the galactosidase: H^+ cotransport in S.lactis.

Using either whole cell or everted vesicle preparations, artificial gradients of charge ($\Delta \psi$), pH (Δ pH) and Δ p can be achieved by use of ionophores and pH shifts (the methodology has

recently been reviewed by Kashket, 1985). These have enabled a study of fluxes of H^+ and galactosides (Flagg and Wilson 1978; Ahmed and Booth, 1981, a, b). Reagents which abolish Δp leave the lac permease to operate in facilitated diffusion mode (Overath and Wright, 1982); reagents which reduce Δp also reduce transport (Lancaster et al., 1975). Foster et al., (1982) used a proteoliposome reconstitution system, with trapped lac permease, to demonstrate lactose induced H^+ flux. This represents the final confirmation of Mitchell's chemiosmotic theory.

The binding of galactosides to the lac carrier is a spontaneous reaction, with a stoichiometry of 1.2 - 1.3 galactoside per carrier (Overath and Wright, 1982). It is not clear whether H^+ and galactoside binding is independent of one another. One model sees the lac permease as converting between forms with high and low K_m values (Robillard and Konings, 1982). In this model, changes in the affinity of the substrate-binding site occurs by a dithiol-disulphide interchange. Such a transition is in response to a change in the redox state of the carrier, which can be brought about by $\Delta\psi$ or by Δ pH. The $\Delta\tilde{\mu}_{H+}$-induced changes in affinity are thought to be sufficient to drive active transport (symport or antiport). In evidence, it has been reported that in the absence of Δp, the K_m for lactose influx increases 100-fold from 0.2 mM to 20 mM (Kaczorowski et al., 1979; Robertson et al., 1980). Studies on the effect of $\Delta\tilde{\mu}_{H+}$ on the dissociation constant K_S and the half-saturation constant for active transport, K_T have been reviewed by Wright et al., (1986). Lipophilic oxidising agents inhibit lactose uptake driven by artificially generated membrane potential (Robillard and Konings, 1982). The inhibition is reversed by reducing agents. Oxidising

agents also protect the carrier from inactivation by N-ethylmaleimide, which is specific for -SH groups. The K_m of the oxidised and reduced carrier are the same as the K_m of the non-energised and energised carrier respectively (Robillard and Konings, 1982). These authors suggest that these data prove dithiol-disulphide inter-conversions play a role in determining the K_m of the carrier. It has also been noted that the cysteine residue 148 of the lactose carrier is a critical SH group which is in some way involved in substrate binding (Overath and Wright, 1982).

1.4.1.1.2 Binding-protein-dependent systems

These are osmotic shock-sensitive and are absent from membrane vesicles because they require a periplasmic binding protein which is lost during vesicle preparation (Boos, 1974; Wilson, 1978; Ahmed, 1983; Ames, 1986). Transport involves the interaction of membrane-bound proteins with a periplasmic binding protein. The energy source for active transport is unknown although phosphate compounds have been proposed (Hunt and Hong, 1982). Of these, measurements of ATP or acetylphosphate during transport have generally not been made, and available data suggests little correlation; a tentative conclusion made by Ames (1986) is that the proton-motive force plus another factor are involved (probably not ATP).

The histidine permease of Sal.typhimurium is a well studied example of this system. Of the four proteins of the histidine permease of S.typhimurium, one is periplasmic (protein J), and three are membrane-bound, Q, M, and P (Higgins et al., 1982). The his binding protein J has been purified and extensively characterised (Kush and Ames, 1974). The two operons responsible for the his

permease have been isolated, cloned and sequenced (see Ahmed, 1983).

Maltose transport in E.coli is another example of binding-protein-dependent transport (Wright et al., 1986).

1.4.1.1.3 Anaerobiosis in active transport

Under anaerobic conditions, three different energy yielding processes may be involved in generating the electrochemical potential gradient. These are: a), electron transfer in so-called anaerobic transfer systems with fumarate or nitrate as terminal electron acceptor (e.g. Boonstra and Konings, 1977); b), hydrolysis of intracellular ATP by the membrane-bound ATPase leads to proton efflux, establishing both the membrane potential and Δ pH gradient required (Maloney, 1982); and c), by a more recent proposal that excretion of charged metabolic end products, in symport with protons, generates an electrochemical gradient (Michels et al., 1979; Otto et al., 1982). Under aerobiosis, the role of c) is possibly nil. Direct evidence for the generation of a Δ p coupled to anaerobic electron transfer has been obtained by studies with membrane vesicles from E.coli, induced for nitrate respiration or the fumarate reductase system (Michels et al., 1979).

In fermenting bacteria the Δ p is maintained by the H^+-ATPase from glycolytically generated ATP (Kashket, 1985). Measurements of Δ pH and $\Delta\psi$ in batch grown C.thermoaceticum indicated that the Δ pH is short-circuited by the acetic acid produced by the cell, rather than by an external pH of 5.0 (Baronofsky et al., 1984). The Δ pH of C.pasteurianum is sensitive to ionophores and ATPase inhibitors (Booth and Morris, 1975; Riebeling et al., 1975; Kell et al., 1981). The proton-translocating ATPase (BF_0F_1) of S.lactis

has a stoichiometry of 3 H^+/ATP during glycolysis, determined in part from data of $\Delta\psi$ and Δ pH (Maloney, 1983). Measurements of Δ p parameters have also been made in other streptococci and clostridia; strict anaerobic assay conditions are required to assay Δ p in Methanobacterium barkeri (Kashket, 1985).

1.4.1.2 The Bacterial Phosphoenolpyruvate-Sugar Phosphotransferase system

Kundig et al., (1964) first described a novel phosphotransferase reaction in E.coli cell-free extracts that involved transfer of the phosphoryl moiety of phosphoenolpyruvate (PEP) to hexose, catalysed by two enzymes, enzyme I and enzyme II, and a histidine-containing heat-stable protein (HPr). Subsequently, this group recognised the sugar uptake step, with concomitant phosphorylation, as being a phosphotransferase reaction (Kundig et al., 1966). It is now well known that the PEP: phosphotransferase system (PEP:PTS) is widespread among prokaryotes, though most extensively studied in E.coli, Sal.typhimurium and K.pneumoniae (Roseman et al., 1982; Saier et al., 1985; Postma, 1986).

Generally, only three or four enzymes are required for the transport and phosphorylation of PTS substrates. The overall functional interrelationship of these PTS proteins is shown in Fig. 1.4.1.1. Two general non-specific proteins of the PTS, enzyme I and HPr, initiate the sequence of phosphoryl transfer reactions. The earlier designation of enzyme I and HPr as cytoplasmic proteins has been questioned; Saier et al., (1982) suggest that these enzymes may exist, in part, as peripheral membrane constituents associated with the integral membrane enzyme II complexes. However, enzyme I and HPr are not directly involved in

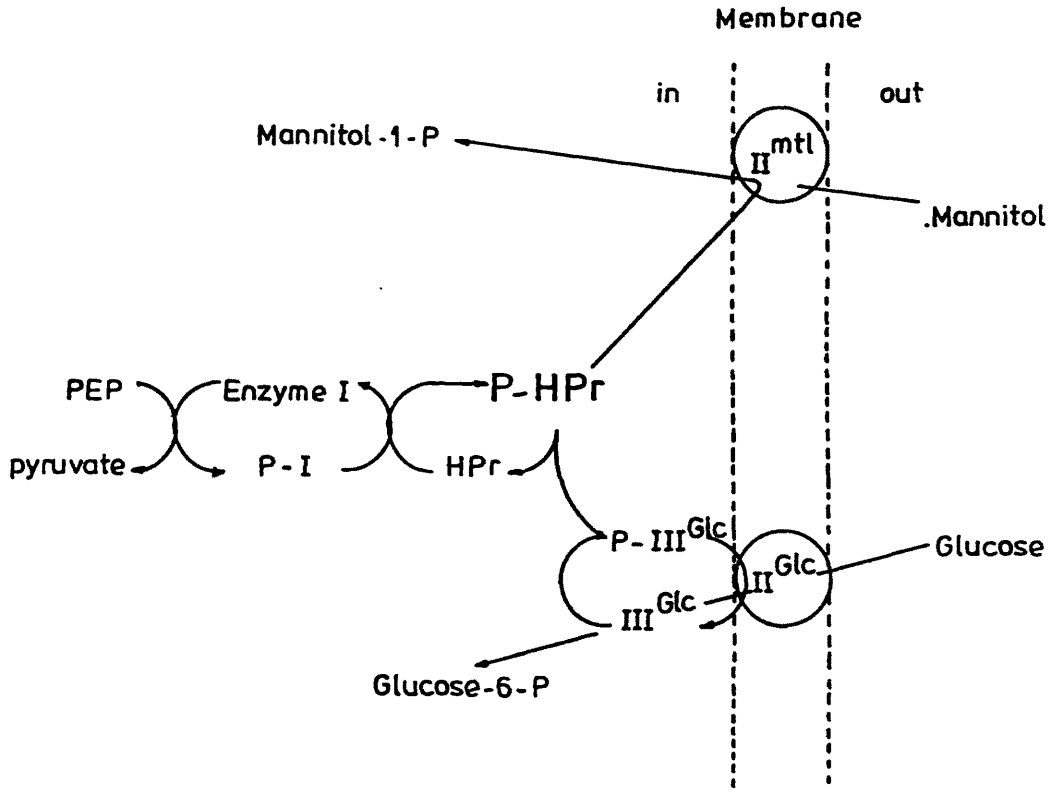


Fig. 1.4.1.1

The PTS Enzyme I and HPr are the general PTS proteins. Two specific enzymes II are shown as examples: II^{Mtl} is specific for mannitol, and II^{Glc} together with III^{Glc} is specific for glucose. P~I, P~HPr and P~III^{Glc} are the phosphorylated forms of enzyme I, HPr and III^{Glc}, respectively (from Postma and Lengeler, 1985).

the translocation of substrates through the membrane (Postma and Lengeler, 1985). In the evolutionary divergent bacterium, Ancalomicrobium adetum, both enzyme I and HPr of the PTS proteins are membrane bound (Saier and Staley, 1977). The fructose specific PTS in Rhodospirillum rubrum is similarly found to be membrane associated (Saier et al., 1971). Enzyme III may be cytosolic or in the membrane fraction, depending upon source organism; III^{Glc} is of particular importance in regulating transport of non-PTS carbohydrates (Section 1.4.2). Enzyme II is an inducible and generally sugar-specific protein or complex of proteins (Kundig and Roseman, 1971) which reacts with one or two phosphoryl group donors, phospho-HPr or phospho-enzyme III. Differing affinities of enzyme II to a range of sugars mean that, for example, glucose uptake is possible via several PTS mechanisms (e.g. Robillard, 1982). In contrast, strains of K.pneumoniae have a plasmid-encoded II^{Lac} in addition to the lactose proton symport system encoded on the chromosome (Hall et al., 1982).

1.4.1.2.1 Distribution of the PTS

In general, the PEP:PTS is limited to bacteria which metabolise sugars via the Embden-Meyerhof-Parnas pathway (EMP) (Saier, 1977; Table 1.4.1.2.1). The rationale for this is the yield of two molecules of PEP from hexose dissimilation by the EMP, thereby supplying one molecule of PEP for sugar transport and another for energy and anabolic metabolism (Saier, 1977). This stoichiometry is not met by other catabolic pathways, the pentose and hexose phosphoketolase pathways, or the Entner-Doudoroff pathway, as the yield is only one molecule of PEP per hexose metabolised. One genus illustrates this hypothesis: homofermentative species of lactobacilli possess the PTS whereas the heterofermentative lactobacilli (which channel hexose via the pentose

Table 1.4.1.2.1 Distribution of the PTS among prokaryotes

General PTS present	PTS absent
Beneckea	Rhodomicrobium
Escherichia	Caulobacter
Klebsiella	Hyphomicrobium
Photobacterium	Prosthecomicrobium
Salmonella	Azotobacter
Serratia	Thiobacillus
Bacteroides	Micrococcus
Staphylococcus	Mycobacterium
Streptococcus	Nocardia
Bacillus	Zymomonas
Clostridium	Lactobacillus
Lactobacillus	
Arthrobacter	
Mycoplasma	
Ancalomicrobium	
Fructose-specific PTS present	
Rhodopseudomonas	
Rhodospirillum	
Thiocapsa	
Thiocystis	
Pseudomonas	
Alcaligenes	
Mannitol-specific PTS present	
Spirochaeta	

From Saier (1977) and Romano et al., (1979)

phosphoketolase pathway) do not (Romano et al., 1979). However, in the case of Bacteroides thetaitaomicron which ferments glucose by the EMP scheme, an exception seems to exist, as this sugar is not transported by the PTS. Whether this is an exception or a more general phenomenon awaits clarification.

From the point of view of the human gut environment, the genera Bacteroides, Clostridium, Streptococcus, Lactobacillus and members of the Enterobacteriaceae are noteworthy in possessing the PTS (specific examples are cited in Chapter 5); it offers important advantages to organisms carrying out anaerobic glycolysis (Roseman, 1969). First, the system provides a tight linkage between the transport of the sugar and subsequent metabolism; second, under conditions where energy supply is limited, the system allows for conservation of ATP, since the product of the transport event is a phosphorylated sugar which can enter catabolic and anabolic pathways directly.

The presence of the PTS in strict aerobes appears limited to specific sugars that are metabolised exclusively or largely via the EMP scheme, such as the PEP:fructose PTS in Arthrobacter pyridinolis or a number of Pseudomonas species (Romano et al., 1979).

Recently, an inducible PEP:dihydroxyacetone PTS system has been described in E.coli (Jin and Lin, 1984). This is the first example of a triose, a central metabolite in glycolysis, being translocated by the PEP:PTS.

1.4.1.2.2 Enzymology of PTS components

The first step in the phosphotransfer reaction sequence catalysed by the PTS is the phosphorylation of the general protein enzyme I. Purified enzymes I from E.coli and S.typhimurium,

molecular weights (MWs) in the range 57000 - 70000, have similar biochemical properties, but differ significantly from S.faecalis enzyme I (Postma and Lengeler, 1985). The pts I gene of E.coli K-12 has been cloned; the pts H-ptsI-crr genes are apparently transcribed as a polycistronic operon (De Reuse et al., 1985). Discrepancies are apparent in the reports of the oligomeric nature of enzyme I. Recently, Grenier et al., (1985) isolated covalently linked enzyme I dimers and trimers using SDS-PAGE and high-pressure liquid chromatography; each oligomer showed catalytic activity. Inhibition by N-ethylmaleimide but not p-chloromercuri-phenylsulphonate suggested two distinct classes of sulphydryl groups in enzyme I. The influence of PEP or pyruvate on the relative amounts of the three species was minimal. In contrast, Missett et al., (1980) suggested a requirement for both PEP and Mg^{2+} for enzyme I dimerisation. Hoving et al., (1982) reported that dimerisation occurred in the absence of Mg^{2+} and PEP. The phosphoenzyme intermediate of this protein, akin to similar intermediates for HPr and enzyme III, carries the phosphoryl group as a phosphohistidine (Hengstenberg, 1977; Anderson et al., 1971; Kalbitzer et al., 1981). Conflicting data has been reported on the stoichiometry of phosphoryl group to active enzyme I; Hoving et al., (1981) found only one subunit per dimer to be phosphorylated, whereas Weigel et al., (1982) reported two phosphoryl groups per mole of enzyme I dimer.

HPr is a low molecular weight protein, 9600 for E.coli, which is active in its monomeric form (Anderson et al., 1971). The nucleotide sequence of pts H in E.coli has recently been reported, the pts H promoter contains a nucleotide sequence similar to the

consensus site for cAMP receptor protein (De Reuse et al., 1985); HPr amino-acid sequence analyses show virtually identical patterns for E.coli and Sal.typhimurium proteins (Powers and Roseman, 1984). In addition to the PTS catalysed phosphorylation, an ATP-dependent protein kinase that phosphorylates HPr to give phospho-seryl-HPr has been described in S.faecalis, S.lactis, and S.pyogenes and in Staph.aureus and Bacillus subtilis, but not in E.coli or Sal. typhimurium; in S.salivarius, the product is 3-phosphohistidinyl HPr (Waygood et al., 1986). Streptococci HPr have almost the same MW as that from Staph.aureus (MW, 7685). The ATP-dependent phosphorylation of HPr may play a regulatory role in the preferential uptake of one PTS substrate over another (Postma and Lengeler, 1985).

Preparation of (^{32}P)-labelled HPr, starting with (^{32}P)-PEP and enzyme I, resulted in incorporation of(^{32}P)at the N-1 position of a histidine residue (Anderson et al., 1971). A similar assignment has been made to Staph.aureus phospho-HPr by means of ^{1}H-n.m.r. studies measuring the pK of the phosphohistidine (Gassner et al., 1977; Kalbitzer et al., 1981). The tertiary structure and active site of phospho HPr in E.coli has been characterised by using various deaminated forms of HPr (Bruce et al., 1985). The phosphate-carrier protein FPr catalyses transfer of PEP during fructose uptake in E.coli by enzyme II^{Fru}, but HPr is involved when fructose is translocated via II^{Man} (Kornberg, 1986).

The phosphoryl moiety of the phospho-HPr protein is generally transferred to the membrane-bound enzyme II of the PTS (Fig.1.4.1.1) although in certain cases transfer occurs to a third soluble protein, enzyme III. In the Gram-positive bacteria, a number of soluble III^{sugar} phosphocarrier proteins have been

described for various sugar-specific PTSs: lactose, Staph.aureus (Hays et al., 1973), S.mutans (Vadeboucoeur and Proulx, 1984) and L.casei (Chassy et al., 1983); gluconate, S.faecalis (Bernsmann et al., 1982); fructose, S.mutans (Gauthier et al., 1984); and xylitol, L.casei (London et al., 1983). Enzyme III^{Lac} from Staph. aureus is trimeric, each subunit binding one phosphoryl group (Deutcher et al., 1982). The molecular weight of the trimer is 35000 (Simoni et al., 1968, 1973). Two proteins are inducible by growth of Staph.aureus on lactose: the soluble III^{Lac} and a membrane-bound II^{Lac}.

Enzyme III^{Glc} is required for the transport of glucose and its nonmetabolisable analogues (e.g. methylα-glucoside) in E.coli and Sal.typhimurium (Saier and Roseman, 1972). III^{Glc} from Sal.typhimurium has been purified to homogeneity (Scholte et al., 1981); it accepts one molecule of phosphate from PEP at His-91 (Meadow and Roseman, 1982). Peptides containing the phosphohistidine active site of phospho-III^{Glc} from Sal.typhimurium have been isolated by reverse-phase high performance liquid chromatography and sequenced (Alpert et al., 1985). Different mobilities of III^{Glc} and phospho-III^{Glc} on SDS-PAGE were used to estimate the proportions of these two forms in intact cells; wild-type cells contain predominantly phospho-III^{Glc} in the absence of PTS sugars in Sal.typhimurium (Nelson et al., 1986). III^{Glc} is also phosphorylated by an ATP cataysed reaction. III^{Glc} is coded by the crr gene, which has been sequenced in E.coli and Sal.typhimurium (Nelson et al., 1984). Two forms of enzyme III^{Glc} have been discerned, differing in their electrophoretic mobility; a fluorescein derivative of III^{Glc}_{Slow}, purified to homogeneity, appeared only 20% active in catalysing

methyl- α-glucoside phosphorylation via the membrane-bound II-B^{Glc} as the native molecule (Jablonski et al., 1983). The existence of different III^{Glc}-related proteins in enteric bacteria has been assayed using antibody probes and by means of gels in which it is possible to detect PEP-dependent protein phosphorylation (Postma and Lengeler, 1985).

The cytoplasmic proteins enzyme I, HPr and enzyme III have been isolated from the soluble fraction of broken cell extracts with relative ease as compared to the membrane-bound enzyme II, which requires detergent-based membrane solubilisation procedures (Robillard, 1982). Enzyme II^{Glc}, which forms a complex with III^{Glc}, has been purified to homogeneity from Sal.typhimurium (Erni et al., 1982); purification of II^{Mtl}, part of the mannitol-PTS in E.coli, to homogeneity, its properties and intramembrane topography have also been reported (Jacobson et al., 1983a; 1983b). Its structural gene is sequenced (Lee and Saier, 1983) and the native enzyme in inverted cytoplasmic membrane vesicles is composed of two identical subunits linked by a disulphide bridge (Roossien and Robillard, 1984). The two distinct components of the mannose-PTS, II-A and II-B in E.coli (Kundig and Roseman, 1971), the II^{Lac} in Staph.aureus (Schäfer et al., 1981) and the II^{Glc} from S.faecalis (Huedig and Hengstenberg, 1980) have also been solubilised and characterised to varying extents.

The phosphorylation of each PTS protein is the key element in the function of that enzyme function. Two aspects have been studies, (1) the site of phosphorylation (discussed above), and (2) mechanism of phosphoryltransfer. A general mechanism for the transfer of the phosphoryl group to and from the active site

histidine residue in relevant PTS proteins is suggested, based on high resolution ^{1}H-NMR data (Robillard, 1982). At physiological pH the active site histidine is deprotonated, - - and can abstract the phosphoryl group from the donor while protonation destabilises the phosphohistidine facilitating passage of the phosphoryl group to the following enzyme intermediate. The change in protonation state accompanies a phosphorylation induced conformation change in the carrier.

The integral membrane enzyme II serves as the sugar recognition component of the system, i.e. in chemotaxis (e.g. Dills et al., 1980; Black et al., 1983). It also mediates sugar uptake: the question has been whether enzyme II catalyses both transport and phosphorylation, or whether the phosphoryl group transfer proceeds directly between phospho-HPr or phospho-enzyme III and substrate, with enzyme II responsible for transport. Kinetic studies on the PTS uptake of glucose or its analogues in Bacillus subtilis, S.faecalis and E.coli have suggested a phospho-enzyme II intermediate (see Begley et al., 1982; Peri et al., 1984). Direct evidence has also been obtained for II^{Glc} and II^{Mtl} phosphorylation in E.coli and Sal.typhimurium (Waygood et al., 1984). Using PEP chiral at phosphorous, the stereochemical transfer of the phosphoryl group occurred five times from PEP to the product α-methylglucose, implicating a covalent phospho-enzyme II^{Glc} (Begley et al., 1982). The occurrence of a phospho-IIB^{Glc}/III^{Glc} has been physically demonstrated in vitro during α-methylglucose transport in Sal.typhimurium, the reaction proceeding according to a ping-pong mechanism (Millett et al., 1983). However, a bi-bi mechanism for phosphoryl group transfer during the uptake stage has

been suggested in some reports, but is less favoured (see Postma and Lengeler, 1985). Mutants lacking enzyme II, such as the II^{Mtl} in Enterobacter (Aerobacter) aerogenes (Tanaka and Lin, 1967), or the general proteins of the PTS, enzyme I and HPr (Tanaka et al., 1967) do not utilise the appropriate PTS substrate. This implies that a PTS substrate can only be transferred by enzyme II if it can be phosphorylated. By loading liposomes with reconstituted E.coli II^{Mtl} internally with sugar phosphate, it has been shown that enzyme II catalyses both phosphorylation and translocation; however, transphorylation was assayed, where (^{14}C) $carbohydrate_{out}$ exchanges with $carbohydrate\ phosphate_{in}$ to produce (^{14}C) $carbohydrate\ phosphate_{in}$ (Jacobson et al., 1979; Robillard, 1982). Mutated enzymes II can catalyse limited facilitated diffusion, such as the altered enzyme $II\text{-}B^{Glc}$ in pts HI deletion strain of Sal.typhimurium, which transported glucose in the absence of PEP:sugar-PTS mediated phosphorylation (Postma, 1981). Facilitated diffusion in some nonmutated enzymes II has also been noted (e.g. Postma, 1976; Kornberg and Reardon, 1976). Another facet of enzyme II function is the phenomenon of PTS-mediated phosphorylation of carbohydrates within the cell. In membrane vesicles from Sal.typhimurium, II^{Man} phosphorylated its substrates from both sides of the membrane, but II^{Glc} was asymmetric (Beneski et al., 1982). A similar effect is apparent in intact cells (Postma and Lengeler, 1985). A mutant of S.lactis 133, deficient in both glucokinase and PEP-dependent mannose-PTS, accumulated high intracellular glucose levels (100 mM) during growth on lactose, suggesting that phosphorylation of glucose is by, (i) ATP dependent glucokinase and (ii) by (internal) PEP-dependent mannose-PTS (Thompson and Chassy, 1985). Although phosphorylation required an intact II^{Man}, exit of the dephosphorylated

analogue did not. In Gram-positive bacteria, expulsion of accumulated phosphorylated intermediates in the cell is linked to phosphorylation of the displacing substrate; the hexose 6-phosphate: phosphohydrolase from S.lactis may be involved in this process (Thompson and Chassy, 1985).

A model has been proposed to explain sugar binding, and subsequent release, which explains key membrane protein function as a redox reaction involving affinity changes mediated by dithiol-disulphide interconversion (Robillard, 1982; Robillard and Konings, 1982). Imposition of a $\Delta\tilde{\mu}_{H+}$ in inverted membrane vesicles where enzyme II was rate limiting raised the K_m of the enzyme by a factor of 200 - 1000 (Robillard and Konings, 1981). Changing the redox potential of the membrane with oxidising or reducing agents caused a similar effect. However, Grenier et al., (1985) recently reported that oxidising agents, to the contrary, inhibited methyl α-glucoside phosphorylation by the PEP-sugar PTS; instead, 2 phosphotransferase systems were thought to be involved, II^{Glc} with low K_m and II^{Man} with high K_m, both catalysing methyl α-glucoside translocation but with different sensitivites to SH group oxidation.

1.4.2 Fermentation of Sugars

Although a wide range of polysaccharides and oligosaccharides are utilised by saccharolytic gut bacteria (Section 1.3.4), intracellular fermentation of these substrates is channeled through monosaccharide units. Intracellular, or possible extracellular hydrolysis of lactulose similarly yields hexoses or hexose phosphate for fermentation, depending on the mode of disaccharide uptake.

Whilst an oxidative metabolism of lactulose by bacteria may occur in certain intestinal niches, fermentation of the disaccharide is more likely. This discussion looks at sugar dissimilation in selective Bacteroides and Clostridium strains and lactic acid bacteria.

1.4.2.1 Central pathways

The fermentation of intracellular monosaccharides in gut anaerobes is mediated by a number of pathways: the Embden-Meyerhof-Parnas (EMP) system, three pathways involving 6-phosphonogluconate (hexose monophosphate shunt, the pentose phosphoketolase pathway or heterolactic fermentation, and the Entner-Doudoroff pathway) and a specific pathway for glucose breakdown in Bifid. bifidum (Cooper, 1977; Goltschalk, 1979; Cooper, 1986). In the 6-phosphonogluconate pathway (Fig. 1.4.2.1), the ATP yield is only one ATP per mol of glucose fermented (Prins, 1977) whereas it is 2.5 mol of ATP per mol glucose in the bifidum pathway (Goltschalk, 1979). In the EMP sequence, i.e. glycolysis, hexose is converted to 2 mol each of pyruvate, ATP and reduced pyridine nucleotide (Stanier et al., 1972). Most anaerobic bacteria, excepting heterolactic acid species which use the pentose phosphoketolase route employ the EMP pathway for hexose metabolism, (Zeikus, 1980). The role of these central pathways in sugar catabolism by Enterobacteriaceae is not discussed as it is described elsewhere (Cooper, 1977; 1986). In E.coli and Sal.typhimurium, which is not part of the normal gut flora, 75 - 80% of the glucose metabolised passes through the EMP pathway (Prins, 1977).

Of the clostridial species endogenous to the gut, details of glucose fermentation by C.perfringens have been reported. The

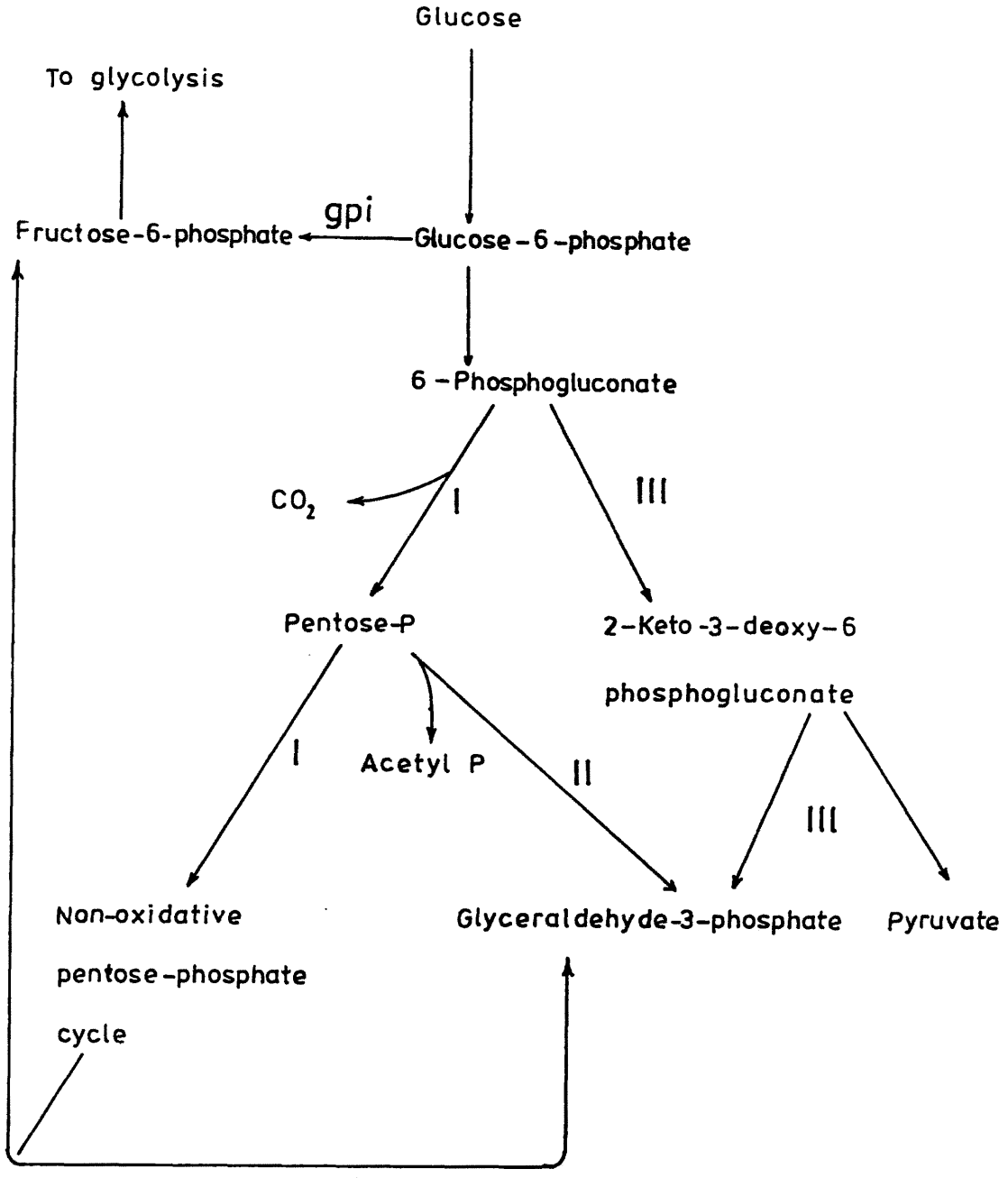


Fig. 1.4.2.1

Schematic representation of the three 6-phosphogluconate pathways. I: entry to the pentose phosphate cycle; II: pentose phosphate phosphoketolase; III: Entner-Doudoroff scheme; gpi: glucose phosphate isomerase (from Prins, 1977).

shift from a predominantly acetic/butyric acid (also ethanol, H_2 and CO_2 produced) to a homolactic fermentation of glucose by C.perfringens in iron-deficient medium (Pappenheimer and Shaskan, 1944). is mediated via a metallo-aldolase (Bard and Gunsalus, 1950). Triose phosphate isomerase (EC 5.3.1.1) and glyceraldehyde-3-phosphate dehydrogenase (EC 2.7.2.3) activities were also detected in the latter report. Further evidence of glycolysis was obtained from labelling studies (Palge et al., 1956; Cynkin and Gibbs, 1958), and assays for EMP enzymes in cell-free extracts, including lactic acid dehydrogenase (EC 1.1.1.27) and the pyruvate clastic systems; but not glucose 6-phosphate dehydrogenase (EC 1.1.1.49) or 6-phospho-gluconate dehydrogenase (EC 1.1.1.43) could not be demonstrated, suggesting the absence of the conventional hexose monophosphate shunt (Groves and Gronlund, 1969b).

The studies on sugar fermentation by Bacteroides strains are relatively recent. The presence of hexokinase (EC 2.7.1.1), glucose 6-phosphate isomerase (EC 5.3.1.9), fructose, 1,6-biphosphate aldolase (FbP.Ald; EC 4.1.2.13) and other glycolytic enzymes indicated the presence of the EMP scheme in B.thetaiotaomicron (Hylemon et al., 1977). FbP-Ald activity was observed in all Bacteroides species examined, notably in B.fragilis, a psedominant organism in the human intestinal tract (Macy and Probst, 1979). B.fragilis also possesses a pyrophosphate-dependant 6-phosphofructokinase to form fructose 1,6-biphosphate without recourse to a molecule of ATP, thereby increasing ATP yield from glucose degradation. The latter report also presented evidence from labelling studies to show that B.fragilis ferments glucose via the EMP pathway (Macy et al., 1978).

The proposed pathway of glucose degradation in B.fragilis is shown in Fig. 1.4.2.2. The formation of a polyglucose hexose storage product by B.thetaitaomicron is noteworthy (Hylemon et al., 1977) as polysaccharide storage may be an important survival strategy in the gut where monosaccharides fermentation is rapid (Savage, 1977).

Glucose is fermented predominantly to lactic acid by all streptococci and a large number of lactobacilli (Wood, 1961). Homofermentative streptococci and lactobacilli yield $\geq$ 85% lactic acid as the predominant end-product, with no gas production, whereas heterofermentative strains produce about 50% lactate, and considerable amounts of CO_2, acetic acid and ethanol (Buchanan and Gibbons, 1974). Evidence reviewed by Wood (1961) from biochemical studies indicates that the EMP pathway is the quantitatively significant mechanism functioning in the homolactic fermentation (for L.casei, L.plantarum and S.faecalis, each a gut isolate). In the final step, homofermentative bacteria regenerate NAD^+ in the conversion of pyruvate to lactate. Heterolactic fermentation follows a hexose monophosphospate pathway (Wood, 1961; Goltschalk, 1979).

1.4.2.2 Initial sequences leading to central pathways

Entrance points, in general, for monosaccharides and related compounds into the main metabolic systems have been reviewed by Wood (1981).

Lactose fermentation has been studied extensively in lactobacilli and several streptococci (see below), and is of particular interest as a model for the fate of lactulose in gut anaerobes.

Strains of S.mutans transport lactose via an inducible

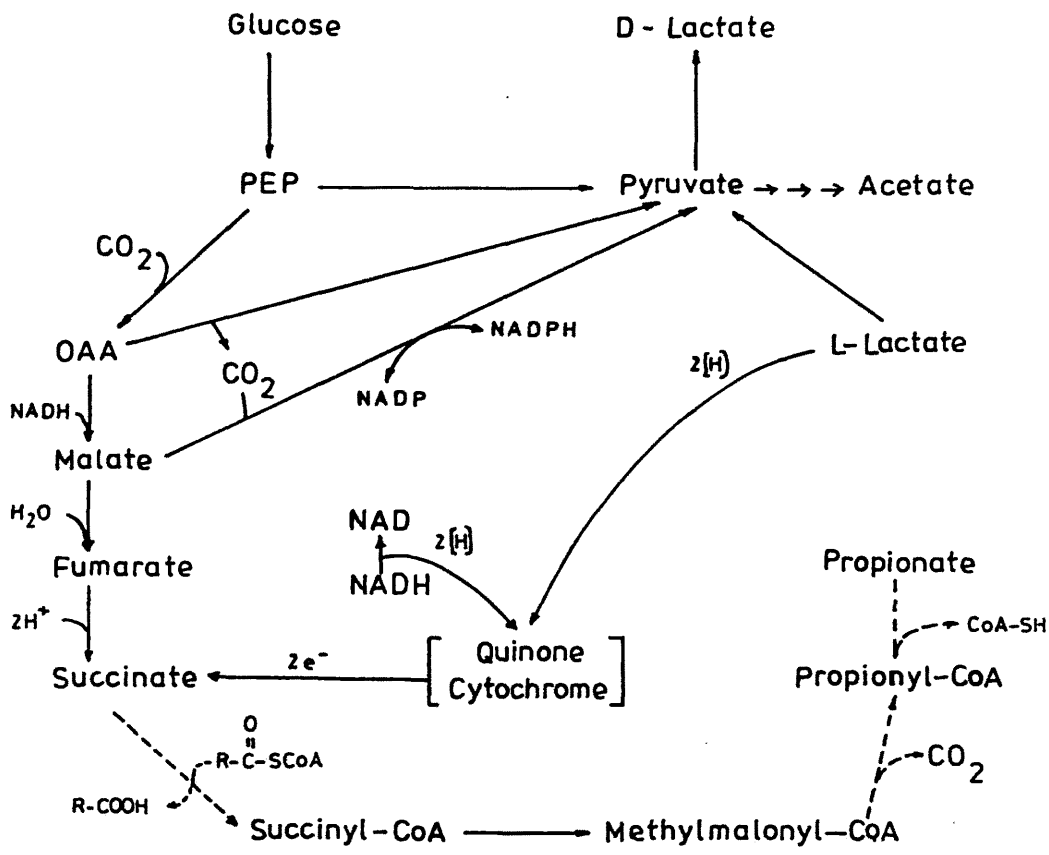


Fig. 1.4.2.2

Pathway of glucose degradation in Bacteroides fragilis (from Macy and Probst, 1979).

PEP:lactose-PTS, which results in the phosphorylation of lactose in the 6 position of the galactose moiety during translocation (Hamilton and Lo, 1978). The phosphorylated disaccharide is cleaved by an inducible β -D-phosphogalactoside galactohydrolase (EC 3.2.1.85; P- β -Glase) to produce glucose and galactose 6-phosphate intracellularly (Calmes and Brown, 1979; Hamilton and Lo, 1978). However, of the five S.mutans examined by Calmes (1978), strain HS6 apparently transported lactose by an active transport-type system. The other four strains exhibited PEP:lactose-PTS transport, with a strict requirement for PEP and Mg^{2+} for transport of ONPG and methyl- β -D-thiogalactopyranoside (TMG) analogues into decryptified cells. Both intact and decryptified cells accumulated TMG-phosphate. Another gut isolate, S.salivarius, utilises both the lactose-PTS and lac operon enzymes, as P- β -Glase and β -galactosidase (EC 3.2.1.23; β -Glase) were co-induced (Hamilton and Lo, 1978), with the latter the predominant metabolic system. Other studies of streptococci using this approach are discussed in Section 5.3.1.3. The active transport of lactose, after hydrolysis, yields glucose and galactose, with galactose being channelled into the EMP scheme via the Leloir pathway (Cooper, 1977): galactose → galactose 1-phosphate → glucose 1-phosphate → glucose 6-phosphate by means of uridine nucleotide intermediates. By contrast, the metabolism of galactose 6-phosphate from cleavage of the lactose 6-phosphate translocated by the PTS of Staph.aureus (Hengstenberg et al., 1970; Bissett and Anderson, 1973) or of group N streptococci (Bissett and Anderson, 1974) to triose phosphates of the glycolytic pathway occurs through the tagatose 6-phosphate pathway: galactose 6-phosphate → tagatose 6-phosphate → tagatose 1,6-diphosphate → glyceraldehyde 3-phosphate plus dihydroxyacetonephosphate, and

involves the enzymes galactose 6-phosphate isomerase, tagatose 6-phosphate kinase, and tagatose 1,6-diphosphate aldolase. Similarly, lactose-PTS induction in S.mutans was paralleled by induction of the tagatose 6-phosphate pathways (Hamilton and Lebtag, 1979), and lactose uptake in S.faecalis is also PTS-catalyzed (Heller and Roschenthaller, 1979).

From the foregoing, two models for lactulose (LL) breakdown by gut bacteria are possible.

a) LL $\xrightarrow[\text{P-}\beta\text{-Glase}]{\text{PTS}}$ [galactose phosphate + fructose] or [galactose + fructose phosphate]

b) LL $\xrightarrow{\beta\text{-Glase}}$ galactose + fructose

Galactose metabolism needs to be assessed for tagatose 6-phosphate or Leloir pathway involvement, and fructose may be fed into the central pathways by a non-specific hexokinase or specific fructokinase. In C.perfringens, a non-specific hexokinase was shown to have a lower affinity for fructose than glucose (Groves and Gronlund, 1969b). Fructose uptake in clostridia is mediated by the PTS, and 1-phosphofructokinase (EC 2.7.1.56;PFK) has been detected in cell-free extracts (Hugo and Goltschalk, 1974). The presence of PFK may be indicative of the PTS uptake of lactulose.

Studies on the regulation of the PEP-dependant lactose-PTS and P-β-Glase, mediating lactose utilisation in L.casei, are of particular interest here (Chassy and Thompson 1983a). Data obtained from transport studies with whole cells and from in vitro PTS assays with permeabilised cells revealed that the lactose-PTS had a high affinity for β-galactosides (e.g. lactose, lactulose, lactobionic acid and arabinosyl-β-D-galactoside). Both the lactose-PTS and P-β-GLase were inducible on each of these β-galactosides. This suggests lactulose uptake in L.casei to be via a PEP:PTS mode (however, lactulose grown cells were not used in

assaying for a lactulose-PTS). Furthermore, cells of L.casei grown on galactose or lactulose were induced for high levels of PEP-dependent galactose PTS activity; lactulose (5 mM) produced < 5% inhibition of (^{14}C) galactose uptake by this transport system (Chassy and Thompson, 1983b).

1.4.2.3 Secondary fermentation reactions

Pyruvate is one of the major products from the fermentation of monosaccharides and can be further metabolised by a number of different pathways. These are generalised in Fig. 1.4.2.3.

Three closely related butyric fermentations are recognised for clostridia. The butyric type fermentation exemplified by C.butyricum (a gut anaerobe) yields butyric and acetic acids, carbon-dioxide and hydrogen (Woods, 1961). C.perfringens in addition produces lactate and ethanol. An examination of the fermentation balances in the literature by Wood (1961) revealed considerable fluctuation in the type and quantity of end-products. For example, the addition of iron to whey fermentation by C.acetobutylicum (a species not found in the human gut) decreased the lactate, formate, and ethanol content, and increased the amount of hydrogen, carbon dioxide, acetone, acetoin, butyrate and acetate. Towards the middle of glucose fermentation by C.acetobutylicum, when the pH of the medium drops to 4.5, two new products are formed: acetone and butanol, with a decrease in the concentration of butyrate. This shift to neutral compounds apparently occurs to prevent a further decrease of pH (Gottschalk, 1979). Large quantities of CO_2 and H_2 are produced by several clostridia (McKay et al., 1982), a quantitation of which is necessary to fully assess carbon recovery (Wood, 1961).

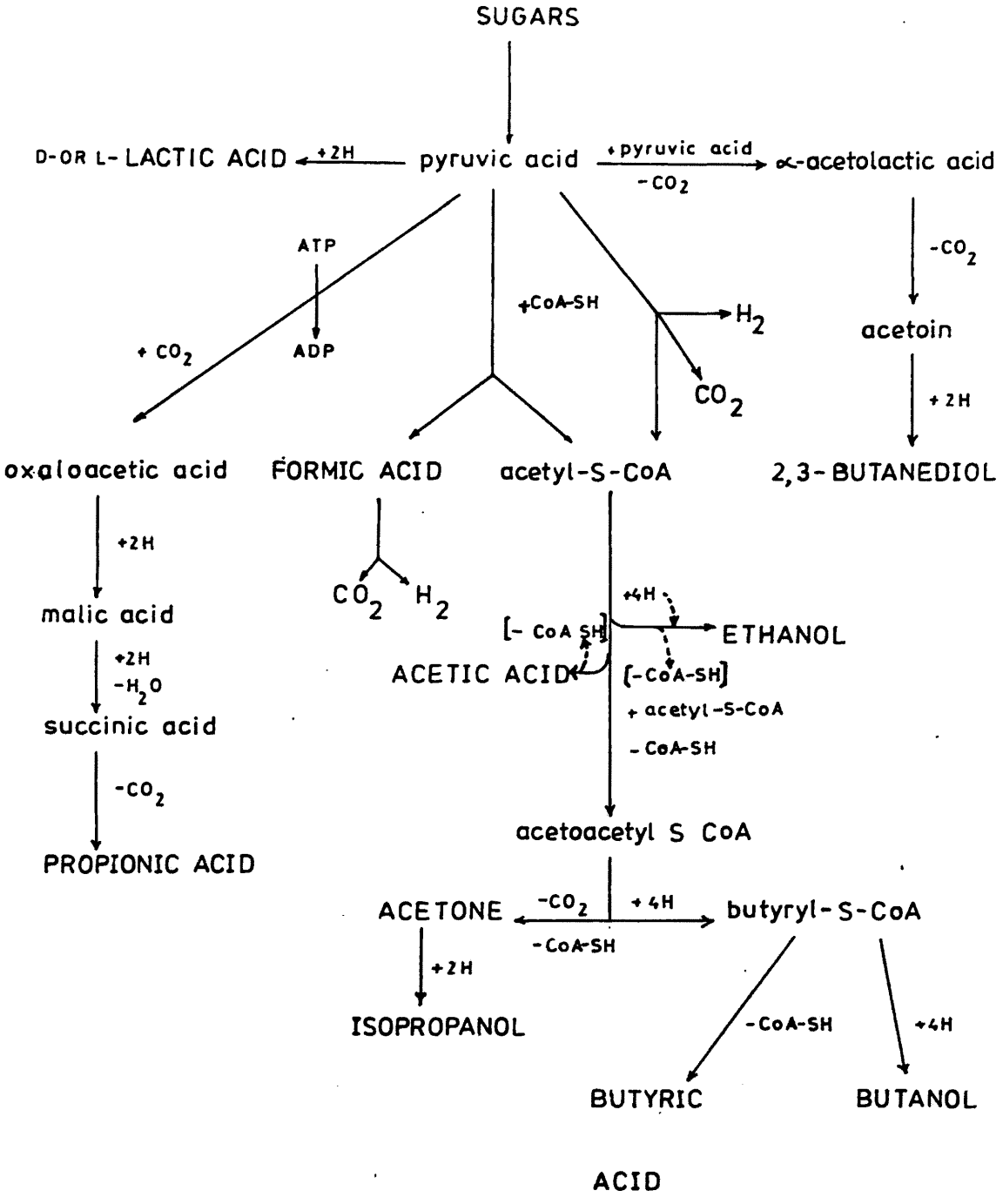


Fig. 1.4.2.3

Derivations of some major end-products of the bacterial fermentations of sugars from pyruvic acid.

Pyruvate occupies the branch point for secondary fermentation in several clostridia, but not all, for instance, in C.pasteurianum (not indigenous to the human gut), it is acetate (Thauer et al., 1977). Oxidation of pyruvate to yield CO_2, H_2 and acetyl CoA occurs in clostridia by the pyruvate-clastic reaction (Prins, 1977). The pyruvate-clastic split may be a misnomer, as the first products of the pyruvate-cleavage reactions include acetyl CoA, and acetyl phosphate is formed subsequently in a separate reaction but is a term still used to describe the reaction (Prins, 1977). The pyruvate-clastic reaction of clostridia resembles that of E.coli only in the formation of acetyl phosphate, and not in the subsequent formate production and utilisation (Wood, 1961). An overall description of the pyruvate cleaving reaction in C.pasteurianum has been reported (Gottschalk, 1979).

The pyruvate-clastic system in C.perfringens was assessed by the formation of acetyl phosphate after incubating cell-free lysates with pyruvate and coenzyme A (Groves and Gronlund, 1969b). Lactate dehydrogenase (LDH: EC 1.1.1.27) reported in the latter investigation, reduces pyruvate to lactic acid, and uses NAD^+/NADH as coenzyme.

Synthesis of butyrate in anaerobes occurs by a reversal of β -oxidation, and formation of butyryl CoA from acetyl CoA involves the enzymes acetoacetyl CoA thiolase, β -hydroxybutyryl CoA dehydrogenase, crotonase and butyryl CoA dehydrogenase (Prins, 1977). In clostridia (e.g. C.butyricum) the reduced pyridine nucleotides produced in the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) reaction is regenerated by converting acetyl-CoA into butyrate or other reduced compounds, such as ethanol (Hugo et al.,

1972; Gottschalk, 1979). $NADH_2$ is consumed by the reduction of acetoacetyl-CoA to β -hydroxybutyrl-CoA and by butyryl-CoA formation from crotonyl-CoA, probably with the participation of flavoproteins (Hugo et al., 1972).

Ethanol can be produced by reduction of acetyl phosphate by acetaldehyde dehydrogenase (EC) and alcohol dehydrogenase (EC 1.1.1.1), requiring four reducing equivalents (Stainer et al., 1972).

In a hemin-deficient medium, and in the presence of CO_2, B.fragilis ferments glucose to predominantly fumarate and lactate, with approximately 1 mol of fumarate formed per mol of glucose used (Macy et al., 1975). A shift of end-products to propionate, succinate and acetate takes place only in the presence of hemin (2 µg/ml). It was also found that the presence of hemin in the growth medium is necessary for the formation of reduced NADH: fumarate oxidoreductase (EC 1.3.1.6) and a membrane-bound cytochrome b. It was suggested that hemin is needed for the synthesis of the cytochrome, which is part of a primitive electron transport system that functions in the generation of energy during the flow of electrons from NADH to fumarate. Thus, in the absence of hemin, B.fragilis is forced to subsist on ATP generated solely via substrate-level phosphorylation, whereas in the presence of hemin additional energy is formed by electron transport phosphorylation. The presence of malate dehydrogenase (EC 1.1.1.38), fumarate reductase, and methylmalonyl-coenzyme A mutase in cell extracts, supported by data from labelling experiments, indicated that propionate is formed from succinate via succinyl-methymalonyl- and propionyl-coenzyme A (Macy et al., 1978). The pathway is unique in that CO_2 is fixed into oxaloacetic acid via an ADP-dependent PEP

carboxykinase with concomitant formation of ATP (Macy et al., 1978).

Butyrate is probably formed in a manner similar to that found in clostridia (Macy and Probst, 1979). Formation of butyrate is not found in the presence of vitamin B_{12} in B.splanchnicus (non-gut species).

In lactic acid bacteria fermentation (review, Kandler, 1983), pyruvate reduction to lactate can occur via NAD^+ dependent and NAD^+ independent lactate dehydrogenases (Garvie, 1980). The NAD^+- linked dehydrogenases (EC 1.1.1.27 and 28) function in the formation of lactate from pyruvate, whereas the NAD^+- independent enzymes (containing cytochrones or flavins) would appear to function primarily in the oxidation of lactate to pyruvate (Prins, 1977). The isomers of lactate formed are used in the classification of the lactic acid bacteria; L.casei produces mainly L(+)-lactate, and small amounts of D(-)-lactate (Garvie, 1980). No lactobacilli form only L(+)-lactate. Streptococci, in contrast, synthesise only L(+)-lactate, and have no D(-)-NAD-linked lactate dehydrogenase (EC 1.1.1.28).

The homolactic fermentation of galactose employed by group N streptococci (S.cremoris, S.lactis, both non-indigenous to the gut) undergoes a phenotypic change to heterolactic metabolism under conditions of carbon limitation (Thomas et al., 1980). This was shown to be due to reduced intracellular levels of both the LDH activator (fructose, 1,6-diphosphate) and pyruvate-formate lyase inhibitors (triose phosphates). Yamada and Carlsson (1975) also reported a fermentation transition to produce formate, acetate, and ethanol, but no lactate by cariogenic S.mutans under conditions of glucose-limitation, as contrasted to homolactic fermentation when

glucose was supplied in excess. Similar LDHs have been reported for S.faecalis and Bifid.bifidum (Prins, 1977) and in L.casei (de Vries et al., 1970).

In the fructose 6-phosphate phosphoketolase pathway found in Bifid.bifidum (Cooper, 1977), pyruvate was reduced to lactate to permit the reoxidation of the reduced NAD^+, and the lactate dehydrogenase involved had a specific requirement for fructose 1,6-diphosphate for activity. Bifidobacteria are also lactic acid bacteria, producing the L(+)-lactate form as a major product (Gottschalk, 1979). Energy conservation in the bifidum pathway is 2.5 mol of ATP/mol of glucose and is somewhat greater than the EMP yield of ATP. The phosphoclastic cleavage of pyruvate has also been shown in Bifid.bifidum (de Vries and Stouthamer, 1968). This type of oxidative decarboxylation of pyruvate via pyruvate-formate lyase has also been demonstrated in S.faecalis (Prins, 1977).

1.4.3 Regulation of carbohydrate metabolism

A bacterial cell can derive the basic elements, S, N, P, and C, from various sources, but some are better than others. When confronted with a mixture of these substrates the cell is able to choose among them, a process requiring priority regulation. This involves regulatory mechanisms effective at two levels (i) at the level of enzyme synthesis and/or (ii) at the level of enzyme activity. Priority in the utilisation of carbon sources provided one of the earliest examples of regulation in bacteria. Referred to as the glucose effect, Monod (1947) observed the preferential utilisation by E.coli of glucose from a mixture with lactose, resulting in a biphasic pattern of growth termed diauxiae. Whilst several regulatory pathways may be involved in this process, to

date three distinct inhibitory effects are known to comprise the glucose effect: (i) catabolite repression, (ii) transient repression and, (iii) inducer exclusion (review, Ullman and Danchin, 1983). These effects have only been studied to any detail in the family Enterobacteriaceae, in particular E.coli and Salmonella typhimurium (review, Ullman, 1985). Mediated at the level of transcription (e.g. Pastan and Adhya, 1976) catabolite repression denotes the permanent repression, varying in severity, of catabolic enzymes elicited by glucose (Magasanik, 1970). Other rapidly metabolisable substrates, such as galactose or mannitol or even glycerol under conditions of nitrogen limitation, can produce this effect (Magasanik, 1961), and in general,the preferred compound serves more efficiently in causing an excess of catabolism over anabolism, and reduces the synthesis of a catabolic enzymes by virtue of causing a rapid and large increase in intermediate metabolite levels. Catabolite repression is now recognised as a general phenomenon, as it is involved in the regulation of some enzymes not involved in carbon and energy metabolism (Wanner et al., 1978). Transient repression is identical to catabolite repression, but differs in kinetic terms (Pastan and Perlman, 1970). In contrast, inducer exclusion is mediated at the level of enzyme activity, by inhibition of the activity of the transport system by the secondary substrate or enhancing its exit from the cell (Ullman and Danchin, 1983) Absence of inducer also causes inhibition of enzyme synthesis, requiring careful observation in differentiating the glucose effects. The importance of the various glucose effects vary, depending upon the regulon in question. For instance, constitutive mutants of the galactose operon exhibit a sole inducer exclusion aspect which

is responsible for the glucose effect, whereas similar mutants of the maltose regulon reduce the glucose effect by a factor of 15, representing a significant inducer exclusion phenomenon (Ullman and Danchin, 1983). Gratuitous inducers also permit the dissociation of the inducer exclusion effect from other regulatory glucose effects since no specific transport system is necessary for their uptake and internal concentration.

The precise mechanisms of the glucose effect components are not yet fully understood. The presence of adenosine 3 ,5 -cyclic monophosphate (cAMP) in E.coli, coupled with a correlation of low intracellular cAMP levels with severe reduction in synthesis of catabolic enzymes brought about by glucose suggested a possible explanation (Makman and Sutherland, 1965). Indeed, exogenous cAMP largely overcame the glucose effect on lac enzymes (Perlman and Pastan, 1968), an observation reiterated in subsequent work: cAMP relieved inducer exclusion and catabolite repression effects on the maltose system (Debarbouille and Schwartz, 1979; Schwartz, 1976), but not the inducer exclusion effect on the gal operon (Joseph et al., 1981). Since the early work, most authors have evaluated a direct relationship between levels of cAMP and degree of repression, a model now challenged to the extent that glucose effects are viewed as independent of cAMP mediation (discussed later in this section).

Evidence of a role for cAMP in glucose repression is implicitly linked mechanistically to the molecular role of cAMP in transcription of catabolic operons. Genetic and in vitro studies have clearly established that cAMP acts in complex with catabolite activator protein (CAP), product of the crp gene, to stimulate an early step during transcription initiation (reviews, Glass, 1982; McClure, 1985). Details

of cAMP-CAP action are very well characterised for the lac operon, but less certain in the case of ara and gal operons (Botsford, 1981a; de Crombrugghe et al., 1984; Raibaud and Schwartz, 1984). However, the involvement of the cAMP-CAP complex in the termination of transcription has cast some doubt on the exclusive role that is ascribed to this complex at transcription initiation. A prominent feature of prokaryote gene expression is the phenomenon of polarity, i.e. the reduced expression of promoter distal genes with respect to the proximal genes, for example in the gal or lac operons (Kennel and Reizman, 1977; Ullman et al., 1979). One protein at least, the rho factor, is known to act in transcription termination, but its action is probably only one aspect of the whole process (Zwaski et al., 1978). Experiments performed with an E.coli mutant bearing a thermosensitive rho factor activity indicate that cAMP relieves polarity by interfering with transcription termination of the lac operon (Ullman et al., 1979).

It is clear that the cAMP-CAP complex is a prerequisite for efficient transcription of catabolic operons. The relationship between intracellular levels of cAMP and modulation of the glucose effect has produced little agreement. Cellular cAMP was 20-fold higher in E.coli cells grown on L-proline compared to glucose (Buettner et al., 1973) but a several fold variation in bacterial growth rates had no effect on intracellular cAMP (Wright et al., 1979). Good correlations between the extent of catabolite repression and cAMP levels have been found; however, no such relationship was found between adenylate cyclase activity and the degree or inhibition (Ullman and Danchin, 1983). CAP levels do not vary under different growth conditions nor with the available carbon source.

Attention has also focused on how the cellular cAMP pool size is regulated during catabolite repression. Possible mechanisms include regulation of adenylate cyclase, cAMP phosphodiesterase, or by a change in the rate of release of cAMP from bacteria. A combination of all three is also a possibility.

Phosphodiesterase has been isolated in a number of organisms, in K.pneumoniae it has a K_m in the order of 0.1 to 0.5 mM, about two orders of magnitude greater than the intracellular concentration of the nucleotide (Botsford, 1981 a, b). The specific activity of the enzyme in E.coli is not affected by the growth medium (Botsford, 1981a), and in mutants lacking phosphodiesterase, the cellular content of cAMP varies with the source of carbon (Buettner et al., 1973). Consequently, cAMP phosphodiesterase appears not to play a major role in catabolite repression.

Cyclic AMP efflux is an energy-dependant process, one stimulated by metabolisable sugars (Botsford, 1981a). Epstein et al., (1975) observed that the rate of cAMP excretion did not vary with carbon source; contrary to this, Crenon and Ullman (1984) found that in a cya strain, (see p.72) cAMP efflux from pre-loaded cells was rapid and caused a decrease in β -galactosidase synthesis. Cellular excretion of cAMP as a regulatory device in catabolite-sensitive operons requires further work.

In view of these findings, it seems likely that the activity, and possibly the concentration, of adenylate cyclase (AC) is crucial in modulating the glucose effects. Previously, it was noted that E.coli AC cannot be released from the membrane without a simultaneous loss of activity (Janecek et al., 1980). However, Young and Epstein (1983) reported a near homogeneous preparation of the E.coli enzyme, which was largely soluble. AC

from S.salivarius is membrane bound (Khandelwal and Hamilton, 1972). The membrane associated AC presents methodoligical difficulties in assay, and involves cell permeabilisation treatment (Pastan and Adhya, 1976).

Isolation of E.coli mutants deficient for AC (coded by the cya gene) are phenotypically pleiotropic carbohydrate-negative (Perlman and Pastan, 1969). Earlier experiments indicated that sugars and non-metabolisable analogues, e.g. α-methylglucoside, inhibited AC activity in whole and toluene-treated cells (Peterkofsky and Gazdar, 1974; Harwood and Peterkofsky, 1975; Saier et al., 1976). By inference, therefore, the transport of the sugar rather than a metabolite produced inhibition. In support of this conclusion, it was noted that glucose 6-phosphate did not mimic the inhibitory effect of glucose in intact cells and that inhibition was not observed in cell-free extracts, indicating a requirement for an intact membrane (Peterkofsky, 1976). More recently, it has been observed that in intact or permeabilised E.coli cells, inhibition of AC activity by methyl α-glucoside took place only in strains expressing genes for the proteins of the PEP:PTS (Reddy et al., 1985). This inhibition was not observed in cell-free extracts. The range of sugars producing modulation of AC activity include both PTS and non-PTS substrates (Peterkofsky, 1976), more specifically the PTS for glucose and mannitol (Postma and Roseman, 1976), proton symport mechanisms such as for lactose (Saier, 1977; Peterkofsky and Gazdar, 1979) and the facilitated diffusion of glycerol (Botsford, 1981a). A pre-requisite for an effect on AC activity is that the appropriate transport system is induced. The regulatory significance of these non-PTS effects awaits further work, although Postma and Lengeler (1985) suggest that electrochemical

proton gradient mediated regulation of AC activity occurs in an indirect way.

Evidence of PTS-directed regulation in catabolite and transient repression and inducer exclusion comes from extensive studies with PTS mutants of the Enterobacteriaceae (Ullman and Danchin, 1983; Ullman, 1986). Tight pts H,I mutants are unable to induce catabolic operons for non-PTS substrates, a defect corrected by exogenous cAMP (Pastan and Perlman, 1969). A leaky enzyme I mutation resulted in virtually a complete loss of AC activity (Peterkofsky and Gazdar, 1975; Peterkofsky, 1976), suggesting a direct action of enzyme I on AC activity. However, measurements of cAMP levels in a mutant thermosensitive for enzyme I showed no change upon increases in temperature (Dahl et al., 1972). An important development came with the isolation of a suppressor mutation in Sal.typhimurium (Saier and Feucht, 1975) and E.coli (Kornberg and Watts, 1978; 1979) termed crr for carbohydrate repression resistant, enabling growth of tight pts mutants on a series of non-PTS carbohydrates. The crr gene is known to encode III^{Glc} (Postma and Lengeler, 1985). On the basis of these and other results (see Ullman and Danchin, 1983), a model for regulation was advanced in which III^{Glc} plays a crucial role (Fig. 1.4.3.1). Two reactions are particularly important: (i) phospho-III^{Glc} activates AC, thereby regulating transcription of catabolic operons by levels of intracellular cAMP and activated CAP; (ii) nonphosphorylated III^{Glc} is an inhibitor of non-PTS permeases, and present in tight pts H,I mutants. Sholte and Postma (1980) found a pts^{+} crr double mutant of Sal.typhimurium utilised a number of non-PTS substrates, in agreement with this model. Nelson et al., (1984) isolated an iex mutant of E.coli, the iex locus being an allele of the crr gene,

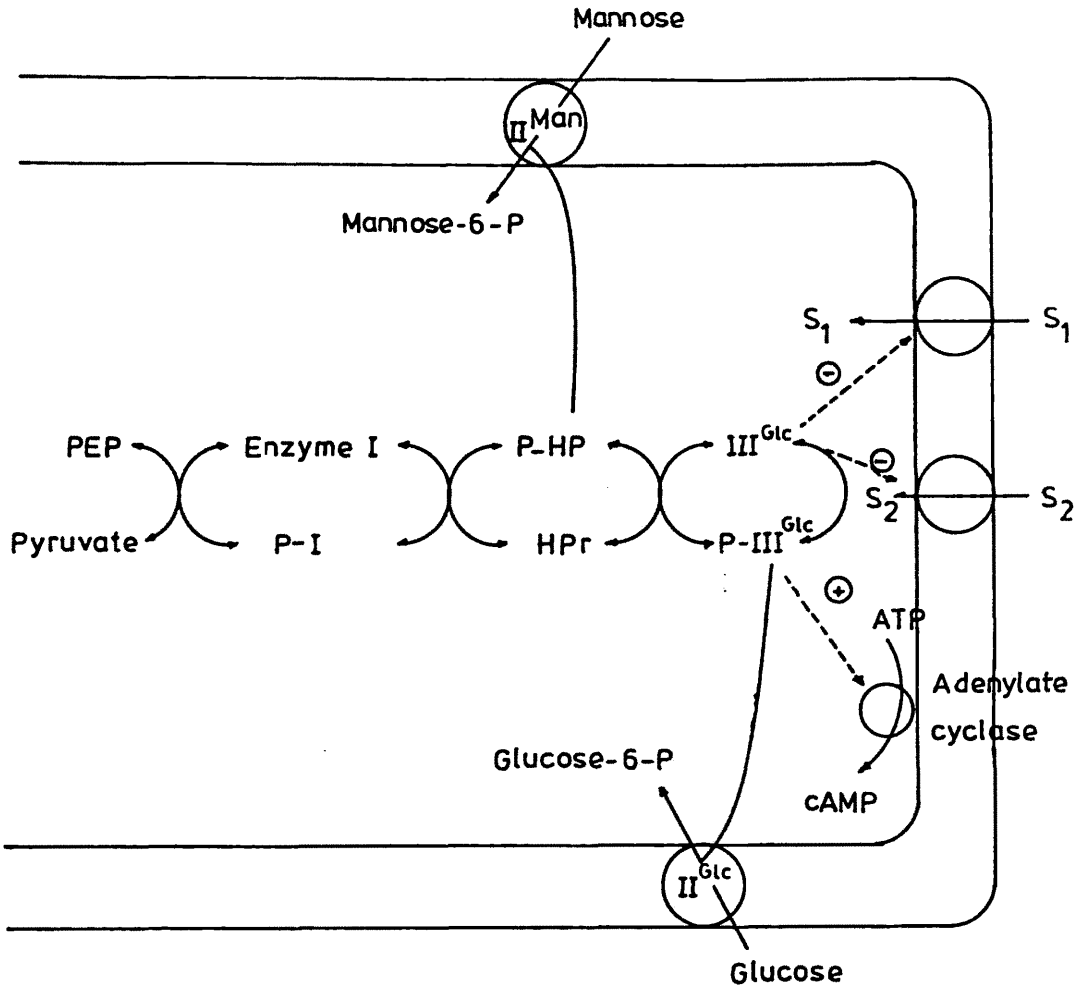


Fig. 1.4.3.1

Model for the regulation by the PTS. Components of the PTS shown are the general proteins of the system, and two specific enzymes II, for mannose (II^{Man}) and glucose (II^{Glc}) respectively. Activation (+) of adenylate cyclase by phosphorylated III^{Glc} ($P\sim III^{Glc}$) and inhibition (-) of two different non-PTS uptake systems by III^{Glc} are illustrated. S_1 and S_2 represent lactose, melibiose, maltose, or glycerol (from Postma and Lengeler, 1985).

and coding for an altered III^{Glc} which cannot bind the lactose carrier. Consequently, uptake of non-PTS in an iex strain was resistant to PTS sugars. The introduction of a plasmid containing a wild-type crr^+ allele into the iex train restored the iex phenotype to that of the iex^+ parent. Either a pts HI deletion or a crr::Tn10 mutation in crp strains of Sal.typhimurium resulted in low levels of cAMP synthesis, whereas a leaky pts I mutation exhibited a high level of cAMP synthesis which was inhibited by PTS carbohydrates. Thus, phospho-III^{Glc} rather than III^{Glc} is apparently required for full cAMP synthesis (Den Blaauwen and Postma, 1985). Recently, it has been shown that wild-type Sal.typhimurium cells contain predominantly phospho-III^{Glc} in the absence of sugars (Nelson et al., 1986). However, some anomalous data on the above model has been reported (Ullman and Danchin, 1983; Postma and Lengeler, 1985).

In addition, AC regulation now appears to be multifactorial and a number of cAMP-CAP effects have been reported. A crp strain of E.coli producing more cAMP than its crp^+ parent (Botsford and Drexler, 1978; Majerfeld et al., 1981) and inhibition of cya expression by cAMP or CAP (Makoto et al., 1985) suggests that CAP regulates cya transcription in a negative way. Different authors, on the other hand, provide evidence suggested CAP acts on the level of adenylate cyclase activity (Wang et al., 1981; Guidi-Rontani et al., 1981; Joseph et al., 1982). For example, Bankaitis and Bassford (1982) isolated cya-lac operon and protein fusions in E.coli, and reported that neither cAMP nor CAP were found to play a major role in transcription or translational regulation of cya expression in these fusion strains, suggesting cAMP-CAP regulation of adenylate cyclase as post-translational, i.e. at the level of enzyme activity. Furthermore, Roy and Danchin (1982) have characterised

the *cya* locus of *E.coli* by cloning techniques. When truncated *cya* genes (with 60% of their C-terminal end detected) are present on a multicopy plasmid in a *cya*$^-$ host, the synthesis of β -Glase is still regulated by glucose. The *crp* gene has also been cloned and its nucleotide sequence determined; the transcription of the *crp* gene was found to be inhibited antogenously by its gene product, CAP and by cAMP (Alba, 1983).

Clearly, demonstration of the precise mechanism by which AC activity is controlled will require detailed reconstitution studies. To this end, the solubilised AC of *E.coli*, purified 17,000-fold to near homogeneity (Yang and Epstein, 1983) is of interest. To further characterise AC, *E.coli* plasmids carrying *cya-lac* fusions were constructed (Danchin and Ullman, 1983). This data suggested that AC probably consists of two functional domains: the NH_2-terminal carrying ATP → cAMP catalytic activity, and the COOH-terminal being the target of PEP:PTS mediated regulation. Structurally, the results also suggest that AC is bound to the cytoplasmic membrane via the COOH-terminal domain.

Finally, a number of studies suggest that cAMP-CAP is not the unique regulator of catabolite repression and other glucose effects. Therefore, these phenomena may be mediated by several distinct mechanisms. Differing lines of approach have been used. Genetic experimentation has relied on double mutations: several mutations suppressing deletion mutations of adenylate cyclase map in the *crp* region and result in making CAP independent of cAMP for activity (Dessein *et al.*, 1978; Takebe *et al.*, 1978). In some of these strains, *lac* enzyme synthesis is still sensitive to catabolite repression. Another category of mutants, *alt* mutants affecting RNA polymerase, and isolated as *Ara*$^+$ (arabinose operon) revertants from

a cya strain synthesised reduced cAMP levels (reviewed by Guide-Rontani et al., 1980). These mutants partially overcame cAMP deficiency for β -galactosidase synthesis at low temperatures. The latter authors isolated a specific class of double mutants, rho crp, exhibiting catabolite repression, therefore possible in strains lacking a functional CAP provided they are also deficient in rho protein. Therefore, rho protein may itself be involved in regulation of catabolite-sensitive operons.

Another line of evidence comes from a search by Ullman et al., (1976) for mediators other than cAMP. Water-soluble extracts of E.coli cells exerted an extremely strong repressive effect upon the expression of catabolite sensitive operons. The compound responsible for this activity was partially purified and proved to be a heat stable compound of low molecular weight (termed the catabolite modulator factor; CMF). CMF causes catabolite repression of the lac operon in strains having a cya deletion or the mutation in crp that suppresses the cya deletion (Botsford, 1981b).

Wanner et al., (1978) found that E.coli cells with a wild-type lac promoter showed an 18-fold variation in lac expression whereas cells with the lac P37 promoter exhibited several hundred-fold variation. Exogenous cAMP could not abolish catabolite repression, even though controls demonstrated that cAMP was entering the cells in significant amounts. The authors concluded that catabolite repression is a general effect, and may be cAMP-mediated or independent or cAMP-CAP involvement.

In addition, some Gram-positive bacteria, e.g. Bacillus megaterium, do not synthesise cAMP but still exhibit catabolite repression (Ullman and Danchin, 1983). Although cAMP, adenylate

cyclase and cyclic phosphodiesterase are present in Pseudomonas aeruginosa, cAMP does not appear to influence catabolite repression of histidase in either Psd.aeruginose or Psd.putida (Botsford, 1981a). Cyclic AMP has not been detected in Bacteroides fragilis; addition of dibutyryl cAMP or sodium cholate to cultures of this strain growing on lactose did not significantly affect β -galactosidase specific activity, although nucleotide uptake was not tested (Hylemon and Phibbs, 1974). Nor was there a diauxic growth pattern in medium containing glucose and lactose. In surveys of cAMP in bacteria (Peterkofsky, 1974; Botsford; 1981a), this nucleotide appears virtually absent in obligate anaerobes. Spore formation though, was enhanced by cAMP in Clostridium botulinum (Emeruwa and Hawirko, 1975). A search for cAMP in Lactobacillus plantarum also suggested its absence, as well as related enzymes (Peterkofsky, 1974, 1976). Diauxic growth of L.plantarum on glucose-lactose mixtures was observed. Studies of catabolite repression in this bacterium indicated that glucose, but not α -methyl glucoside, inhibited β -galactosidase synthesis. Glucose exerted its effect through its ability to exclude galactose or lactose entry into the cell, therefore indicating an inducer exclusion phenomenon not catabolite repression. Cyclic nucleotides did not relieve the inhibition.

Little is known about the occurrence of guanosine 3 , 5 -cyclic monophosphate in prokaryotes (Peterkofsky, 1974). Its presence in a limited number of bacterial species has been surveyed by Botsford (1981a).

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1. Chemicals

Both "Analar" and "Laboratory Reagent" grade chemicals were obtained from B.D.H. Ltd., Poole, Dorset. Crystalline lactulose (4-O-β-D-galactopyranosyl-α-D-fructofuranose) was generously donated by Philips-Duphar Ltd., Weesp, Holland; other carbohydrates were obtained from either Sigma Chemical Co., Poole, Dorset, or B.D.H. Ltd. Sugar phosphates were supplied by Sigma Chemical Co. Chromatographically pure alcohols and carboxylic acids were purchased from B.D.H. Ltd., and diethyl ether and chloroform from May and Baker Ltd., Eccles, Manchester. Diethyl ether was dried over sodium-lead alloy and then redistilled prior to use. (D-glucose-1-^{14}C) lactose, (U-^{14}C) glucose, (1-^{14}C) galactose, and n-(1-^{14}C) hexadecane were obtained from the Radiochemical Centre, Amersham, Bucks. 2,5-Diphenyloxazole (PPO) was obtained from International Enzymes Ltd., Windsor, Berkshire.

Ingredients for culture media were purchased from Oxoid Ltd., London, S.E.1, Difco Laboratories Ltd., East Molesey, Surrey, and Becton, Dickinson and Co. (U.K), Wembley, Middlesex. Microbiological filters for sugar media (0.22μm and 0.45μm) were supplied by Oxoid Ltd. Sterile outdated human blood was obtained from a local hospital. Glass beads (2mm. diameter) employed in the storage technique for anaerobes were supplied by Ellis and Farrier, Hanover Square, London, and Merck narrow range non-leaching pH strips by B.D.H. Ltd. Sachets of palladium coated alumina pellets for anaerobic incubations were purchased from Becton Dickinson (U.K) Ltd., Cowley, Oxford.

The gas mixture used for anaerobic cultivations, comprising $N_2/H_2/CO_2$ (80:10:10, by vol.), helium (grade A), and all chromato-

graphic gases (CO_2-free N_2, H_2, and air, respectively) were provided by The British Oxygen Co. Ltd., Wembley, Middlesex. A gas mixture used for calibration (CH_4 1.05%/C_2H_6 0.96%/C_3H_8 1.52%/n-C_4H_{10} 0.86%, in N_2) was purchased from Phase Separations Ltd., Queensferry, Flintshire, CH5 2LR.

The following suppliers were used for column chromatography material: DEAE-52 cellulose and DE81-cellulose discs (2.5cm diameter) from Whatman Ltd., Maidstone, Kent; Bio-Gel A-0.5m (100-200 mesh) from Bio-Rad Laboratories Ltd., Watford, Hertfordshire; and, Blue-Sepharose CL-6B from Pharmacia Fine Chemicals (G.B) Ltd., Hounslow, Middlesex. Schleicher and Schüll silica-gell G F1500 t.l.c. plates were obtained from Anderman and Co. Ltd., East Molesey, Surrey. Glass columns and Diatomite C (60-80 mesh; washed with 3% phosphoric acid to neutralise acid absorbing sites) coated with 10% polyethylene glycol 20M for g.l.c. analyses were purchased from Phase Separations Ltd.

All enzymes, NADH and NADPH were from Sigma Chemical Co., or The Boehringer Corporation (London) Ltd., Lewes, Sussex. The cAMP assay kit (code TRK.432) was purchased from The Radiochemical Centre, Amersham, Bucks.

2.1.2 Organisms

Strains of lactobacilli were purchased from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. All other organisms were kindly provided by Dr. A. Vince, The Rayne Institute, University College Hospital Medical School, London; Dr. M. Hill, Bacterial Metabolism Research Laboratory, Central Public Health Laboratories, London; and Professor I. Phillips, Department of Microbiology, St. Thomas's Hospital Medical School, London. Identification tests to characterise anaerobes were carried out by

Ms. E. Taylor in the Department of Microbiology, St. Thomas's Hospital Medical School.

2.2 GENERAL TECHNIQUES

2.2.1 Storage of organisms

Facultative anaerobes were maintained on nutrient agar (Oxoid CM3) slopes and stored at 4°C. Lactobacilli were grown to early stationary phase on skimmed milk, and also stored at 4°C. All obligate anaerobes were maintained on Oxoid "Cooked Meat" base (CM349) with peptone-yeast basal medium, described below, and kept at -70°C after the addition of sterile glycerol to a final concentration of 15% (v/v). This complex storage medium was also found useful for perpetuating the viability of lactobacilli stock cultures. Maintenance procedures for anaerobes were subsequently supplemented with a "glass-beads" storage technique described by Feltham et al., (1978). However, of the two methods, the "Cooked Meat" technique was found to be more reliable. Stocks were subcultured at 6-monthly intervals, and the purity of strains verified by visual examination and Gram-staining of cultures grown on appropriate nutrient or blood agar (Oxoid CM271) plates.

2.2.2 Media and growth conditions

The basal liquid medium used for aerobes was 1% (w/v) peptone water (Difco Proteose Peptone No. 3) and peptone-yeast (PY) for anaerobic investigations (Holdeman and Moore, 1972). Basal PY medium contained, per litre:

Difco Proteose Peptone No. 3	10g
Yeast Extract (Difco, L21)	10g
Salts solution (see below)	40ml
Resazurin solution (1mg/ml)	1ml
Sodium thioglycollate	0.5g
Sodium formaldehydesulphoxylate	0.3g

The ingredients were suspended in distilled water (990ml) and heated with mixing until the resazurin changed hue from orange to colourless. An aliquot (10ml) of a stock hemin-vitamin K solution (H-VK), described below, was added, as well as L-cysteine hydrochloride (0.5g). The pH of the basal medium was adjusted to 7.2, dispensed into bottles or other vessels and autoclaved (15 min at 120°C and ~103.5 k Pa) Anaerobic incubation of the sterile medium, ≥ 24h, was necessary to insure optimal anaerobiosis of the liquid medium.

The salts solution stock contained:

$CaCl_2.2H_2O$	0.25g
$MgSO_4$	0.20g
K_2HPO_4	1.00g
$NaHCO_3$	10.00g

Initially, $CaCl_2$ and $MgSO_4$ were dissolved in distilled water (800ml) and the remaining salts added gradually to insure a minimal precipitate, which inevitably formed. The volume was made up to a litre, and the solution stored at 4°C.

For the H-VK stock, two solutions were prepared:

(1) Menadione (100mg) in 95% ethanol (20ml), sterilized by aseptic filtration.

(2) Hemin stock solution: hemin (50mg) dissolved in 1 N NaOH (1ml) and made up to 100 ml with distilled water, and autoclaved.

The H-VK stock was obtained by mixing the sterile menadione solution (1ml) with hemin stock solution (100ml).

When necessary, lactulose or other sugars were added to the sterile basal medium through a bacteriological filter (0.22μm pore size).

To achieve an adequately oxygen-free environment, standard anaerobic procedures were employed. Two types of gas replacement anaerobic jars were used: a) domestic pressure cookers (Megfeesa and Tefal brands, obtained locally) with a 4 - 6 litre

capacity. Firm tubing was attached to the safety valve outlet, and the second outlet sealed airtight; b) commercial anaerobic jar (Gallenkemp, ANF-250-V model) with a volume of 5 litres. Each jar was fitted with 3 sachets of palladium coated alumina pellets which had been reactivated before use by heating to 160^{o}C for 2h in a hot air oven. The lid was then secured, and the outlet vent of the jar connected to a vacuum pump (Edwards High Vacuum, Crawley, Surrey) with a commercial vacuum gauge (Gallenkamp; scale ~ 103.3 k Pa) and evacuated to about -93 k Pa. A rubber bladder (6 litre anaesthesia bag) was filled with the gas mixture $N_2/H_2/CO_2$ (80:10:10, by vol.) and allowed to slowly enter the evacuated jar. The bladder with residual gas was left attached to the jar for 20 min in order to replace trace quantities of oxygen which were converted to water by the palladium catalyzed reaction with hydrogen. To test anaerobiosis in the jar, a biological indicator, Pseudomonas aeruginosa inoculated on Simmonds citrate agar (Oxoid, CM 155) plates, was used as suggested by Gargan (1976). The agar contains methylene blue, which is colourless when reduced and blue when the citrate is oxidised. A plate for aerobic incubation was inoculated as a control. Several studies have shown that the anaerobic procedure described above is as effective as other, more elaborate, techniques (Rosenblatt et al., 1974; Willis, 1977).

2.2.3 Monitoring bacterial growth

The standard inoculation procedure was as follows. Stock cultures were initially revived on either nutrient or blood agar plates using loop inocula from nutrient agar slopes or thawed anaerobic stock culture preparations. After a 24h incubation ($\pm$ O_2) colonies were loop transferred aseptically to sterile basal medium and incubated for a further 16 - 24h. Cell cultures thereby

adapted to the liquid medium were used as inocula (5%; $^{v}/_{v}$) for basal medium ± sugar (s) and grown for the required test period. The incubation temperature in all studies was 37°C.

Two types of procedures were required: a) to assess growth of a number of species after a fixed incubation period, and b) a time-course evaluation of the growth characteristics of a selected species.

For procedure a), inocula from overnight basal medium cultures of different species were transferred individually to basal medium ± sugar (15ml), and incubated for 48h. The basal medium inoculations served as controls. Inverted gas sample tubes were included in the sugar medium to determine gas production.

For the time-course experiments, a set of basal medium ± sugar (15ml) bottles were inoculated with a selected species grown overnight on basal medium, and the set incubated in a anaerobic jar. At allotted time periods, a sample bottle was removed for various analyses and the jar re-gassed. It was subsequently found that an incubation period of about 2h in the anaerobic jar was sufficient to enable lactobacilli and C . perfringens cultures to be further incubated on the bench at 37°C, without significantly affecting their growth characteristics.

Time-course experiments over a 24h period required two inoculated sets of the same culture. The first was assayed from 0 - 12h, after which time the second set was inoculated, incubated for 12h and then assayed from 12 - 24h. Mean values for the 12h points were recorded.

Growth was assessed by turbidity measurements at A_{612}, and pH determinations by microelectrode or narrow range pH strips. Gaseous fermentation products were indicated by gas collected in

the inverted sample tubes. Supernatants from cultures centrifuged at 2000g for 15 min at 4°C were stored at -20°C until required, and the harvested cells used for β-galactosidase assays (Section 2.2.5).

2.2.3.1 Large-scale anaerobic cultivation

A suitable volume of PY-medium (1875ml) was autoclaved in a conical flask (2 x 1) and a sterile liquid paraffin: petroleum jelly mixture (1:1 w/w; 200g) added aseptically whilst both preparations were hot. A sterile magnetic flea was also placed in the medium. After cooling, filter-sterilised PY-sugar (25ml) was added to a final concentration of 5mm. The medium was inoculated through its air-excluding seal and incubated in the stoppered Erlenmeyer flask. This ensured a minimal headspace. For harvesting, the cell suspension was siphoned through the jelly layer, and suitable aliquots centrifuged at 12000g for 20 min at 4°C. The cell sediments were pooled, washed (x2) with buffer appropriate to the next stage of study, and the final pellet stored at -20°C until required.

2.2.4 Determination of carbohydrates

Carbohydrates in culture supernatants were estimated either by t.l.c. or colourimetric procedures.

The t.l.c. method described by Menzies et al., (1978) was used and is based on the separation of sugars on silica gel G with subsequent quantitation by locating and scanning the appropriate bands. The solvent mixture used to separate lactose and lactulose was ethyl acetate/pyridine/4% (w/v) sodium tetraborate (60:30:10, by vol.; Mount, J. N. and Menzies, I.S., personal communication). When fructose was present in the supernatants containing the two disaccharides, the monomer appeared as an indistinct band with the borate solvent system. Consequently, these samples, and all other carbohydrate mixtures, were separated by the solvent system ethyl

acetate/pyridine/acetic acid/water (60:30:10:10, by vol.). Mean values of carbohydrate concentrations were obtained from two separate t.l.c. determinations.

The colourimetric assay developed by Vachek (1971) for lactulose estimation is based on the reaction of the disaccharide with dilute sulphuric acid, followed by heating to produce a yellow product which is stable and is measured at A_{400}. For the calibration curve, linear for 0 - 10mg lactulose in assay, standard lactulose solutions were prepared in basal culture medium to correct for interference. Samples (0.5ml; 0 - 10mg lactulose) were placed in large boiling tubes (25mm x 150mm), deionised water (2.5ml) added, followed by dil. H_2SO_4 (H_2O:H_2SO_4; 3:2, by vol.; 5ml). After incubation at 100°C for 15 min, the tubes were cooled in a tap-water bath for 10 min, and A_{400} recorded. The presence of lactose and galactose was shown by Vachek (1971) not to cause any interference in the assay. Fructose was not tested in the latter report, and its inclusion with lactulose in the assay mixture significantly increased A_{400} values. In the absence of fructose, however, this procedure is both rapid and reproducible. The sensitivity of the technique was investigated by preparing various lactulose concentrations in basal medium (5.1 - 6.9mg lactulose in assay). The results, shown in Table 2.1, indicated that a ±5% sensitivity limit is imposed by the assay. This may be due to lack of adequate control over the heating stage or lack of sensitivity to the colour intensity at this wavelength.

2.2.5 β-galactosidase assays

The rapid and effective procedure developed by Putman and Koch (1975) to assay β-galactosidase (β-Glase) using permeabilised cells was followed. Bacterial cells, harvested as described

Table 2.1 Sensitivity of the lactulose quantitation procedure (Vachek, 1971)

Lactulose in assay (mg)	A_{400} $\bar{x}$ ± S.D. (3)	Lactulose determined by calibration (mg)	% recovery (test/expected values)
5.5 (20%)[a]	0.68 ± 0.02	5.4	98
6.2 (10%)	0.79 ± 0.03	6.3	102
6.5 (5%)	0.84 ± 0.02	6.7	103
6.9 (PYL)	0.91 ± 0.00	7.2	104

[a] Values in parenthesis are differences (expressed as %) between the amount of lactulose in peptone-yeast lactulose medium (PYL; 40mM) and test preparations.

Assay procedures are described in Section 2.2.4

previously (Section 2.2.3), were washed (x1) with 50 mM sodium phosphate buffer, pH 7.3, containing D-chloramphenicol (50µg/ml) and resuspended in the same buffer to the original culture volume. An aliquot (1.0ml) of this suspension was treated with 25µl of a lysis mixture containing 10% ($^{w}/_{v}$) sodium lauryl sulphate/0.02M manganous sulphate/toluene/2-mercapto ethanol (1:1:1:5, by vol.). After thorough shaking on a vortex mixer for 15 s, samples (0.1 ml or 0.5 ml) were removed forβ-Glaseassay using a kinetic assay procedure (Sigma product information, No. G-6008) with o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The assay mixture contained (in a 3ml plastic cuvette of 1 cm lightpath):

0.1M sodium phosphate buffer, pH 7.3	2.6 ml
3.36M 2-mercaptoethanol	0.1 ml
0.03M $MgCl_2.6H_2O$	0.1 ml
Enzyme (permeabilised cells)	0.1 ml

The mixture was incubated for 3 minutes at 37°C to activate the enzyme, and 0.068M ONPG (0.1 ml) added. The change in A_{410} was recorded with H_2O as reference (2.84 is the millimolar extinction coefficient used for calculations).

2.2.6 Protein determination

Protein was measured in cell-free extracts by the method of Lowry et al., (1951) using bovine serum albumin (BSA) as a standard. A procedure for estimating the total protein content of whole cells has been described by Herbert et al., (1971). The technique is based on heating whole cell suspensions to 100°C in 1.0M NaOH, which extracts the cell proteins in a soluble form to be assayed by the Folin-Ciacalteau reagent. BSA (0 - 200µg) was used as the protein standard, and subjected to the same heat treatment.

2.2.7 Sonication procedures

Depending on the species, rupture of the bacterial cell

envelope required variable sonication periods (between 5 and 30 min; amplitude 20 microns). Washed cell suspensions were sonicated (MSE 150W Ultrasonic Disintegrator) in the double-walled glass chamber of a Churchill-Chiller/Thermo circulator (Chem Lab Instruments Ltd., London), the circulating fluid being maintained at $< 0^{o}C$.

2.2.8 Column chromatography

DEAE-52 cellulose, Bio-gel A-0.5m and Blue-Sepharose CL-6B were prepared as described in the manufacturer's instructions. Details of column dimensions, flow rates, etc., are given in the appropriate sections. Columns were either obtained commercially or made in the laboratory by fitting a porous polyethylene gel support disc to one end of glass tubes of required sizes. Unless stated otherwise, all columns were run at $4^{o}C$ using downward-flow, gravity feed elution.

2.2.9 Counting radioactivity

Low background glass vials were used. DEAE-cellulose discs or microbiological filters with radioactive samples were dried at temperatures indicated in the relevant sections. On cooling, 10ml of a toluene/PPO mixture (5g of PPO per litre toluene) was added, and the vials placed in a Packard Tricarb liquid scintillation spectrometer. After an equilibration period of about 4h, the radioactivity was counted. Efficiency determinations were made by the internal standard method, using n-(1-^{14}C) hexadecane, and are indicated in relevant sections.

CHAPTER 3: GAS-LIQUID CHROMATOGRAPHY OF BACTERIAL FERMENTATION PRODUCTS

3.1 INTRODUCTION

A range of alcohols, volatile and nonvolatile fatty acids, as well as gases occur as end-products in different types of microbial fermentation. Their separation and quantitative determination in culture media is relevant to both diagnostic and more detailed metabolic studies. This chapter describes g.l.c. procedures to quantitate non-gaseous fermentation products and a qualitative method to identify some common gaseous end-products.

3.2 ASSAYS FOR NON-GASEOUS FERMENTATION PRODUCTS

Various gas-liquid chromatographic methods are employed for fatty acid analyses, but consist of time-consuming or extensive procedures for both sample preparation and assay (e.g. Salanitro and Muirhead, 1975). The simple and rapid method used in the present studies is a modification of a qualitative procedure (J. A. Hine, 1978; personal communication) and was developed for the purpose of handling a large number of samples in minimum time.

3.2.1 Gas chromatograph

A Pye-Unicam chromatograph (Series 204) equipped with a hydrogen flame ionization detector was used. This was connected to a digital integrator (Pye-Unicam, model DP88) capable of printing retention time (midpoint of the peak area) and set for area sensitivity above a threshold of 100$\mu V\ s^{-1}$ was used. A flat-bed chart recorder (Linseis, model D480L) at a chart speed of 5 - 10 mm min^{-1} recorded the elution profile.

3.2.2 Column and chromatographic conditions

Alcohols, volatile fatty acids (VFA) and methyl esters of non-volatile fatty acids were separated on coiled glass columns packed with Diatomite C (60 - 80 mesh) coated with 10% polyethylene glycol 20M. The columns were conditioned prior to connection to

the detectors by heating to 190°C for 24h under a flow of nitrogen. Reference and injection columns were not interchanged throughout the investigations.

The following conditions were employed for estimating non-gaseous metabolites: (i) gas flow rates of 50, 750, 45 ml min^{-1} for H_2, air and N_2 respectively; (ii) injection port temperature 120°C, and detector temperature 180°C; (iii) temperature programme: initial column temperature was set at 60°C, immediately rising by 8°C min^{-1} to 160°C, and held constant for 15 min for VFA and 10 min for non-volatile fatty acids (NVFA). The total time for chromatographic separation and integration of each sample was 30 min, (and 25 min for NVFA), followed by a 4 min cooling period prior to injection of next sample. Attenuation for peak area integration was varied between 4 - 8 x 10^2 but held constant for each batch of standards and test samples.

3.2.3 Preparation of VFA samples

3.2.3.1 Extraction, elution profiles and calibration curves for VFA

A standard aqueous mixture containing each of the following (at a final concentration of 50µmol ml^{-1}, and 100µmol ml^{-1} for acetic acid) was prepared: alcohols (methanol, ethanol, isobutanol, n-propanol, isopentanol, n-butanol, pentanol) and VFA (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, iso-caproic, caproic). Samples (2.5ml) of the standards mixture, individual fatty acid, or culture supernatants were placed in glass tubes with ground glass stoppers (13mm x 100mm) and acidified with 0.5M H_2SO_4 (0.4ml) to give a pH of 2.0 or less. Diethyl ether (1.0ml) was then added to the free acids, the sample inverted (40 - 45 times) and the ether layer removed with a Pasteur pipette and dried over anhydrous sodium

sulphate. After 10 min, the ethereal extract was transferred to brown glass vials (2ml size; Pye-Unicam Ltd) with air-tight stoppers, and stored at $4^{o}C$ until required. Suitable volumes (3 - 10µl) were injected onto the column using a gas-tight syringe. Duplicate extractions of standards were co-chromatographed with each batch of test samples analysed.

A typical elution profile of alcohols and VFA is shown in Fig. 3.1 and retention times in Table 3.1. Repeated extractions of a single solution of standards gave acceptable and reproducible data as typified by Table 3.1. Extraction reproducibility in culture samples was assumed to be similar.

In both standard and test samples, methanol concentrations could only be determined accurately when $\geqslant$ 30µmol ml^{-1}, and consequently could not be assayed in culture supernatants when below this detection limit.

Calibration curves for alcohols and VFA are shown in Figs. 3.2 and 3.3 respectively. Linear correlations between concentration and peak area were observed for each standard, except ethanol and propanol (Fig. 3.2.B). This reflects extraction or chromatographic variations.

3.2.3.2 Use of internal marker for quantitative analyses

The use of an internal standard is necessary to compensate for variations in column temperature, gas flow rates, detector sensitivity, sample preparation, and injection volume, since both sample and the internal standard are co-chromatographed and thus are influenced by these factors to the same extent.

The absence of butanol in any extracts of culture supernatants suggested that it would be suitable as an internal marker (1M) for quantitative analyses.

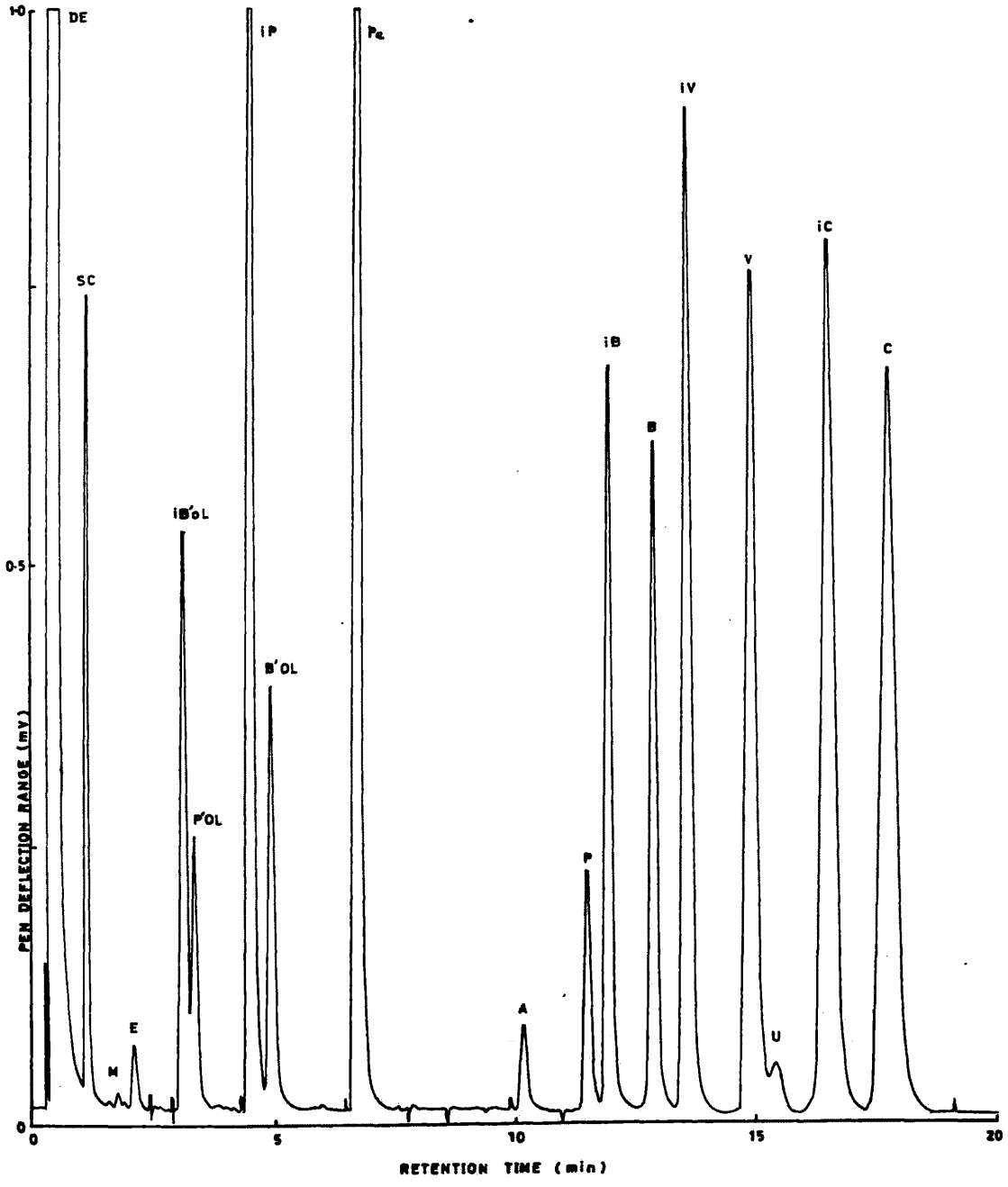


Fig. 3.1 Chromatographic separation of a standard mixture of alcohols and VFA

Each standard was prepared at 20 μmol ml^{-1}, except acetic acid at 40 μmol ml^{-1}. Extraction and assay procedures are described in Section 3.2.3.1 (1 μl injection; attenuation 4 x 10^{2}). Peak symbols: DE, diethyl ether; M, methanol; E, ethanol; i B'ol, isobutanol; P'OL, propanol; iP, isopentanol; B'OL, butanol; Pe, pentanol; A, acetic; P, propionic; iB, isobutyric; B, butyric; iV, isovaleric; V, valeric; U, unknown component, iC, isocaproic; C, caproic.

Table 3.1 Retention times and reproducibility of ether extractions from aqueous mixtures of alcohol and volatile fatty acid standards

Standard[a]	Retention time (min)	Peak area[b] $\bar{x}$ ± S.D. (%)
Methanol	2.00	0.661 ± 0.035 (5.3)
Ethanol	2.30	1.461 ± 0.031 (2.4)
Isobutanol	3.35	15.770 ± 0.350 (2.2)
n-Propanol	3.57	1.601 ± 0.040 (2.9)
Isopentanol	4.75	39.363 ± 1.220 (3.1)
n-Butanol	5.18	26.927 ± 0.754 (2.8)
Pentanol	6.95	44.950 ± 1.708 (3.8)
Acetic	10.40	6.993 ± 0.195 (2.8)
Propionic	11.75	17.258 ± 0.704 (4.1)
Isobutyric	12.18	44.885 ± 2.039 (4.5)
Butyric	13.12	43.867 ± 2.100 (4.8)
Isovaleric	13.82	70.785 ± 3.511 (5.0)
Valeric	15.17	68.652 ± 3.571 (5.2)
Isocaproic	16.77	90.227 ± 5.400 (6.0)
Caproic	18.02	87.535 ± 5.404 (6.2)

[a] Alcohols and VFA standards were prepared separately at a final concentration of 50µmol ml^{-1} (100µmol ml^{-1} for acetic acid). Each was extracted (n = 3 for alcohols and n = 4 for VFA; 1µl injections) as described in Section 3.2.3.1.

[b] Peak area in µvolt sec^{-1} integrator units (x 10^4); attenuation at 4 x 10^2; S.D. is also given as a % of $\bar{x}$ in parenthesis.

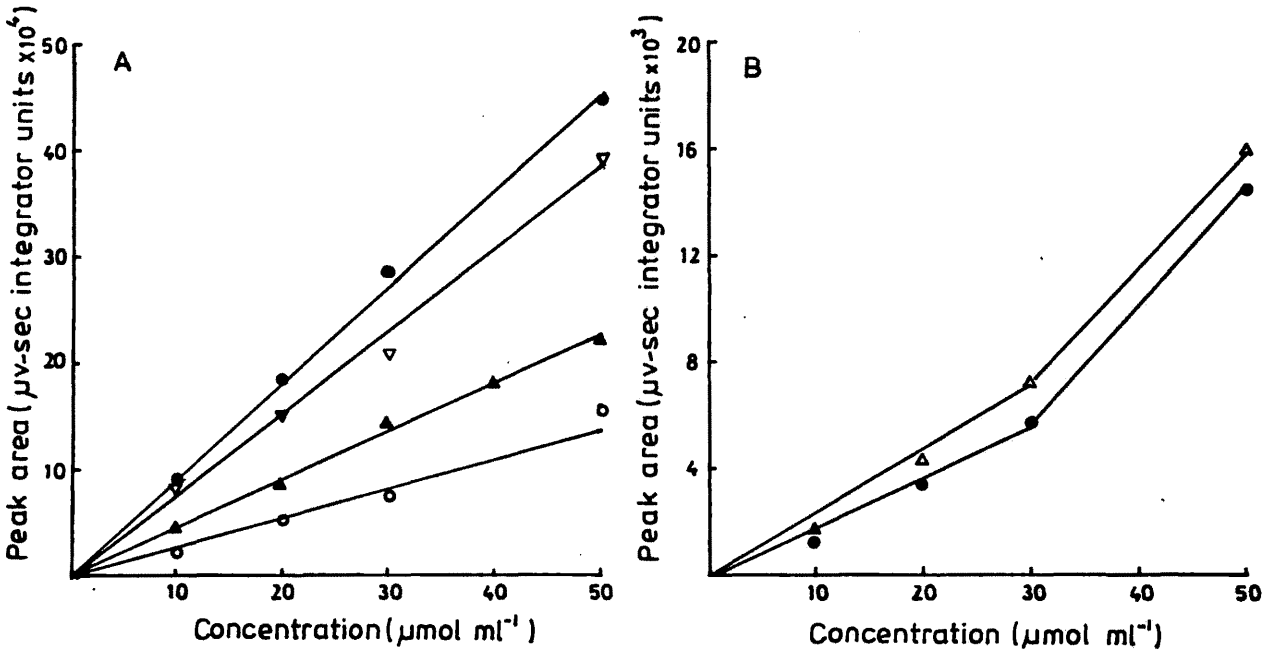


Fig 3.2 Calibration curves (peak area versus concentration) of alcohol standards

Assay conditions and preparative details are given in Sections 3.2.2 and 3.2.3 respectively; 1 ul of the ether extract was injected (integrator attenuation 4 x 10^2). Part A: (o) iso-butanol, (▼) isopentanol, (▲) n-butanol, (●) n-pentanol. Part B: (●) ethanol, (▲) propanol.

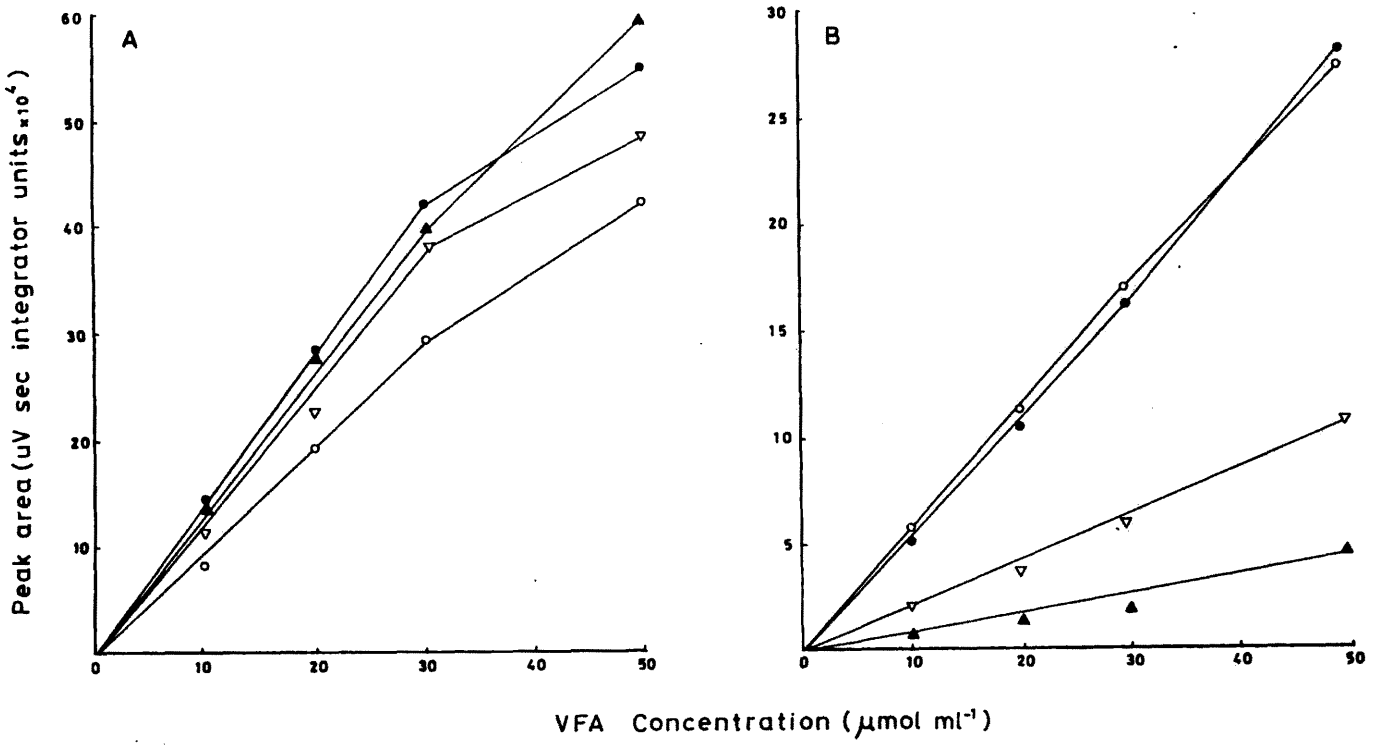


Fig 3.3 Correlation curves of peak area versus variable concentrations of volatile fatty acids

Assay conditions and preparative details are given in Sections 3.2.2 and 3.2.3 respectively; 1 μl of the ether extract was injected (integrator attenuation 4 x 10^{-2}). Concentrations of acetic acid are x2 the values indicated (from 20-100 μmol ml^{-1}). Part A: (○) isovaleric acid, (▽) valeric acid, (●) isocaproic acid, (▲) caproic acid; Part B: (▲) acetic acid, (▽) propionic acid, (●) isobutyric acid, (○) butyric acid.

A suitable aliquot (0.1ml) of aqueous butanol (0.625M) was added to each test sample (2.4ml) prior to extraction, and a comparison of the ratios of peak areas from standard (S) and test (T) samples enabled the concentration of each fermentation product (FP) to be calculated:

$$\text{Concn. FP} = \frac{\text{area FP}_T}{\text{area IM}_T} \times \frac{\text{area IM}_S}{\text{area FP}_S} \times \frac{\text{concn. IM}_T}{\text{concn. IM}_S} \times \text{concn. FP}_S \times \text{diln.}$$

This calculation is applicable when FP_S exhibits a linear calibration curve.

3.2.3.4 Enzymic determination of formic acid

Formate was estimated using formate dehydrogenase (EC 1.2.1.2; FDH) and change in A_{340} according to the following equation:

$$\text{formate} + NAD^+ \xrightarrow{FDH} CO_2 + NADH + H^+$$

Assays were carried out as described by Schutte et al., (1976)., and in Product Information for FDH (The Boehringer Corporation, London).

3.2.4 Non-volatile fatty acids

3.2.4.1 Comparison of methylation procedures

Methylation of non-volatile fatty acids was examined using an acid-methanol procedure catalyzed by boron trifluoride, obtained commercially as a BF_3-methanol (BF_3-MeOH) reagent. Five alternate conditions were tested experimentally in order to find the most efficient methylation reaction (Table 3.2). Based on its simplicity and on its high recovery of lactate (Table 3.2), method E was adopted routinely. Lactic acid added to peptone-yeast basal medium at various concentrations (10 - 50μmol ml^{-1}) was estimated reproducibly, with 91 - 96% recoveries (data not shown; n=5).

3.2.4.2 Elution profiles and standard calibration curves

Standard NVFA were initially chromatographed in order to obtain retention times and to assess any by-products of the

Table 3.2 Comparison of NVFA methylation conditions

NVFA	Methylation conditions[b]				
	A	B	C	D	E
Pyruvic	0.928[a]	4.912	5.661	5.586	6.303
Lactic	0.570	2.385	2.973	3.272	4.477
Oxalic	0.113	1.610	2.441	2.690	2.696
Malonic	0.231	5.888	10.660	14.490	13.800
Fumaric	0.342	0.922	2.135	2.613	2.349
Succinic	0.630	16.965	26.670	33.200	31.955

[a] Areas (mean of 2 determinations) in μvolt sec^{-1} integrator units ($\times 10^4$)

[b] Methylation conditions: in all cases a standards mixture (1.0 ml) prepared as described in Section 3.2.4.2 (final concentration 100μmol ml^{-1} except fumaric and succinic acids at 50μmol ml^{-1}) was used:

A Standards + 50% H_2SO_4 (0.4 ml) + MeOH (2 ml), heated 60°C for 30 min, extracted by inverting 40 - 45 times with H_2O (1.0 ml) and $CHCl_3$ (0.5 ml); $CHCL_3$ layer drawn off and injected (2μl).

B Standards + 50% H_2SO_4 (0.4 ml) + BF_3-MeOH (1.0 ml), heated 60°C for 30 min, extracted as in A.

C Standards + 2 drops concentration H_2SO_4 + BF_3-MeOH (1.0 ml), heated 60°C for 30 min, extracted as in A.

D Standards + 2 drops concentration H_2SO_4 + BF_3-MeOH (1.0 ml), left overnight at room temperature, extracted as in A.

E As for D, except H_2O omitted during extraction.

the methylation reaction. A by-product of pyruvic acid corresponding to the methyl-ester of oxaloacetic acid (retention time 9.38 min) was found, but did not alter the reproducibility of the main peak (Table 3.3). The following NVFA were used for standards: pyruvic, lactic, oxalic, malonic, succinic (at 100μmol^{-1}) and fumaric (50μmol^{-1} due to its relatively lower solubility). After derivatization and extraction an aliquot of the $CHCl_3$ layer (2µl) was chromatographed (Fig. 3.4). The calibration curves indicated linearity between peak area and the concentration of NVFA used (Fig. 3.5). Derivatization and extraction reproducibility using a single preparation (Table 3.3) was acceptable; between sample reproducibility was < 6%.

3.2.4.3 Internal marker for quantitative analyses of NVFA

Procedures similar to those employed for quantitating VFA (Section 3.2.3.2) were used to select malonic acid as the internal marker; an aqueous stock solution of malonic acid (0.5M; 0.1 ml) was added to culture supernatants (0.9 ml) prior to methylation and extraction.

3.3 QUALITATIVE ANALYSES OF GASEOUS FERMENTATION PRODUCTS

Gas chromatography was used to analyse the gases formed during fermentation, namely CO_2, H_2 and methane. The method used in the present study separated various components of a gaseous mixture by elution with N_2 from an absorption column packed with silica gel, and their subsequent detection with a katharometer.

3.3.1 Gas chromatograph

A Pye-Unicam chromatograph (Model 104) equipped with a katharometer was used.

Table 3.3 Retention times and reproducibility of methylation and chloroform extraction of aqueous NVFA standards

Standard[a] NVFA	Retention time (min)	Peak area[b] $\bar{x}$ ± S.D. (%)
Pyruvic	6.98	5.773 ± 1.780 (3.1)
Lactic	8.33	4.626 ± 0.541 (1.2)
Oxalic	9.70	2.504 ± 0.320 (1.3)
Malonic	11.27	11.043 ± 6.027 (5.5)
Fumaric	11.95	13.990 ± 0.227 (2.0)
Succinic	12.55	28.723 ±10.113 (3.5)

[a] Standard NVFA were prepared at concentrations of 100µmol ml^{-1}, or 50µmol ml^{-1} for fumaric and succinic acids. Methylation and extraction procedures (n = 4) are described in Section 3.2.4.2 (1µl injections).

[b] Peak area in µvolt sec^{-1} integrator units (x 10^4); attenuation 4 x 10^2; S.D. is also given as a % of $\bar{x}$ in parenthesis.

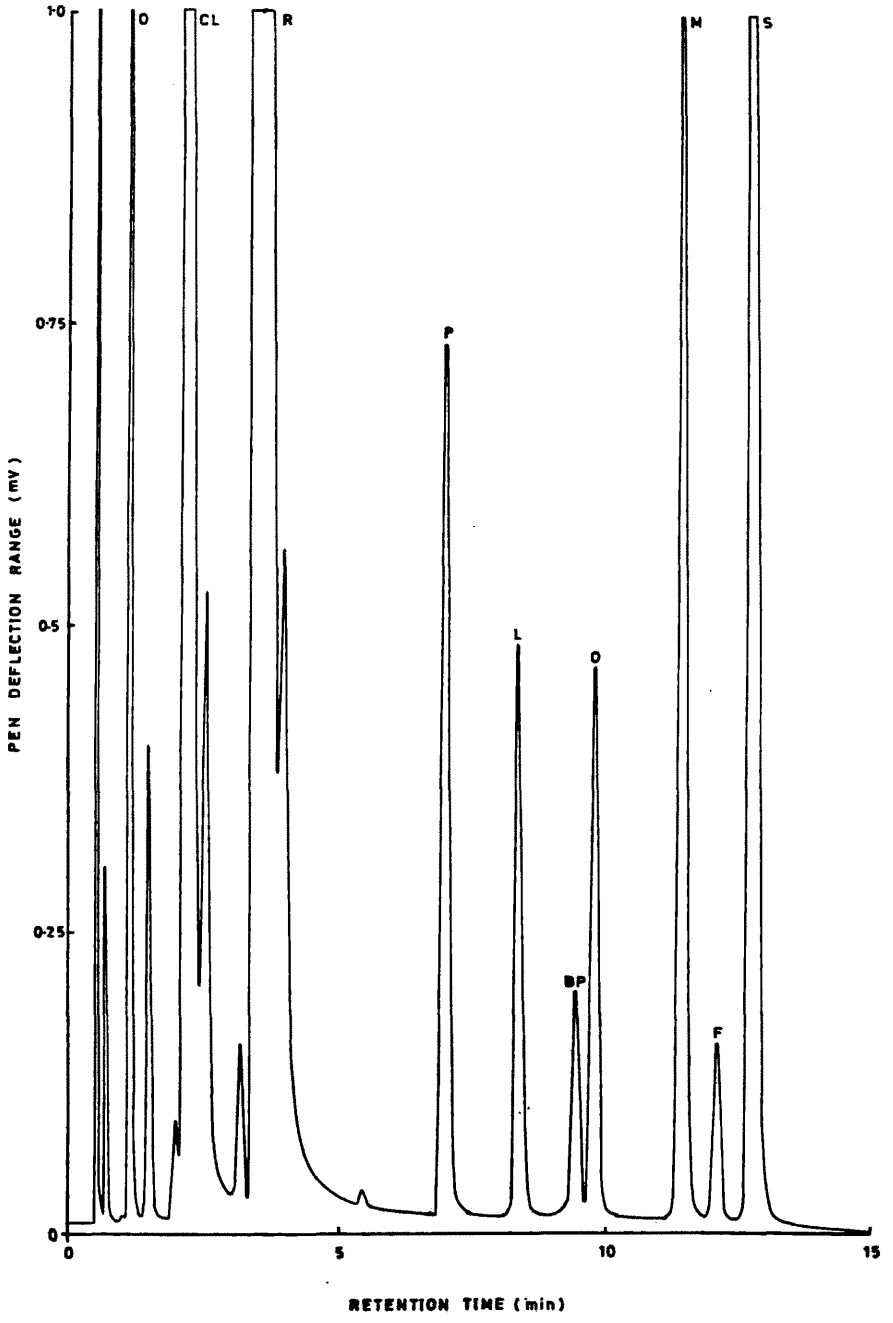


Fig 3.4 Elution profiles of methylated NVFA

NVFA (at 100 μmol ml^{-1}) were methylated using a BF_3-MeOH reagent described in Section 3.2.4.2. Peak symbols: R, reagent; CF, chloroform; P, pyruvic; L, lactic; O, oxalic; M, malomic; S, succinic; U, unknown component.

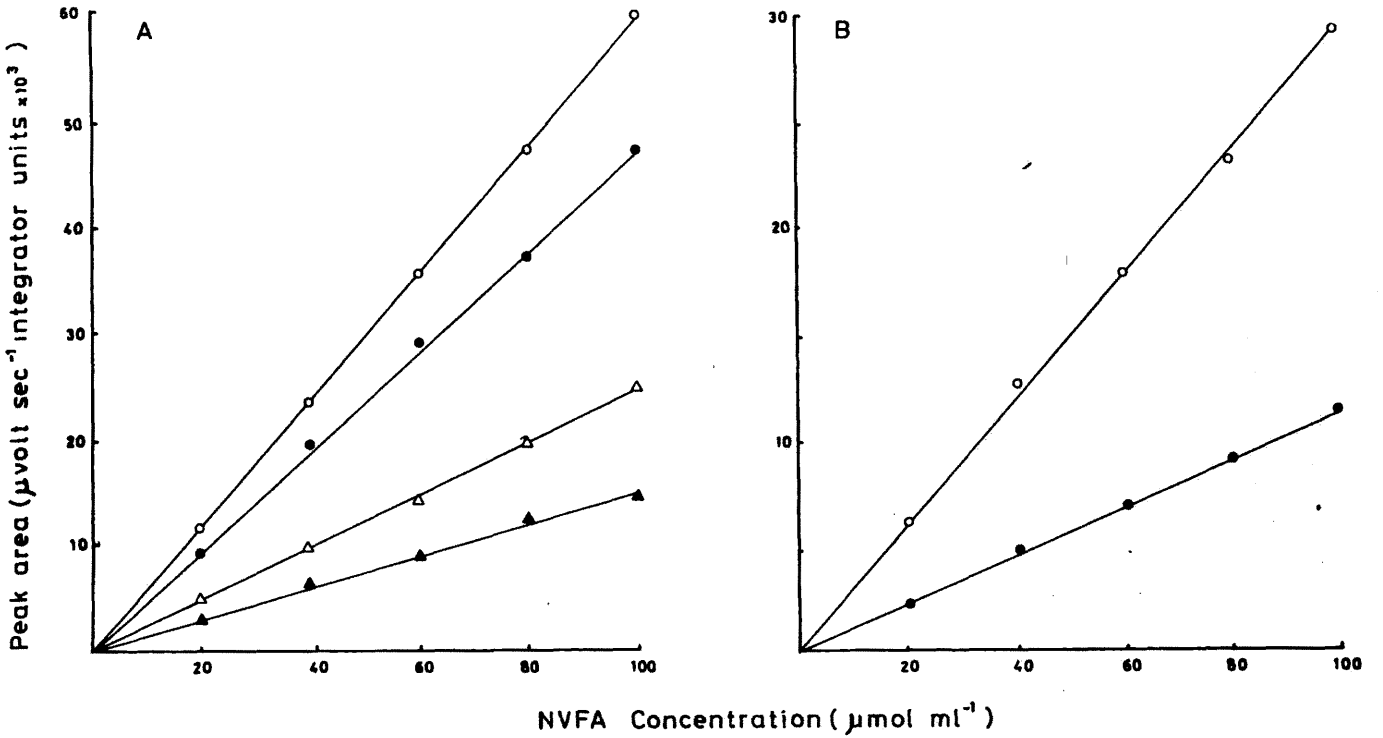


Fig. 3.5 Linear calibration curves (peak area versus concentrations) of methyl esters of NVFA standards

Assay conditions and preparative details are given separately in Section 3.2.2 and 3.2.4.2; 1 μl of the chloroform extract was injected (integrator attenuation 4 x 10^{-2}). Part A: (o) pyruvic acid, (●) lactic acid, (Δ) oxalic acid, (▲) fumaric acid. Part B: (o) succinic acid, (●) malonic acid.

3.3.2 Column and chromatographic conditions

Reference and injection columns, packed with silica gel (80/100 mesh), were purged overnight at 100°C using a flow of nitrogen gas. The following conditions were preset for sample analyses: (i) N_2 carrier gas at a flow rate of 50 ml min^{-1}; (ii) injection port temperature, 50°C; (iii) detector temperature, 50°C; (iv) column temperature, 50°C; (v) bridge current, 95 mA. A gas tight syringe with a maximum volume capacity of 1.0 ml (Precision Sampling Corp., L.A.70815, U.S.A.) was used to inject gas samples.

3.3.3 Calibration gas standards

A mixture of gases comprising CH_4 (1.05%, by vol.), C_2H_6 (0.96%), C_3H_8 (1.52%), n-C_4H_{10} (0.86%) in N_2 was flushed through a gas tight vial for approximately 15 min. (Hypo-vials, Pierce Chemical Co., IL.61105, U.S.A.) containing 10 ml distilled water. Other standards, H_2 (10%) in N_2 and CO_2 (10%) in N_2, and laboratory tap-gas (CH_4 and H_2) were collected using the same procedure. Samples (1.0 ml) were removed from the head space for chromatographic analyses; representative standard chromatograms showing retention time and elution profile are depicted in Fig. 3.6.

3.3.4 Experimental samples

Basal medium (± 40 mM lactulose; 9.5 ml) was transferred aseptically to sterile gas vials, which were then sealed and gassed with N_2. Strict aseptic techniques were used at all stages. An inoculum of a test culture (0.5 ml), prepared as described in Section 2.2.3, was injected into the vial, and incubated at 37°C for the required period. Fermentation gases were collected from the head space (1.0 ml) and chromatographed as described above (Section 3.3.2).

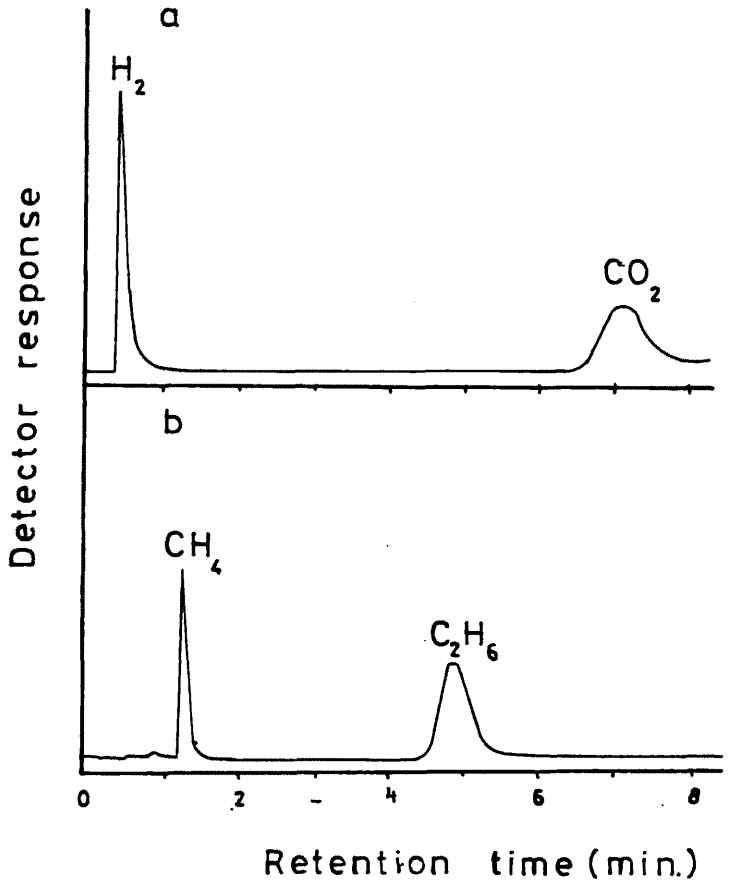


Fig. 3.6 Elution profile of standard mixtures of gases

Gas mixtures (a) and (b) were prepared in gas-tight vials (Section 3.3.3) and 1.0 ml samples injected (attenuation x 50). Retention time (min): H_2 (0.5), CO_2 (7.1), CH_4 (1.3), C_2H_6 (4.9).

CHAPTER 4: THE *IN-VITRO* FERMENTATION OF LACTULOSE BY COLONIC BACTERIA

4.1 INTRODUCTION

A study of lactulose fermentation by intestinal bacteria is of importance in understanding its mode of action in clinical use, and of interest in characterising the carbohydrate fermentation pathways operative in these adaptive microbes.

The only previous report in the literature of a screen for lactulose metabolising bacteria was by Hoffman et al., (1964). Sugar-free brain-heart infusion broth containing bromocresol-purple pH indicator and lactulose to a final concentration of 1% (w/v) was used. Results from the latter study are shown in Tables 4.1 and 4.2. In cultures where the indicator showed acid pH, fermentation of lactulose was assumed, and lactic acid shown to be invariably produced (Table 4.2). Bifidobacterium bifidum (Lactobacillus bifidus), L. acidophilus, Clostridium perfringens (C. welchii) and Streptococci (Lancefield group D) readily fermented lactulose (Table 4.1), whereas weak metabolism was shown by E. coli and Staph. aureus, yielding negligible quantities of lactic acid (Table 4.2). In addition to acidic end-products, C. perfringens produced gas vigourously. In the case of Bacteroides (Ristella), Proteus, Salmonella, and Shigella strains, no acid formation was detected in the lactulose medium. Gas chromatography indicated that, apart from small amounts of lactic acid, o.4 - 1.2 mg formic acid and 2.0 - 3.1 mg acetic acid per culture were produced under the described experimental conditions by four E. coli strains. Gas chromatogric analyses were limited to these stains, and not performed with other organisms.

In a different type of study, Ruttloff et al., (1967) examined the disaccharidase activity in cell-free lysates from E.coli, Bifid. bifidum &undefined Bacteroides spp. Each extract contained a β-galactosidase capable of hydrolysing lactulose and lactose. This enzyme was absent in extracts from P. mirabilis and S. faecalis.

Table 4.1 Lactulose metabolism by intestinal bacertia (data reproduced from Hoffman et al., 1964)

Organism[a]	No. of Strains	Period of Incubation (Days)[b]							
		1	2	3	4	5	6	7	8
Bifid.bifidum (adult)	7	-	±	+	+	+	+	+	+
Bifid.bifidum (child)	3	-	±	+	+	+	+	+	+
L.acidophilus, Type I	3	-	-	±	±	+	+	+	+
L. acidophilus, Type II	1	-	-	-	-	-	±	+	+
Streptococci (Group D)	7	+	+	+	+	+	+	+	+
E. coli	5	-	-	±G	±G	±G	±G	±G	±G
Salmonella	5	-	-	-	-	-	-	-	-
Shigella sonei	1	-	-	-	-	-	-	-	-
Proteus	2	-	-	-	-	-	-	-	-
Bacteroides	10	-	-	-	-	-	-	-	-
C. perfringens	3	+G	+G	+G	+G	+G	+G	+G	+G
Staph. aureus	6	-	±	±	±	±	±	±	±

[a] Bifid.bifidum was quoted as L.bifidus; Bacteroides as Ristella; C.perfringens as C.welchii. Abbreviations of genera are as in text.

[b] Lactulose concentration 1% (w/v) and anaerobic incubations used. Notation as follows: + = clear indicator change to acid; ± = weak indicator change to acid; - = no change.

Table 4.2 Formation of lactic acid by intestinal bacteria following growth on a lactulose-containing medium (Hoffman et al., 1964)

Organism	Lactic acid (mg) [a] formed/culture
Bacteroides	0
Bifid.bifidum	2.7 - 3.4
L. acidophilus	3.3
E. coli	0 - 0.8
Streptococci (Group D)	2.0 - 7.0
C. perfringens	2.0
Staph. aureus	0.3 - 0.5

[a] Lactic acid values were corrected for amounts in uninoculated medium and range of values obtained from different strains. The configuration of lactic acid was not determined.

Lactulose metabolism and a intracellular β-galactosidase were also observed in Bacillus megaterium (Leuchtenberger and Ruttloff, 1978).

A systematic and quantitative approach to the study of lactulose utilisation by colonic bacteria is therefore lacking. Consequently, the aim of this section of the present study was to establish an improved screening procedure. This study differs from previous work (Hoffman et al., 1964; Ruttloff et al., 1967) in several respects: a) virtually all the bacterial isolates examined were identified at the species level; b) the extent of bacterial growth and pH changes were defined; c) lactulose utilisation was estimated using a specific assay; d) major non-gaseous fermentation products were quantified and the gases produced identified in selected strains; and, e) β-galactosidase activity in control and lactulose grown cells was assayed. The concentration of lactulose used (40 mM cf. about 30 mM used by Hoffman et al., 1964) represents an estimated amount typically achieved in the colon during oral therapy (Vince et al., 1978). The data obtained is indicative of the terminal reactions of fermentation, and its usefulness in identifying the central pathway of fermentation is briefly discussed.

4.2 MATERIALS AND METHODS

Materials and general methods were described previously (Chapters 2 and 3). Bacterial growth and fermentation metabolites were assessed after a 48 h incubation using basal medium ± 40 mM lactulose (15 ml) as described in Sections 2.2.3 and Chapter 3. The growth experiments were duplicated. Data is from one experiment.

4.2.1 Faecal homogenate samples

Faecal homogenate samples were kindly provided by Dr. A. Vince, The Rayne Institute, University College Hospital Medical School,

London. Samples had been incubated anaerobically for 48 h at 37°C with 30 mM or 90 mM lactulose respectively, as described elsewhere (Vince et al., 1978). After centrifugation at 47500 g for 90 min at 4°C, the supernatants were removed and analysed for fermentation products by standardised techniques (Chapter 3).

4.3 RESULTS AND DISCUSSION

Changes in growth parameters (A_{612}, pH, β-GLase, fermentation products) due to sugar fermentation were assessed by inoculating control and lactulose containing media. Lactulose utilisation generally increased turbidity at A_{612}, with a fall in culture pH to between 4.0 - 6.0, and β-GLase activity indicated induction in the absence of corresponding activity in basal medium. Less than 5% lactulose uptake was regarded as not significant. Disaccharide transport occurred without extracellular hydrolysis, as constituent monosaccharides were generally not detected in culture supernatants. The two exceptions (Table 4.10) were B.vulgatus (S1) and B.distasonis (S1), probably due to excretion of galactose and fructose. Monosaccharide excretion by streptococci has been observed during metabolism of lactose (Thompson, 1978). Differences in non-gaseous fermentation products from control and lactulose grown cultures were largely quantitative.

Representative species of each of the following important genera were examined: eubacteria, bacteroides, bifidobacteria (all approximately 10^{10} per g faeces; Drasar and Hill, 1974), streptococci, enterobacteria (both approximately 10^{8} per g faeces), and lactobacilli and clostridia (10^{5} - 10^{6} per g faeces).

4.3.1 Facultative anaerobes

Collectively, the ability of each genus to degrade lactulose was in the following order (Table 4.3): lactobacilli > streptococci > staphylococcus ≃ enterobacteria.

Table 4.3 Anaerobic cultivation of facultative anaerobes on lactulose

Organism[a]	Basal Medium		Basal Plus Lactulose			
	Growth[b]	pH	Growth	pH	% sugar used	Gas[c]
Lactobacillus sp.[d] (S1)	0.22	5.9	1.32	4.0	47.4	-
Lactobacillus sp. (S2)	0.37	5.9	1.93	4.0	36.1	-
Lactobacillus sp. (S3)	0.64	5.9	1.87	4.7	26.3	-
Lactobacillus sp. (S4)	0.60	5.9	1.60	4.0	21.1	-
L.casei sub casei	0.21	5.7	1.02	4.0	33.5	-
L.acidophilus	0.08	6.5	0.88	4.4	13.1	-
L.brevis	0.12	6.2	0.95	4.4	13.2	-
L.buchneri	0.11	6.7	0.12	6.7	<5.0	-
Staph.aurens	0.13	5.9	0.45	5.0	14.9	-
Streptococcus sp.	0.54	5.9	1.51	4.0	8.8	+
S.intermedius (S1)	0.52	5.9	1.33	4.4	6.6	+
S.intermedius (S2)	0.35	6.5	1.12	4.8	14.3	+
S.faecalis[e]	0.51	6.8	1.18	5.4	6.0	+
S.viridans[e]	ND		0.55	5.7	6.0	+
E.coli[e]	0.45	6.1	0.72	5.1	<5.0	+
K.aerogenes[e]	0.41	6.0	1.11	5.6	6.0	-
Ent.aerogenes[e]	0.14	5.5	0.46	6.0	<5.0	+
P.vulgaris[e]	0.71	6.2	1.33	4.8	16.1	2+
P.mirabilis[e]	0.46	6.6	0.69	6.2	<5.0	2+
N.catarrhalis	0.11	6.0	0.18	6.0	<5.0	-

[a] Abbreviations for organisms are as in text, and Ent.aerogenes is Enterobacter aerogenes

[b] 48 h culture growth (± 40 mM lactulose) assayed at A_{612}. Sugar utilised is given as a percentage of original concentration.

[c] Visual determination of gas quantity (+ to 3+) using inverted gas tubes as described in Section 2.2.3

[d] Strains of each species are numbered for reference as S1, S2 etc.

[e] Basal medium used was nutrient broth with 0.05% (w/v) L-cysteine hydrochloride; PY basal medium was employed otherwise; pH of sterile medium was 6.7 - 7.0 in each case.

A range from 13.1 - 47.3% lactulose was utilised by lactobacilli, with marked acidification of the culture pH to 4.0 - 4.7 (Table 4.3). Notable fermentative species were strains of Lactobacillus sp., L.casei sub casei, L.brevis and L.acidophilus. These organisms also ferment lactose efficiently (Holdeman and Moore, 1977). Only L.acidophilus had previously been shown to dissimilate lactulose (Table 4.1; Hoffman et al., 1964). This organism is the prominent lactobacillus species in the gut (Drasar and Hill, 1974). L.buchneri failed to utilise lactulose, and does not metabolise lactose (Holdeman and Moore, 1977). The lactulose utilising species produced lactic acid as the main fermentation metabolite (Table 4.4). Acetic and other acids were generated in trace quantities. The low levels of C_4 - C_5 volatile fatty acids from Lactobacillus sp. strains may represent amino-acid fermentation, since they were also detected in basal medium cultures. Gaseous end-products were not observed (Table 4.4). This pattern of end-products is similar to the homofermentation of glucose by lactobacilli (Wood, 1961). Although fermentation balance calculations indicated anomalies (data not shown), lactulose catabolism appears to be homofermentative in L.casei sub casei, L.acidophilus and four strains of the unidentified Lactobacillus sp. Lactic acid was produced during each phase of growth of a Lactobacillus sp. strain (Fig. 4.1). The continued utilisation of lactulose during a protracted stationary phase (e.g. for maintainence energy) produced lactic acid but no further decrease in culture pH (Fig. 4.1). The heterofermentative L.brevis yielded lactic acid alone (considerable amounts of CO_2, acetic acid and ethanol are produced in addition to lactic acid by heterofermentative lactobacilli grown on glucose; Buchanan and Gibbons, 1974). Homofermentation typically proceeds by the EMP-pathway in lactic acid bacteria (Wood, 1961). The presence of

Table 4.4 Quantitative analyses of non-gaseous products of lactulose fermentation by facultative anaerobes

Organism [a]		Fermentation product (µmol(ml supernatant)$^{-1}$)[c]							
		AC	Prop	iBut	But	iVal	Val	Pyr	Lac
Lactobacillus sp.(S1)	BM[b]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.21
	BML	0.90	0.0	0.0	0.0	0.0	0.0	0.0	76.13
Lactobacillus sp.(S2)	BM	0.0	0.0	0.0	0.0	0.01	0.0	0.0	4.34
	BML	0.0	0.0	0.0	0.0	0.03	0.0	0.0	58.24
Lactobacillus sp.(S3)	BM	1.78	0.13	0.0	0.0	0.03	0.02	0.0	5.78
	BML	1.59	0.17	0.0	0.0	0.04	0.0	0.0	51.43
Lactobacillus sp.(S4)	BM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.94
	BML	0.70	0.0	0.0	0.01	0.18	0.21	0.0	48.97
L.casei sub casei	BM	1.76	0.0	0.01	0.0	0.0	0.0	0.0	4.99
	BML	0.0	0.0	0.00	0.0	0.0	0.0	0.0	49.88
L.acidophilus	BM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.22
	BML	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.22
L.brevis	BM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.73
	BML	3.50	0.0	0.0	0.0	0.0	0.0	0.0	27.22
Staph.aureus	BM	ND							
	BML	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.81
Streptococcus sp.[d]	BM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.47
	BML	0.0	0.0	0.0	0.0	0.30	0.51	0.0	23.56
S.intermedius (S1)	BM	5.85	0.0	0.0	0.29	0.0	0.0	0.09	4.07
	BML	5.83	0.0	0.0	0.0	0.0	0.0	0.12	23.65
S.intermedius (S2)	BM	0.90	0.0	0.0	0.0	0.02	0.01	0.0	4.36
	BML	2.10	0.0	0.0	0.0	0.01	0.01	0.0	17.63
P.vulgaris[e]	BM	8.42	0.0	0.0	0.0	0.0	0.0	0.0	3.34
	BML	12.90	0.0	0.0	0.0	0.0	0.0	0.0	10.90

[a] Growth conditions described in Table 4.3. Culture supernatants were extracted and assayed as described previously (Section 3.2).

[b] BM = basal medium; BML = basal medium with 40 mM lactulose.

[c] AC = acetic acid; Prop = propionic acid; iBut = iso-butyric acid; But = butyric acid; iVal = iso-valeric acid; Val = valeric acid; Lac = lactic acid.

[d] Fermentation products of S.faecalis and S.viridans not determined.

[e] Basal medium was nutrient broth (see Table 4.3)

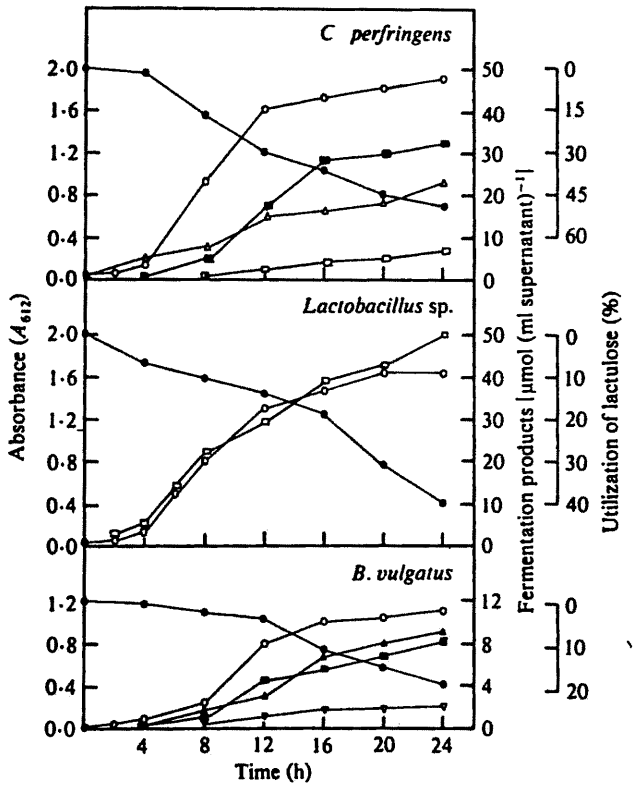


Fig. 4.1 Growth of selected organisms on 40 mM-lactulose.

Absorbance (○), utilisation of lactulose (●), concentrations of fermentation products - lactic acid (□), acetic acid (■), butyric acid (△), succinic acid (▲), propionic acid (▽). pH not shown.

this scheme for lactulose fermentation requires investigation.

β-Glase activity was absent in lactobacilli grown on basal medium or lactulose (Table 4.6). Enzyme lability is unlikely, as 48h lactose-grown cells of unidentified Lactobacillus spp. hydrolysed p-nitrophenyl-β-D-galactoside (PNPG: Hawksworth et al., 1971). In addition, ONPG is a more sensitive assay substrate than PNPG (Wallenfels and Malhotra, 1961). A ONPG-cleaving β-Glase, induced by galactose, was reported in L.acidophilus (Kachhy et al., 1977). A possible role for phospho-β-galactosidase (P-β-Glase) hydrolysis of translocated lactulose is implicated in lactobacilli in the present study, analagous to the report of a P-β-Glase in L.casei grown on lactulose (Chassy and Thompson, 1983a).

Of the four species of streptococci examined, S.faecalis predominates in the gut (Helferich and Westhoff, 1980). Except for S.intermedius (S2), utilising 14.3%, the amount of lactulose catabolised by other strains was comparable (6.0 - 8.8%), with significant increases in turbidity at A_{612} (Table 4.3). A variable decrease in pH from 4.0 - 5.7 reflected the different buffering capacities of the PY and mutrient broth media. Unspecified streptococci of the Lancefield group D were previously found to ferment lactulose (Hoffman et al., 1964). Lactulose was fermented predominantly to lactic acid, with variable amounts of acetic acid and trace C_4 - C_5 volatile acids (Table 4.4). Gas produced by S.faecalis and S.viridans during growth on lactulose was found to be CO_2 (Table 4.5). This pattern of end-products is typical of heterofermentative streptococci, which yield L(-) lactic acid, CO_2, ethanol and/or acetic acid from glucose fermentation by the hexose monophosphosphate pathway (Buchanan and Gibbons, 1974). However, a quantitative analysis of gaseous products in a separate study would determine the significance of CO_2 as a lactulose fermentation metabolite. Since homolactic streptococci form other products in major amounts

Table 4.5 Qualitative analyses of gaseous end-products of lactulose fermentation by selected facultative anaerobes

Organism[a]	A_{612}	pH	Gas produced[b]
S.faecalis	0.73	5.5	CO_2
S.viridans	0.55	5.7	CO_2
P.vulgaris	1.36	5.0	$H_2 + CO_2$

a Each strain grown for 48 h on nutrient broth plus 40 mM lactulose using the gas-vial procedure described in Section 3.3.4. Gas was not detected in corresponding controls.

b Analytical procedures were described in Section 3.3

Table 4.6 β-Galactosidase assays in selected facultative anaerobes grown on glucose or lactulose

Organism[a]	Enzyme activity units (x 10^{-1})[b]		
	Basal Medium	Basal with Glucose	Basal with Lactulose
L.casei sub casei	0.0	ND	0.0
L.acidophilus	0.0	ND	0.0
L.brevis	0.0	ND	0.0
L.buchneri	0.0	ND	0.0
S.intermedius	0.0	ND	0.0
S.faecalis	ND	ND	0.0
E.coli	T	0.0	4.4
K.aerogenes	0.0	0.0	3.1
Ent.aerogenes	0.0	0.0	0.9
P.vulgaris	5.5	0.3	2.5
P.mirabilis	0.0	ND	0.0

[a] Growth conditions as described in Table 4.3. Sugar concentration was 40 mM respectively.

[b] Enzyme activity expressed as µmol o-nitrophenyl released ml^{-1} $A_{612}{}^{-1}$ units (T < 0.01 units). Lysis and assay procedures described in Section 2.2.5

under certain conditions (including CO_2 by S.liquefaciens; Wood, 1961). The relative importance of the EMP and hexose monophosphate as central pathways in metabolising lactulose requires further study.

The absence of a ONPG hydrolysing β-galactosidase in this investigation (Table 4.6) and the inability of cell-free extracts of S.faecalis to degrade lactose or lactulose (Ruttloff et al., 1967) infer disaccharide hydrolysis by P-β-GLase. Intracellular lactose 6-phosphate, accumulated by the PEP:PTS, is cleaved by an inducible P-β-GLase to release galactose 6-phosphate and glucose in group N streptococci (Romano et al., 1979; Thomas et al., 1980).

About 14% of the available lactulose was utilised by Staph.aureus (Table 4.3). Lactic acid was the only fermentation product found (Table 4.4). The lactose PEP:PTS model is well established for Staph.aureus (Postma and Roseman, 1976) and provides a suitable hypothesis for future investigations of lactulose metabolism in this organism.

Of the five species of enterobacteria, P.vulgaris utilised the larger amount of lactulose, compared to K.aerogenes (Table 4.3). P.mirabilis and other enterobacteria did not utilise lactulose, as assessed by the assay procedure used. Hoffman et al., (1964) reported two unspecified Proteus strains which did not metabolise the disaccharide. Comparable amounts of lactic and acetic acids were produced by P.vulgaris (Table 4.4), and H_2 and CO_2 (Table 4.5) were also noted. Enterobacteria display mixed acid fermentations, with lactic and acetic acids, and ethanol, CO_2 and H_2 as major products from glucose catabolism (Wood, 1961). This pattern was similar to that shown by P.vulgaris above. At about pH 6, a hydrogenlyase yields CO_2 and H_2 from formic acid in E.coli (Wood, 1961). Other minor products, acetoin, 2,3-butanediol and glycerol were not assayed in the present study.

Relatively high levels of β-GLase activity were detected in P.vulgaris cells grown on basal medium or lactulose (Table 4.6). Growth on glucose repressed the β-GLase, but incompletely; the mechanism of this effect requires elucidation. β-GLase in lactulose grown cells of E.coli, K.aerogenes and Ent.aerogenes indicated a low degree of lactulose uptake. For E.coli and Ent.aerogenes, this was below the detection limit of Vachek's procedure (Section 2.2.4). It is of interest to examine in another study the spectrum of substrates hydrolysed by enteric β-GLase, as a partially purified β-GLase from Klebsiella sp. (Hall, 1980) possessed similar catalytic properties towards ONPG (K_m, 32 mM), lactose (K_m, 35) and lactulose (K_m, 39). Lactulose uptake is probably by active transport. Sandermann (1977) observed comparable amounts of permease binding inhibition by lactose and lactulose by assaying facilitated diffusion of ONPG into intact E.coli cells.

4.3.2 Obligate anaerobes

4.3.2.1 Gram-positive anaerobes

Several clostridia were screened for lactuloytic activity (Table 4.7). C.perfringens strains utilised lactulose extensively (33.6 - 58.2%), and C.butyricum isolates catabolised between 7 - 19% of the available sugar. C.difficile, C.cadaveris and C.paraputrificum strains did not metabolise lactulose. The proficiency of C.perfringens in fermenting lactulose has been noted previously (Hoffman et al., 1964) but other species of the genus were not examined. The predominant end-products of lactulose fermentation by clostridia were acetic, butyric and lactic acids (Table 4.8). The gases produced were identified in C.perfringens as CO_2 and H_2 (data not shown). This end-product pattern resembles clostridial butyric type fermentation, which yield butyric and acetic acids, CO_2 and H_2 (Wood, 1961). C.perfringens,

Table 4.7[a] Growth of gram-positive obligate anaerobes on lactulose

Organism	Basal medium		Basal with lactulose			
	Growth	pH	Growth	pH	% sugar used	Gas prodn.
C. perfringens (S1)	0.45	6.5	1.45	4.9	58.2	3+
C. perfringens (S2)	0.42	6.6	1.68	4.8	52.2	3+
C. perfringens (S3)	0.67	6.7	1.75	4.8	49.2	3+
C. perfringens (S4)	0.40	6.7	1.53	4.8	46.3	3+
C. perfringens (S5)	0.26	6.6	1.02	4.9	38.5	3+
C. perfringens (S6)	0.34	6.5	1.60	4.5	33.9	3+
C. perfringens (S7)	0.37	6.3	1.22	4.8	33.6	3+
Bifid.bifidum	0.50	5.7	1.20	4.8	30.5	-
C. butyricum (S1)	0.17	6.4	1.01	5.1	19.0	2+
C. butyricum (S2)	0.16	6.3	1.02	4.8	12.2	2+
C. butyricum (S3)	0.46	6.4	1.01	5.0	7.5	2+
C. difficile	0.40	6.6	0.63	6.9	<5.0	2+
C. cadaveris	0.45	6.9	0.42	6.9	<5.0	2+
C. paraputrificum	0.02	6.5	0.27	5.7	<5.0	-
F.nucleatum	0.23	6.7	0.25	6.6	<5.0	-
Pept.anaerobius	0.82	6.7	0.83	6.8	<5.0	2+
Eu.lentum	0.18	6.7	0.18	6.7	<5.0	-
Eu.limosum	0.20	6.7	0.22	6.6	<5.0	-
Prop.acnes	0.48	6.4	0.49	6.7	<5.0	-

[a] Details are as for Table 4.3. Propionibacterium acnes abbreviated as Prop.acnes.

Table 4.8[a] Quantitative analyses of non-gaseous fermentation products from growth of gram-positive obligate anaerobes on lactulose

Organism		Fermentation product (μmol(ml supernatant)$^{-1}$)							
		AC	Prop	iBut	But	Pyr	Lac	Fum	Succ
C. perfringens (S1)	BM	10.64	2.40	0.0	2.76	0.0	0.0	0.88	0.0
	BML	22.29	1.06	0.0	27.64	0.48	0.56	0.30	0.0
C. perfringens (S2)	BM	10.81	2.44	0.0	2.88	0.0	0.0	0.71	0.0
	BML	21.03	0.0	0.0	19.53	0.24	15.43	0.0	0.0
C. perfringens (S3)	BM	11.56	2.97	0.0	1.30	0.0	0.0	1.12	0.0
	BML	23.73	2.01	0.0	28.31	0.28	11.28	0.53	0.0
C. perfringens (S4)	BM	9.90	1.66	0.0	1.45	0.0	0.0	1.59	0.0
	BML	21.73	0.78	0.0	24.24	1.25	14.83	0.0	0.0
C. perfringens (S5)	BM	8.89	2.14	0.0	1.52	0.0	0.0	0.57	0.0
	BML	23.60	1.54	0.0	25.33	0.0	0.0	0.48	0.0
C. perfringens (S6)	BM	12.44	2.71	0.0	1.94	0.0	0.0	1.18	0.0
	BML	17.81	0.0	0.0	15.70	0.35	11.66	0.0	0.0
C. perfringens (S7)	BM	6.25	0.15	0.0	1.92	0.0	0.0	0.0	1.67
	BML	13.53	0.0	0.0	15.35	0.0	3.20	0.0	1.75
C. butyricum (S1)	BM	4.60	0.0	0.0	0.88	0.0	1.50	0.0	0.0
	BML	8.30	0.0	0.0	21.34	0.0	0.0	0.0	0.0
C. butyricum (S2)	BM	6.83	0.0	0.0	0.89	0.0	2.61	0.0	0.0
	BML	10.30	0.0	0.0	2.97	0.0	15.50	0.0	0.0
C. butyricum (S3)	BM	6.01	4.96	0.29	2.60	0.0	0.0	2.16	3.17
	BML	8.78	1.82	0.0	0.54	0.09	10.95	0.40	6.88

[a] Details are as for Table 4.4; Fum = fumaric acid; Succ = succinic acid

in addition, yields lactate and ethanol (Pappenheimer and Shaskan, 1944). However, ethanol was not detected in the present survey. The preferential conversion of acetyl-phosphate to acetate (yielding ATP) instead of reduction to ethanol may be the terminal reaction involved (Stanier et al., 1972), but requires further analysis. Experimental conditions, such as pH of fermentation also influence the type of secondary fermentation observed (Wood, 1961). During the growth of C.perfringens on lactulose (Fig. 4.1), acetic and butyric acids were produced continuously following inoculation, whereas lactic acid excretion was noted during late log-phase. The yield of lactate is increased with iron deficiency in C.perfringens (Pappenheimer and Shaskan, 1944), suggesting that a similar depletion may be responsible for its accumulation in this study.

The use of lysed cells indicated an intracellular β-GLase (Table 4.9); lactulose uptake in C.perfringens and C.butyricum may therefore be via active transport. The comparatively few published reports on carbohydrate transport systems in clostridia have shown the involvement of PEP:PTS in uptake of glucose, mannose and fructose (Groves and Gronlund, 1969a; Patni and Alexander, 1971; Hugo and Gottschalk, 1974) and electrogenic transport in accumulating galactose (Booth and Morris, 1975).

The respective strains of Fusobacterium nucleatum, Eubacterium lentum, Eu.limosum, Propionibacterium acnes and Peptococcus anaerobius were unable to metabolise lactulose (Table 4.7). This result is not surprising in view of the depleted range of carbohydrates, mostly monosaccharides, catabolised by these organisms (Holdeman and Moore, 1977).

Table 4.9[a] Comparison of β-galactosidase activity in obligate anaerobes screened for lactulose utilisation

Organism	Enzyme activity units ($\times 10^{-2}$)	
	Basal medium	Basal + lactulose
C. perfringens (S2)	0.0	1.02
C. perfringens (S4)	0.0	1.12
C. perfringens (S5)	0.0	0.35
C. butyricum (S1)	T	T
C. butyricum (S2)	0.27	0.04
C. butyricum (S3)	0.83	0.23
C. difficile	0.0	0.0
C. cadaveris	0.0	T
F.nucleatum	0.0	0.0
Pept.anaerobius	0.0	0.0
Eu.lentum	0.0	0.0
Eu.limosum	0.0	0.0
Prop.acnes	0.0	0.0
B.oralis	1.21	1.41
B.fragilis (S1)	1.10	ND
B.fragilis (S2)	0.99	3.18
B.fragilis (S3)	0.68	1.17
B.fragilis (S4)	0.24	0.60
B.fragilis (S5)	0.78	1.11
B.vulgatus (S3)	ND	1.68
B.vulgatus (S4)	1.65	1.76
B.distasonis (S1)	4.08	2.35
B.distasonis (S2)	2.52	0.18
B.uniformis (S1)	1.19	1.16
B.uniformis (S2)	1.93	7.15
B.thetaitaomicron	0.98	1.11
B.ovatus	1.69	0.71
B.asaccarolyticus	0.0	0.0

[a] Details are as for Table 4.6

4.3.2.2 Gram-negative anaerobes

B.fragilis, B.vulgatus and B.uniformis are predominant isolates in studies on the faecal flora (Moore and Holdeman, 1974; Moore et al., 1981). These and other bacteroides species utilised varying amounts of lactulose, from 8 - 16.9% (Table 4.10). Hoffman et al., (1964) had previously suggested that bacteroides do not ferment lactulose. Qualitatively similar patterns of fermentation metabolites were produced by bacteroides grown on basal medium or lactulose (Table 4.11). Increases in acetic and fumaric acids, and appearance of lactic acid were observed in lactulose-grown cultures. B.fragilis was found to evolve H_2 during growth on the disaccharide (data not shown). The 24h monitored growth of B.vulgatus (S1) indicated the initial appearance of acetic acid in the medium, followed by both propionic and succinic acids at 8h (Fig. 4.1). Acetic and succinic acids were quantitatively more important. The possible conversion of succinic to propionic acid requires further study. In glucose grown B.fragilis, propionate is produced by decarboxylation of succinate via the intermediates succinyl-coenzyme A (CoA), methylmalonyl-CoA, and propionyl-CoA (Macy and Probst, 1979). Little is known of this pathway in other bacteroides species. In addition to succinate and propionate, other fermentation products normally formed include acetate, butyrate, lactate, and hydrogen (Macy and Probst, 1979). In the latter review, B.fragilis was reported to catabolise sugars to pyruvate by the EMP pathway, whilst the presence of fructose-1,6-diphosphate aldolase activity in B.thetaitaomicron indicates a similar possibility.

Assays for β-GLase in bacteroides indicated an apparently constitutive enzyme (Table 4.9). A hydrolase cleaving both lactose and lactulose was reported by Ruttloff et al., (1967). Active transport for lactulose uptake by bacteroides cells is implicated, and requires further study.

Table 4.10[a]

Growth of gram-negative obligate anaerobes on lactulose

	Basal medium		Basal with lactulose			
	Growth	pH	Growth	pH	% sugar used	Gas Prodn.
B.oralis	0.61	6.7	0.83	5.0	15.8	-
B.fragilis (S1)	0.23	6.5	0.76	5.2	13.5	+
B.fragilis (S2)	0.30	6.5	0.81	5.2	11.0	+
B.fragilis (S3)	0.37	6.5	0.65	5.2	10.3	+
B.fragilis (S4)	0.35	6.5	0.92	5.2	9.7	+
B.fragilis (S5)	0.38	6.5	0.80	5.2	9.0	+
B.vulgatus (S1)	0.32	6.5	1.32	5.0	16.9G/F	+
B.vulgatus (S2)	0.40	6.1	0.92	5.2	10.1	+
B.vulgatus (S3)	0.55	6.6	0.68	5.1	<5.0	+
B.vulgatus (S4)	0.59	6.6	0.72	5.1	<5.0	+
B.distasonis (S1)	0.59	6.6	0.79	5.0	15.7G/F	-
B.distasonis (S2)	0.68	5.9	0.86	5.0	<5.0	-
B.uniformis (S1)	0.39	6.7	0.51	5.4	9.4	+
B.uniformis (S2)	0.22	6.5	0.24	5.6	<5.0	+
B.thetaitaomicron (S1)	0.60	5.5	0.81	4.4	8.0	+
B.thetaitaomicron (S2)	0.94	5.9	1.83	4.9	<5.0	+
B.ovatus	0.55	6.3	0.9	5.3	8.1	+
B.asaccharolyticus	0.50	6.5	0.62	5.4	<5.0	-
V.parvula	0.22	6.7	0.18	6.7	<5.0	-

[a] Details are as for Table 4.3, except G/F indicates the detection of galactose and fructose in the medium

Table 4.11[a]

Quantitative analyses of non-gaseous products of lactulose fermentation by Bacteroides

Organism		Fermentation product (μmol(ml supernatant^{-1}))								
		AC	Prop	iBut	But	iVal	Pyr	Lac	Fum	Succ
B.fragilis (S1)	BM	5.17	4.46	0.0	0.0	1.07	0.0	0.0	1.88	2.52
	BML	8.99	4.52	0.0	0.0	0.93	0.0	5.36	1.67	5.28
B.fragilis (S2)	BM	6.33	5.86	0.0	0.0	1.76	0.0	0.0	2.17	2.83
	BML	8.92	4.01	0.0	0.0	0.97	0.0	7.25	1.78	5.27
B.fragilis (S3)	BM	5.70	5.41	0.0	0.0	0.0	0.0	0.0	1.89	2.82
	BML	9.28	4.74	0.0	0.0	0.0	0.0	5.86	2.00	6.91
B.fragilis (S4)	BM	6.38	5.72	0.26	0.20	1.89	0.0	0.0	0.73	1.55
	BML	15.32	5.73	0.0	0.14	0.91	0.10	5.99	0.34	5.04
B.fragilis (S5)	BM	5.87	5.85	0.29	0.17	2.12	0.0	0.0	0.75	5.27
	BML	12.51	5.09	0.18	0.16	1.38	0.0	3.93	1.05	5.47
B.vulgatus (S1)	BM	6.19	4.20	0.0	1.43	0.0	0.0	0.0	1.53	1.68
	BML	13.37	2.86	0.0	1.14	0.0	0.0	0.0	1.15	17.21
B.vulgatus (S2)	BM	7.23	1.15	0.07	0.16	0.36	0.0	0.0	0.41	3.43
	BML	8.40	3.43	0.18	0.15	1.22	0.0	7.29	0.27	4.49
B.vulgatus (S3)	BM	9.33	6.94	0.25	0.17	1.62	0.0	0.0	0.77	2.81
	BML	8.34	1.49	0.0	0.17	0.17	0.10	5.22	0.54	3.67
B.vulgatus (S4)	BM	8.34	7.17	0.28	0.17	1.81	0.0	0.0	1.02	3.30
	BML	9.04	1.56	0.0	0.15	0.18	0.11	4.63	0.49	4.04
B.distasonis (S1)	BM	8.04	6.76	0.36	0.0	2.26	0.0	0.0	4.14	3.59
	BML	7.48	2.37	0.0	0.29	0.42	0.11	8.86	1.11	6.54
B.distasonis (S2)	BM	3.21	4.72	0.04	0.0	0.59	0.0	0.0	0.51	0.72
	BML	4.32	2.87	0.0	0.0	0.17	0.0	2.8	0.36	1.75
B.uniformis (S1)	BM	4.18	4.67	0.34	0.17	2.15	0.0	0.0	0.54	0.05
	BML	ND								
B.uniformis (S2)	BM	6.27	4.22	0.27	0.12	1.66	0.0	0.0	0.44	0.32
	BML	8.60	1.76	0.10	0.20	0.58	0.0	3.58	1.13	0.68
B.thetaitaomicron (S1)	BM	3.88	3.43	0.04	0.02	0.0	0.0	0.68	0.80	3.99
	BML	0.77	1.07	0.05	0.05	0.55	0.0	0.05	0.30	11.05
B.thetaitaomicron (S2)	BM	4.35	4.46	0.20	0.0	1.55	{Val 0.01}	0.61	0.0	2.31
	BML	7.42	1.77	0.02	0.66	0.40	{0.01}	1.03	0.0	5.80
B.ovatus	BM	7.91	6.39	0.22	0.22	1.59	0.0	0.0	0.73	1.55
	BML	7.52	1.02	0.0	0.18	0.23	0.0	6.58	0.34	5.04

[a] Details are as for Table 4.4

4.3.3 Faecal homogenates

Yields of fermentation products during lactulose therapy have, as yet, not been assessed *in vivo*. Faecal homogenates (FHs) are complex *in vitro* preparations which allow more valid extrapolations to colonic conditions. Consequently, the fermentation products found in FHs incubated with two different concentrations of lactulose were analyzed to supplement data obtained from defined bacterial cultures. High oral doses of lactulose in PSE treatment produce acidification of colonic contents to below pH 5.5. The pH was therefore titrated to $\geq$ 5.5 as one of the experimental regimes (Vince *et al.*, 1978).

Generally, increased amounts of fatty-acids were detected following incubation of FHs with 30 mM or 90 mM lactulose (Tables 4.12 and 4.13). Elevated levels of acetic, propionic and butyric acids, and lactic acid in the untitrated 90 mM lactulose incubation, were most notable. Titration of pH $\geq$ 5.5 produced increments of iso-valeric, valeric and caproic acids in both 30 mM and 90 mM incubations. Under untitrated pH conditions, acid-tolerant lactobacilli, streptococci and clostridia probably account for the high levels of acetic, butyric and lactic acids. All lactulolytic strains produced lactic acid in pure culture; the reasons for absence of lactic acid in the 30 mM lactulose incubation are therefore not clear. Viable cell counts of streptococci and clostridia do not alter significantly over 48 h in untitrated incubations, but bacteroides decrease markedly over the same period (Vince *et al.*, 1978). It is significant, therefore, that on maintainance of a pH $\geq$ 5.5 during incubation, yields of higher fatty acids increased, presumably because bacteroides and other acid-sensitive organisms were more metabolically active at such pH values. However, strains producing iso-caproic and caproic acids from lactulose fermentation were not observed in the present study.

Table 4.12[a]

Non-gaseous products of *in vitro* faecal homogenates incubated with lactulose

Incubation conditions	Product (μmol (ml supernatant)$^{-1}$)										
	AC	Prop	iBut	But	iVal	Val	Cap	iCap	Pyr	Lac	Fum
+ lactulose 0 hours	19.2	3.1	0.6	4.4	1.0	0.8	0.5	0.2	5.8	0.0	6.8
+ lactulose 48 hours	70.3	18.1	1.2	22.4	1.9	0.8	2.6	0.2	0.0	0.0	8.3
+ lactulose 48 hours, titrated to pH $\geq$ 5.5	78.1	18.4	2.0	26.7	3.1	2.8	3.6	0.0	0.0	0.0	8.9

[a] Faecal homogenates were treated as described in Section 4.2.1. Each value is the mean of duplicate incubations. Abbreviations are as for Table 4.4 and Cap = caproic acid; iCap = iso-caproic acid. Lactulose concentration = 30 mM.

Table 4.13[a]

Non-gaseous products of in vitro incubations of lactulose with faecal homogenates

Incubation conditions	Product (µmol (ml supernatant)$^{-1}$)										
	Ac	Prop	iBut	But	iVal	Val	Cap	iCap	Lac	Fum	Succ
+ lactulose 0 hours	19.9	2.4	0.3	2.8	0.4	0.4	0.4	0.4	0.0	0.0	0.0
+ lactulose 48 hours	116.7	9.5	0.3	29.8	0.4	0.5	0.6	0.2	52.6	0.0	0.0
+ lactulose 48 hours, titrated to pH $\geq$ 5.5	99.1	17.2	1.7	65.5	1.7	3.4	9.3	0.4	0.0	2.3	3.6

[a] Experimental details are described in Table 4.12.
Lactulose concentration = 90 mM

Fatty acid pool sizes may have a number of effects *in vivo*. Whilst certain products, e.g. acetic acid, can be used as energy source by bacteria (Stanier *et al.*, 1977), the same or other products (acetic and butyric acids) are selectively toxic to specific bacteria (Lee and Gemmell, 1971). At a lower pH, the proportion of undissociated acid increases, which is directly responsible for microbial inhibition (Hentges and Maier, 1970). The cathartic effect of lactulose may be due to an increase in colon peristalsis caused by acidic products, especially lactate (Bennett and Eley, 1976; Conn and Lieberthal, 1979).

CHAPTER 5: LACTULOSE METABOLISM BY C.PERFRINGENS

5.1 INTRODUCTION

An integral step in the utilisation of carbohydrates by microorganisms is the transport of the carbohydrate across the semi-permeable membrane into the cell interior. Sugar translocation is well characterised in a number of Gram-positive bacteria, in particular lactose uptake by the PEP:PTS in Staph. aureus (Kennedy and Scarborough, 1967; Hays & Simoni, 1982) and a number of lactic acid streptococci (Vadeboucoeur, 1984; Deutcher et al., 1985) and lactobacilli (Chassy et al., 1983). Strain variation may be important, for instance responsible for active transport and PEP:PTS mediated accumulation of lactose in different isolates of S.mutans (Calmes, 1978). Co-induction of both transport mechanisms for lactose uptake in S.salivarius has also been reported (Hamilton and and Lo, 1978). Saccharolytic clostridia transport glucose, fructose and a mumber of disaccharides (lactose not examined) by group translocation (Hugo and Gottschalk, 1974; Booth and Morris, 1982) but galactose by a protonmotive force-dependent transport system (Booth and Morris, 1975). Bacterial uptake of lactose by the PEP:PTS is paralleled by cellular P-β-Glase activity (Calmes and Brown, 1979; Heller and Röschenthaler, 1979) and β-Glase hydrolysis where intracellular lactose accumulates in an unchanged form (see e.g., Hamilton and Lo, 1978).

The purpose of this study was to characterise the system of lactulose transport and the concomitant key catabolic enzymes in C.perfringens. This organism fermented lactulose vigorously

(Chapter 4) and in the laboratory is readily cultured under anaerobiosis. C.perfringens may be significant in vivo in terms of both its numbers and metabolic activities (Hill and Drasar, 1975).

5.2 METHODS

5.2.1 Transport studies

5.2.1.1 Assay for (^{14}C) carbohydrate uptake

Filtration assays, employing radiolabelled substrates, are well established for monitoring nutrient transport. The method described below is a modification of the procedures described by Groves and Gronlund (1969a) and Booth and Morris (1975). All solutions were routinely gassed with helium to minimise O_2 contamination (5 - 10 min).

Cultures (30 ml) were grown under standard conditions on individual sugars (5 mM) to late exponential phase and harvested at 4^{o}C using sealed vessels and anaerobic procedures employing N_2 gas. The pellet of cells was resuspended in chilled 50 mM potassium phosphate buffer pH 7.2 (10 ml), containing D-chloramphenicol (50 ug/ml) and 1 mM mercapto-ethanol, transferred to a tube continuously flushed with N_2, stoppered and stored at 4^{o}C until required. Respective stock solutions of (U - ^{14}C) glucose (291 mCi/mmol), (D-glucose-1-^{14}C) lactose (57.7 mCi/mmol) and (1-^{14}C) galactose (60 mCi/mmol) were diluted in harvest buffer to the required concentrations indicated in Figs. 5.1 and 5.2 (Section 5.3.1.1).

The assay protocol was as follows. A conical flask (25 ml) served as reaction vessel, fitted with a rubber stopper and sealed air-tight except for a fine metal capillary inlet

(i.d. 0.1 cm) for N_2, and an open combined sample and gas outlet port (0.5 cm diameter). The flask was firmly clamped in a shaking water-bath at 37°C, and was flushed with N_2 at a rate which excluded bubbling of the reaction mixture. An aliquot (4 ml) of the chilled anaerobic cell suspension was transferred by syringe as rapidly as possible to the reaction flask under N_2 delivery. A second addition of sodium thioglycollate or L-cysteine hydrochloride in buffer (final concn. 0.05%, w/v ; 100 µl) was then made, and the reaction mixture pre-warmed for 10 min. Various external concentrations of (^{14}C) sugars in buffer (0.5 ml) were added in separate determinations to initiate assay. At timed intervals, samples (100 µl) were removed and filtered through bacteriological membranes (0.22 µm or 0.45 µm pore size; 2.5 cm diameter), followed by several applications of harvest buffer at 22°C (5 x 2 ml) to ensure an adequate wash of filtered cells. The filters were subsequently dried (65°C for 15 min), placed in vials containing toluene/PPO mixture (10 ml; 5 g 2,5-diphenyl-oxazole per litre toluene), and radioactivity measured in a Beckman S7500 scintillation counter.

5.2.1.2 Assay of substrate phosphorylation

Anaerobic handling of cultures and solutions was as described above (Section 5.2.1.1). Cells grown on 5 mM sugar (100 ml) were harvested in mid-log phase, and resuspended in 40 mM sodium potassium phosphate buffer pH 7.2 containing 50 µg/ml D-chloramphenicol (5 ml). An aliquot of the cell cencentrate (1.0 ml) was lysed with toluene/acetone (1:4 or 1:10, by vol.) or toluene/ethanol (1:10 or 3:10, by vol.) mixtures, and a sample (0.1 ml) of the permeabilised cells used for sugar phosphorylation assays. In these assays, 100 mM sodium potassium phosphate buffer

pH 7.2, supplemented with 50 µg/ml D-chloramphenicol, 5 mM $MgCl_2$ and 10 mM sodium flouride, was used.

The following solutions were mixed in a 1.5 ml rubber stoppered plastic cuvette:

Assay buffer	0.5 ml
5 mM phosphenolpyruvate	0.1 ml
10 U lactate dehydrogenase	0.1 ml
0.2 mM NADH (or NADPH)	0.1 ml
permeabilised cells	0.1 ml

After preincubation at 37°C for 5 min, the assay was started by addition of 1 mM glucose or lactulose (0.1 ml). The change in absorbance at A_{340} which occurred prior to and following the addition of substrate was monitored at 37°C using a Beckman 24 recording spectrophotometer. One unit of enzyme activity was defined as the transformation of 1 µmol NADH or NADPH min^{-1} under the above conditions.

5.2.1.3 ONPG-based transport assay

Cultures grown on 5 mM lactulose (10 ml) were harvested according to standard procedures. Cell pellets were washed once in 100 mM sodium phosphate buffer pH 7.3, containing D-chloramphenicol (50 µg/ml), and resuspended to their original volumes. When required, a sample of the washed cell suspension (1.0 ml) was permeabilised with lysis mixture (25 µl/ml), and assayed (0.5 ml lysate) for ONPG hydrolysis as described previously (3.0 ml assay; Section 2.2.5).

5.2.2 Partial purification of β-galactosidase

Combined pellets from a large scale cultivation (2 litre) on 5 mM lactulose, described in Section 2.2.3.1, were washed (x 2) with 50 mM Tris HCl pH 7.5 containing D-chloramphenicol (100 µg/ml).

After combining the pellets, a final centrifugation at 12000 x g for 30 min at 4°C was carried out, and the final pellet (wet weight approx. 43 g) stored at -20°C overnight.

Thawed cells were resuspended in harvest buffer (50 ml) for sonication (20 - 30 min total period). The cell-free extract was centrifuged at 105000 x g for 1.5h at 4°C, and the supernatant concentrated to about 10 ml by ultrafiltration under nitrogen pressure (2 kgf/cm^2) with an Amicon ultrafiltration cell using a PM-10 membrane. The resulting concentrate was designated as the crude cell-free extract.

Column chromatographic procedures were essentially those described by Calmes and Brown (1979) and were conducted throughout at 4°C. Protein determinations were as outlined in Section 2.2.6; β-Glase assay is described below (Section 5.2.2.2).

In the first step, a known volume of crude cell-free extract (8 - 10 ml) was gradually pumped onto a DEAE cellulose column (2 x 26 cm, ex. d.), previously equilibrated with 50 mM Tris HCl buffer pH 7.5. A linear gradient of 0 - 0.7 M KCl in buffer (total volume 500 ml) was then applied using a peristaltic pump (LKB, BROMMA, 1200 Varioperspex) and fractions (9.5 ml) collected (LKB-Ultrorac fraction collector) with a flow rate of 75 ml h^{-1}. Alternate fractions were assayed for A_{280} and β-Glase activity. Fractions with enzyme activity were pooled (total volume, 75 ml) and concentrated to 10 ml by ultrafiltration as described above).

In step 2 of column chromatography, the concentrated sample (9 ml) was gradually layered onto a Biogel A (0.5 M) column (3 x 60 cm, ex. d.) which had been equilibrated as in step 1.

Fractions (4.1 ml) were collected at a flow rate of 18 ml h^{-1} using the previous buffer. Enzyme activity and A_{280} assays were performed on alternate fraction samples, and fractions corresponding to the peak of enzyme activity were pooled (approx. 50 ml) and concentrated by ultrafiltration (7 ml) as described earlier. An aliquot (6 ml) of the concentrated column 2 (C2) enzyme preparation was dialysed against 50 mM sodium phosphate buffer pH 7.3 (1 litre x 3) since Tris buffer was later found to be inhibitory. The dialysed C2 fraction was subsequently used for studies on the properties of β-Glase.

5.2.2.1 Slab gel electrophoresis

The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemli (1970) was used with a slight modification. Slab gel electrophoresis was carried out using a water jacketed BioRad apparatus, cooled by circulating tap water.

Slab gels containing 5% (stacking gel) and 8% acrylamide were prepared from a stock solution of 70% ($^{w}/v$) acrylamide and 0.8% ($^{w}/v$) N,N^{1}-bis-methylene acrylamide. The final concentrations in the separating gel were as follows: 8% ($^{w}/v$) polyacrylamide, 0.375 M Tris HCl buffer pH 8.8, 0.1% ($^{w}/v$) SDS, 2 mM EDTA, 0.025% ($^{v}/v$) tetramethylethylene-diame (Temed) and 0.033% ($^{w}/v$) ammonium persulphate. Gel slabs (12 x 14 cm) with a thickness of 1.5 mm were prepared by polymerising the stacking gel (2 cm in height) on top of the separating gel. The former gel consisted of 5% ($^{w}/v$) acrylamide and contained 0.125 M Tris HCl buffer pH 6.8, 0.1% ($^{w}/v$) SDS and 2 mM EDTA, 0.05% ($^{v}/v$) Temed and 0.1% ($^{w}/v$) ammonium persulphate. About 10 ml stacking gel solution was adequate for 1 slab gel. The electrode buffer (pH 8.3) contained 0.05 M Tris HCl buffer pH 6.8, 0.384 M glycine, 2 mM EDTA and 0.1% ($^{w}/v$) SDS.

Aqueous protein samples (5 - 30 µl containing 5 - 40 µg protein) were mixed with the sample buffer (95 - 70 µl) which contained 0.125 M Tris HCl buffer pH 6.8, 10% (^{v}/v) glycerol, 1.25% (^{w}/v) SDS, 2 mM EDTA and 0.015% (^{w}/v) bromophenol blue. The proteins were dissociated by heating the samples for 3 min at 100^{o}C. After cooling, the samples (20 µl) were applied to the gel (maximum volume applied was 60 µl per track). Pre-run electrophoresis was performed at 12.5 mA/gel slab until the sample entered the stacking gel (40 - 45 min), after which the current was increased to 40 mA/gel. The gels were removed when the dye front was within 10 mm of the bottom of the gel (2 - 2.5h). Gels were fixed in 7% (^{v}/v) acetic acid and stained for protein using 0.2% (^{w}/v) Coomassie brilliant blue R250 in 50% (^{v}/v) TCA for 15 - 30 min at 60^{o}C. Subsequently, the gells were destained with a solution containing 7.5% (^{v}/v) glacial acetic acid and 12.5% (^{v}/v) isopropanol. The time required for destaining could be decreased by placing the gel at 30^{o}C.

5.2.2.2 Assay for β-galactosidase

The reaction was followed as described previously (Section 2.2.5). For β-Glase purification studies, the following assay was used unless otherwise indicated:

100 mM sodium phosphate buffer	0.934 ml
Enzyme	0.033 ml
ONPG (concn. varied as required)	0.033 ml

For inhibitor studies, the volume of buffer was corrected as appropriate.

5.2.3 Galactokinase assay

Each strain of C.perfringens, Staph.aureus and E.coli was grown on 5 mM sugar (250 ml) to late-log phase, and harvested using routine procedures. Both Staph.aureus and E.coli were grown as shake-cultures at 37°C in 1% (w/v) proteose peptone No. 3 (Difco Laboratories) medium. The cells were washed and resuspended in 0.1 M sodium phosphate buffer pH 7.5 containing 50 µg/ml D-chloramphenicol (20 ml). Cell-free extracts were obtained by sonication for a total period of between 10 - 15 min, followed by centrifugation at 105000 x g for 1.5h at 4°C. The supernatants were used for galactokinase (GK) assays.

A comparison of a coupled-enzyme assay system (Bissett and Anderson, 1974) and a DEAE-cellulose disc procedure (Dey, 1980) was made to detect GK activity. The former procedure was relatively insensitive, and was susceptible to NADH oxidase interference, whilst the disc method was both acceptably reproducible and sensitive (picomole of product detected).

The incubation mixture (100 µl) for GK assay (Dey, 1980) consisted of:

Crude extract	10 µl
(1-^{14}C) galactose (0.2 uCi; 60 uCi/umol)	10 µl
50 mM ATP (adjusted to pH 7.3)	5 µl
deionised water	45 µl
0.1 M sodium phosphate buffer pH 7.3 (+ 25 mg/ml bovine serum albumin + 5 mM $MgCl_2$ + 12.5 mM sodium flouride)	30 µl

This was incubated at 25°C and aliquots (20 µl) withdrawn at various time intervals,and applied to DEAE cellulose discs resting on a filtration apparatus attached to a vacuum line. A further

aliquot of water (60 µl) was applied to each disc to ensure that it was fully wet. The discs were immediately rinsed with applications (5 x 20 ml) of water via syringe, and then dried (100°C for 30 min). Finally, the radioactivity was counted using the PPO/toluene cocktail described in Section (5.2.1.1); the counting efficiency was 89% as judged by a (^{14}C) hexadecane internal standard.

5.2.4 Partial purification of phosphofructokinases by affinity chromatography

Cells grown on 5 mM fructose (250 ml) were harvested in late-log phase using routine procedures. The cells were then resuspended in 50 mM sodium potassium phosphate buffer pH 7.2 (5 ml) containing 5 mM mercaptoethanol and 50 µg/ml D-chloramphenicol. The suspension was sonicated for 10 min, and the sonicate centrifuged at 40000 g for 20 min at 4°C. Aliquots were removed for preliminary enzyme assays and for chromatographic procedures.

The procedure for phosphofructokinase assay was modified from the descriptions by Groves and Gronlund (1969b) and Sapico and Anderson (1969). A stock coupled-enzyme solution was prepared in water, of which a 0.2 ml aliquot gave final assay concentrations as follows: 0.90 U D-fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), 1.64 U triose phosphate isomerase (EC 5.3.1.1), and 2.12 U ∝-glycerophosphate dehydrogenase (EC 1.1.9.9.5). Assay reagents in a rubber stoppered cuvette were (final concentrations):

extraction buffer	1.0 ml
4 mM ATP	0.2 ml
12 mM $MgCl_2$	0.1 ml
0.1 mM NADH	0.1 ml
water	0.4 ml
enzyme mix	0.2 ml
4 mM fructose-1-phosphate or fructose-6-phosphate (by syringe)	0.2 ml

The reaction was started by addition of respective sugar phosphate. All assays were carried out at 37°C and followed continuously at A_{340} on a Beckman 25 recording spectrophotometer.

For affinity chromatography, a column (16 cm x 1.5 cm i.d) was packed with Blue Sepharose CL-6B and equilibrated overnight with extraction buffer at 4°C. The same buffer was also used for column elution following the application of an aliquot (1.25 ml) of cell-free extract to the column. The flow rate was adjusted to 18 ml h^{-1} and fractions (3 ml) collected. The elution scheme was as follows: a) elution buffer (25 ml); b) 0 - 1.0 M NaCl (25 ml; as for all gradients); c) 0 - 10 mM ATP; d) 0 - 10 mM NAD^+ or $NADP^+$. Assays for phosphofructokinases were performed on alternate fractions (0.1 ml), and protein determinations were made using Bradford's procedure (Bradford, 1976). Because of the inclusion of chloramphenicol in the buffer, A_{280} profiles could not be recorded.

5.3 RESULTS AND DISCUSSION

5.3.1 Sugar transport studies

5.3.1.1 (^{14}C) Carbohydrate uptake assay

The filtration of cells which have accumulated (^{14}C) sugar provides a versatile technique in differentiating between the two energy-dependant transport mechanisms, active transport and the

PEP:PTS, for instance by identifying the intracellular (^{14}C) sugar phosphate product of PTS uptake (Groves and Gronlund, 1969a), or by use of proton conductors like carbonylcyanide M-chlorophenylhydrazone (CCCP) or valinomycin, together with the membrane ATPase inhibitor N,N^1-dicydohexyl carbodiimide, as specific inhibitors of active transport (Booth and Morris, 1975). In this respect, it has been noted that CCCP, valinomycin, or tetrachlorosalicylanilide caused only small fluxes of protons into C.perfringens cells, whereas 2,4-dinitrophenol caused an instantaneous influx of protons seen as a rise in pH (Daltrey and Hugo, 1974).

One of the basic requirements of the filtration assay is a sufficient cell mass. In the assays shown in Figs. 5.1 and 5.2, $\sim 5 - 10 \times 10^9$ viable cells ml^{-1} were used, cf. $\sim 2 \times 10^8$ viable organisms ml^{-1} in the study of galactose uptake by C.pasteurianum (Booth and Morris, 1975). Various external sugar concentrations have been reported for filtration assays. Groves and Gronlund (1969a) used between 8 - 25 µM final substrate concentration of glucose or mannose (0.18 µCi per assay) in uptake by C.perfringens, whereas the same sugars were supplied at 0.1 mM (0.23 µCi per assay) in estimating B.thetaitaomicron uptake (Hylemon et al., 1977). As radiolabelled lactulose was not available, (^{14}C) lactose uptake by lactulose and lactose grown cells was assessed (Fig. 5.1 a,b,c). This could also indicate affinity of the lactulose permease for its isomer, by assuming a homogeneous presence of permease proteins, in contrast to the possibility of catalytic isoenzymes in the cell. Lactulose induced cells accumulated (^{14}C) lactose (Fig 5.1 a,b), indicating a relatively non-specific permease protein. However,

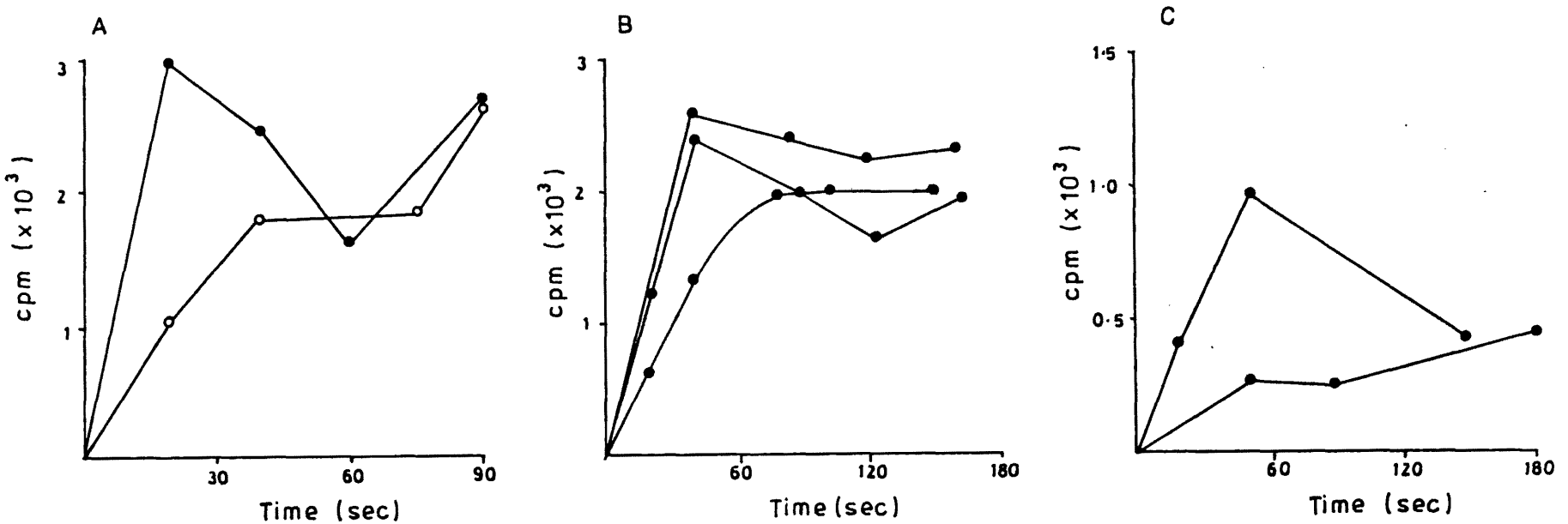


Fig. 5.1

Uptake of [D-glucose-1-^{14}C] lactose (57.7 μCi/umol) by C. perfringens: A) grown on (○) lactulose or (●) lactose (each at 5 mM), external sugar concn. 9.4 μM (0.25 μCi/assay); B) grown on lactulose (5 mM), external sugar concn. 0.4 μM (1.25 μCi/assay); C) as for B) except cells grown on lactose (5 mM). Experimental procedures are described in Section 5.2.1.1.

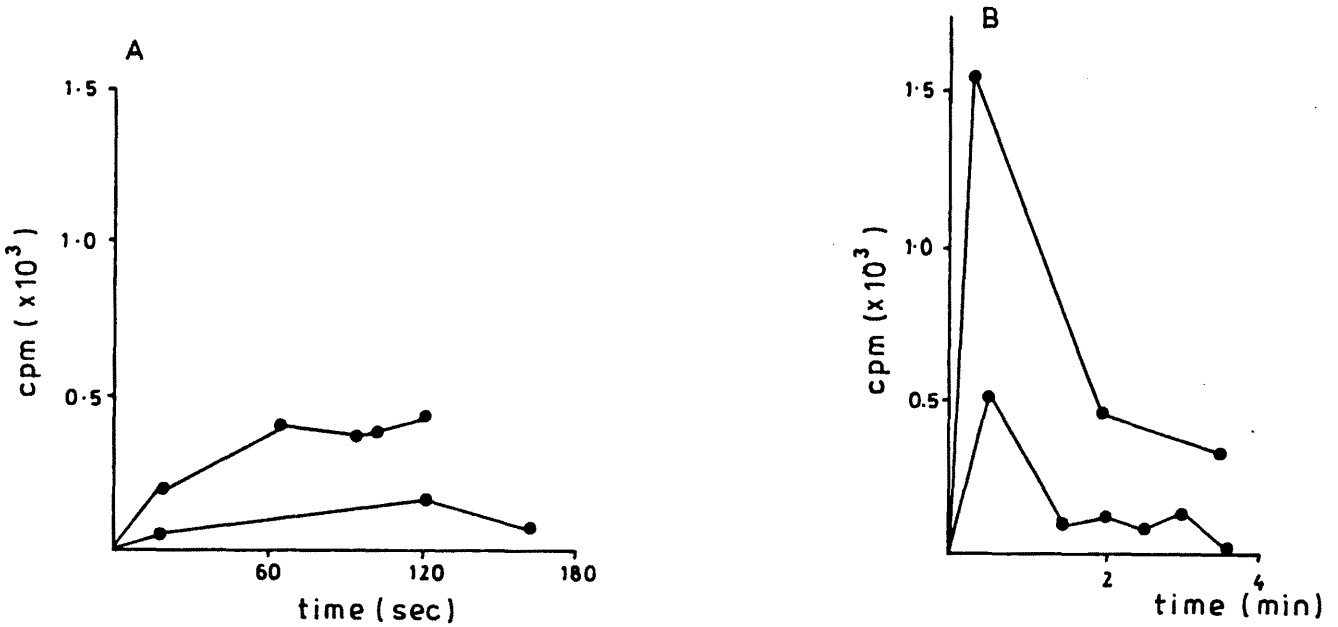


Fig. 5.2

A) Uptake of [1-^{14}C] galactose (60 µCi/µmol) by C. perfringens grown on lactulose (5 mM), external sugar concn. 4.8 µM (1.25 µCi/assay); B) Uptake of [U-^{14}C] glucose (291 µCi/µmol) by C.perfringens grown on glucose (5 mM), external sugar concn. 0.4 µM (0.25 µCi/assay).

the pattern of accumulation over time was found to be variable and not reproducible, a consistent problem with whichever sugar substrate used (Figs. 5.1 and 5.2). In a control assay for (^{14}C) glucose uptake by glucose-grown cells (Fig. 5.2 b), similar results were obtained despite increasing external glucose concentration in assay to 0.1 mM (data not shown).

5.3.1.2 Assay for sugar phosphorylation

Two solvent mixtures were employed to permeabilise cells, toluene/ethanol and toluene/acetone (Tables 5.1 and 5.2). In both experiments, a direct indication of the presence of PEP:PTS linked uptake of glucose, as control, or lactulose by appropriately induced cells was not obtained due to interference by NADH oxidase activity, despite use of NADPH (Table 5.2). Glucose transport in C.perfringens has been characterised as via the PEP:PTS (Groves and Gronlund, 1969a). A toluene/ethanol (3:10, by vol.) permeabilisation treatment yielded higher levels of NADH oxidase activity, indicating a greater degree of cell permeability (Table 5.1).

5.3.1.3 ONPG-based transport assay

ONPG has been used to partially characterise the lac permease in E.coli (Maloney and Wilson, 1978) and the lactose PEP:PTS in streptococci by using permeabilised cells and ONPG ± PEP (Calmes,1978; Thompson, 1979).

The rates of ONPG hydrolyses by whole and lysed cells of C.perfringens harvested during mid- to late-log phase growth on lactulose are shown in Table 5.3. ONPG entry appeared to be the rate-limiting step, as the rates of hydrolyses of this substrate by lysed cells was greater than whole cells. At 8h, the increased

Table 5.1 Masking of phosphenolpyruvate-dependant sugar transport assay by NADH oxidase

Growth Sugar	Assay Sugar	Cell treatment	Units log. viable cells^{-1}
Lactulose	None	Tol/EtOH (1:10,v/v)[a]	0.49[b]
	Lactulose	"	0.35
Glucose	None	"	0.08
	Glucose	"	0.15
Lactulose	None	Tol/EtOH (3:10,v/v)	1.67
	Lactulose	"	1.74
Glucose	None	"	1.60
	Glucose	"	1.68

[a] Cell suspension (1.0 ml) treated with 50 µl toluene/ethanol. (Tol/EtOH) mixture; 0.1 ml decryptified cells used in assay.

[b] Units are nmole NADH oxidised min^{-1} at 37°C

Table 5.2 NADPH based assays for sugar phosphorylation by permeabilised cells and interference by NADH oxidase

Growth Sugar	Assay Sugar	Cell treatment	Units log. viable cells^{-1}
Lactulose	None	Tol/AC (1:4, v/v)[a]	0.47
	Lactulose	"	0.40
	Glucose	"	0.25
Glucose	None	Tol/AC (1:10, v/v)	1.27
	Glucose	"	1.55

[a] Toluene/acetone (Tol/AC) mixture (50 µl) used per 1.0 ml cell suspension; 0.1 ml decryptified cells used in assay.

Table 5.3 ONPG hydrolysis by whole and decryptified cells of C. perfringens

Time of harvest (h)	p mole ONPG hydrolysed min^{-1} $log.\ viable\ cells^{-1}$	
	whole cells	lysed cells[a]
5	1.84 (65.0)[b]	6.66
6	2.07 (50.0)	10.21
8[c]	5.40 (100.0)	25.11

[a] Cell permeabilisation as described in Section 5.2.1.3

[b] Values in parenthesis are expressed as nmole ONPG hydrolysed min^{-1} mg dry $weight^{-1}$ ml^{-1}

[c] Thio-β-D-digalactoside (final concn. 1 mM) produced no inhibition of ONPG hydrolysis by whole cells. (29.6 nmole ONPG hydrolysed min^{-1}/ml cell suspension cf.27.5 units in control).

rate of ONPG hydrolysis reflects an increase in permease concentration. A comparison of the values in parenthesis (Table 5.3) with those in the literature, 0.4 - 2.2 nmoles o-nitrophenol released mg dry weight^{-1} ml^{-1} by S.faecalis cells (Heller and Röschenthaler, 1979) indicates significant uptake of ONPG. These results, together with previous observations of β-Glase activity in several strains of C.perfringens grown on lactulose (Table 4.9), and an absence of phospho-β-galactosidase (Table 5.4), would suggest active transport as the major route of lactulose uptake.

In order to evaluate the non-specific hydrolysis of ONPG, such as a small fraction of external β-Glase and the presence of diffusion pathways for ONPG entry, thio-β-D-digalactoside was added to the standard assay (final concn. 1 mM; Table 5.3). However, no inhibition of ONPG uptake was observed, indicating entry by diffusion, as extracellular hydrolysis of lactulose or lactose was not observed in later studies (Section 6.3.2.4). The thiodigalactoside has an affinity for the lac permease in E.coli and inhibits transport of ONPG but does not inhibit hydrolysis of ONPG by β-Glase (Maloney et al., 1975). Thio-β-D-digalactoside at a concn. below 0.1 mM produced 50% inhibition of ONPG cellular hydrolysis in E.coli (Sandermann, Jr., 1977). A similar affinity for the lactulose transport protein(s) in C.perfringens is assumed for the thiodigalactoside under the conditions used, but requires verification.

The addition of lactose or galactose to S.salivarius co-induced both the lactose PEP:PTS and β-Glase, with the latter the predominant system, although in various strains of S.mutans and S.sangnis, the lactose PTS was the major metabolic pathway with

Table 5.4 Comparison of β-galactosidase and phospho-β-galactosidase in lysed cells and cell-free extract of lactulose-grown C.perfringens

Sample[a]	Specific activity[b]	
	ONPG[c]	ONPG-P[c]
Whole Cells	0.25	ND[d]
Lysed cells	1.03	0.00
		0.01[e]
105K fraction	0.23	0.00

[a] Cells were grown on 5 mM lactulose and harvested at 10h; wash, cell lysis and assay procedures were described in Section 2.2.5. The 105000 x g cell-free fraction (Section 5.2.2) is designated as 105K.

[b] μmole substrate hydrolysed min^{-1} mg protein

[c] Final concn. in assay: ONPG (6.8 mM); ONPG-P (3.4 mM)

[d] ND, not determined.

[e] Final assay concn. ONPG-P was 5.4 mM.

β-Glase induced either too low or negligible levels (Hamilton and Lo, 1978). Of 11 strains of S.lactis, only one isolate expressed β-Glase activity one-tenth that of the P-β-Glase also present (Okamoto and Morichi, 1979). However, Kandler (1983) concluded that lactose permease and β-Glase are cryptic in streptococci and only function in strains lacking the PTS, and that the rate of lactose fermentation and growth are significantly lower in such strains.

Consequently, o-nitrophenol-β-D-galactopyranoside-6-phosphate (ONPG-P) hydrolysis by cells grown on lactulose was examined (Table 5.4). Negligible or nil P-β-Glase activity was noted in lysed cell or cell-free preparations.

Furthermore, the purified β-Glase preparation used in this study (Section 5.3.2) did not catalyse ONPG-P hydrolysis at a concn. of 6.8 mM in assay. The differences in rate of ONPG dissimilation by whole cells and lysed cells again illustrated the rate-limiting step due to entry (Table 5.4).

5.3.2 Characterisation studies of β-galactosidase

β-Galactosidase was present in several strains of C.perfringens grown on lactulose (Chapter 4). Irrespective of whether lactulose or lactose grown permeabilised cells were used, hydrolysis of either disaccharide was marked when assayed by t.l.c. (Section 6.2.1.1). This indicated a non-specific enzyme or possibly iso-enzymic forms. Because of the key position β-Glase occupies in the fermentation of lactulose, it was of interest to study some of its kinetic and regulatory properties using a model substrate, ONPG.

5.3.2.1 Partial purification of β-galactosidase

The cytoplasmic enzyme induced by lactulose was partially purified by a simple, two-step procedure using DEAE-cellulose and Biogel A (0.5 M) column chromatography (Fig. 5.3), as described by Calmes and Brown (1979). In the latter report, the P-β-Glase in S.mutans was purified 13-fold. However, as indicated in Table 5.5, significant enzyme losses occurred during the ultrafiltration steps, possibly by adsorption of protein on the Amicon membrane. Enzyme activity was shown not to be lost in the filtrate of the ultrafiltration cell, despite use of a xM 100 membrane (cut off range for proteins with M.W. 100000). Both columns were shown to be completely eluted of enzyme activity, and negligible losses occurred directly by column gel-bed retention. In two separate determinations, a crude extract (115000 x g at $4^{o}C$ for 15 min) supernatant fraction recovered 94% of enzyme activity detected in lysed cells. Analysis of the partially purified preparation by SDS-PAGE indicated significant loss of contaminating protein, but the final fraction clearly remained impure (Fig. 5.4). However, the enzyme was stable at $4^{o}C$ for up to 7 days.

5.3.2.2 Response of β-galactosidase to H^{+} and Mg^{2+} concentrations

The pH optimum of the β-Glase preparation was between 7.0 - 7.4 in the assay buffers used (Fig 5.5), in accord with the routine assay buffer pH of 7.3. In studies of E.coli β-Glase, Na^{+} ions were found to be better activators for the hydrolysis of ONPG, and K^{+} ions for lactose and p-nitrophenyl-β-D-galactoside (Wallenfels and Malhotra, 1961). In the present study, Mg^{2+} activation was observed for ONPG hydrolysis in sodium phosphate buffer (Table 5.6) as well as in potassium phosphate buffer (data not shown). Varying

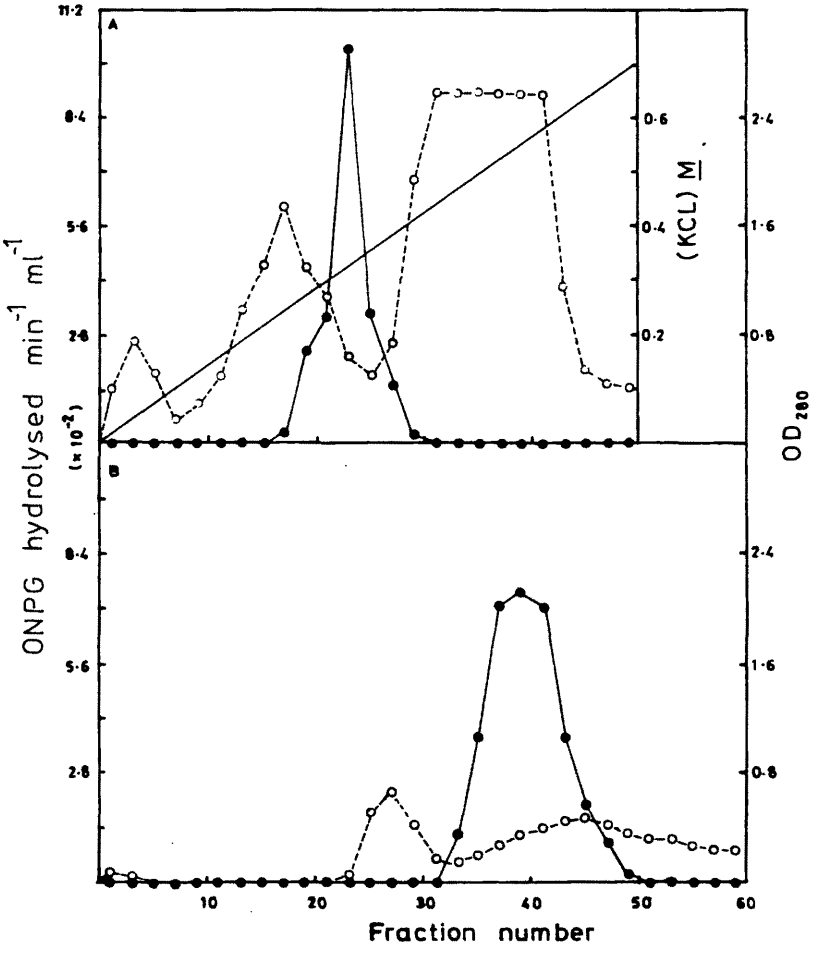


Fig. 5.3 Purification of β-gal from C.perfringens by column chromatography

Methods are described in Section 5.2.2. Effluent fractions were assayed as follows: 0.934 ml 0.1 M sodium phosphate buffer + 0.1 ml fraction + 0.033 ml, 75 mM ONPG, incubated 20 min, stopped by addition of 0.33 ml 0.5 M $Na_2 CO_3$, and absorbance at 410 nm recorded. Symbols: (●)β-Glaseactivity, (o) OD 280 nm, (A) DEAE cellulose chromatography of CE(A), (B) Bio-gel A (0.5 M) chromatography of Column 1 (A) (Table 5.5).

Table 5.5 Partial purification of β-galactosidase from C.perfringens grown on lactulose

Fraction[a]	Total[b] units	Total protein (mg)	Specific activity	% Recovery
CE (U)	15200	166.6	91.2	100
C1 (PF)	11856	62.1	190.9	78
C1 (U)	2800	57.0	49.1	NA[c]
C2 (PF)	1960	8.7	225.3	NA
C2 (U)	700	7.2	97.2	NA

[a] Purification steps are described in the text; abbreviations: CE (U), crude extract after ultrafiltration; C1 (PF), column 1, pooled fractions with enzyme activity.

[b] Nanomoles ONPG hydrolysed min^{-1} at 37°C

[c] NA - not applicable, as discussed in text

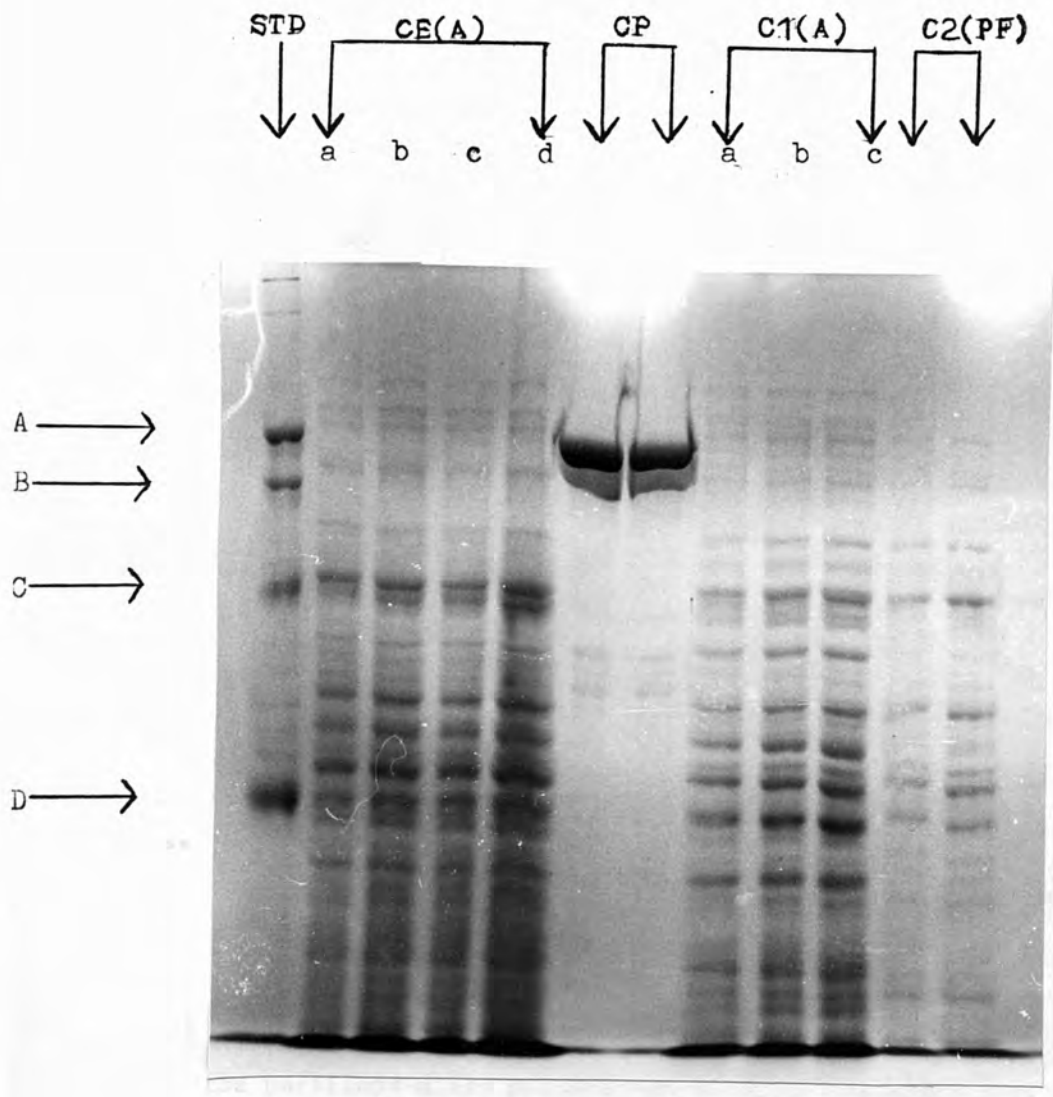


Fig 5.4

SDS-PAGE separation of proteins in fractions obtained during the purification of β-Glase from C. perfringens. Notation as in text: STD, mixture of standard proteins, A (116,000), B (94,000), C (68,000), D (43,000); CE (A) crude extract following ultrafiltration, a = 10.6 µg, b = 15 µg, c = 20 µg, d = 40 µg; CP, commercial preparation of β-Glase from E. coli, a, b = 10 µg; C1(a), column 1 pooled fractions of β-Glase activity following ultrafiltration, a = 10 µg, b = 15 µg, c = 20 µg; C2 (PF), pooled fractions of β-Glase activity following column 2 chromatography, a = 5 µg, b = 7.5 µg.

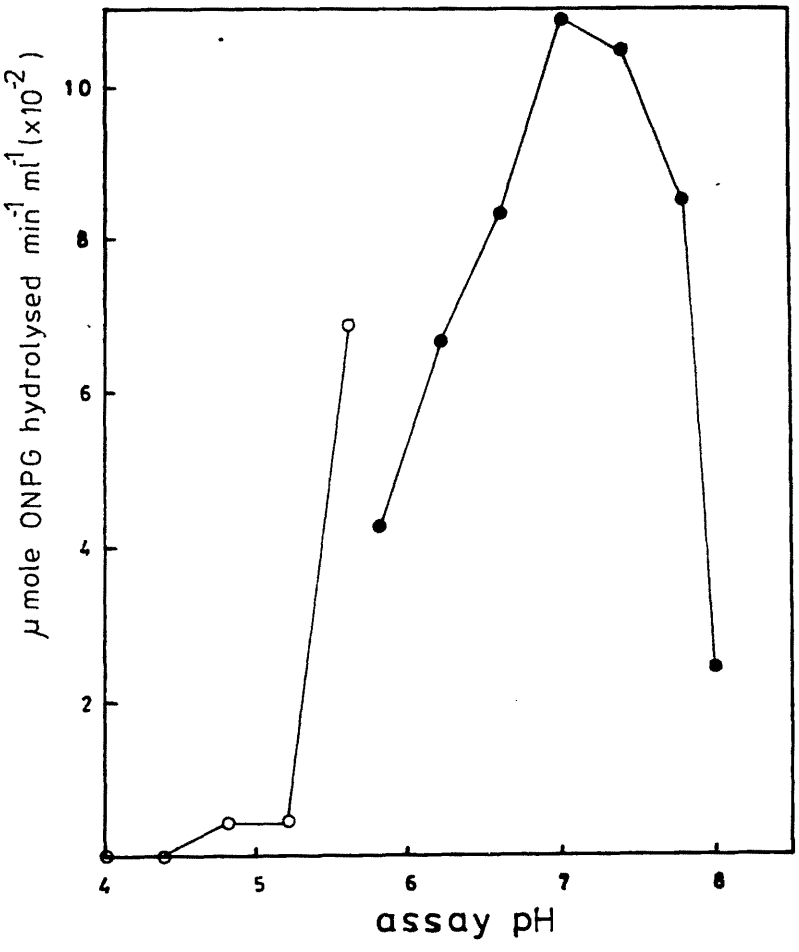


Fig 5.5 Dependence on pH of the rate of ONPG hydrolysis by the purified β-Glase preparation (C2(PF), see Table 5.5 - 19.6 μg/assay). Symbols: (○), 50 mM acetate buffers, (●) 50 mM sodium phosphate buffers. The assay included: 0.081 ml buffer + 0.1 ml fraction + 0.033 ml (3.36 M) mercaptoethanol + 0.033 ml 30 mM $MgCl_2$ + 0.1 ml 68 mM ONPG. After a 20 min incubation, the reaction was stopped by the addition of 0.33 ml 0.5 M Na_2CO_3, and absorbance at 410 nm recorded.

Table 5.6 The effect of Mg^{2+} on β-galactosidase

Final concn. (mM) of Mg^{2+}	% Inhibition[a]
0	41.9
0.5	35.5
1	25.8
2	12.9
3	NIL

[a] β-Glase was assayed as described in text; 5.4 µg of C2 (PF) fraction (Table 5.5) used in each assay.

the concn. of sodium phosphate buffer between 20 - 100 mM had no effect on enzyme activity. However, both lactulose and lactose were hydrolysed only in the presence of K^+ ions (see Section 6.2.1.1), whilst Na^+ ions had no effect.

5.3.2.3 Substrate kinetics of β-galactosidase

β-Glase activity was a hyperbolic function of the ONPG concn., and the apparent K_m value for ONPG was 5.71 mM (Fig. 5.6). The constitutively synthesised β-Glase in a mutant strain of Klebsiella sp. (Hall, 1980) exhibited comparable affinities for the following substrates: lactulose (K_m, 29 mM), lactose (35 mM) and ONPG (32 mM). However, the Vmax values of the free disaccharides were about 0.5% of the estimated value using ONPG as substrate. β-Glase from E.coli has a K_m of 0.95 mM for ONPG (Wallenfels and Malhotra, 1961). β-Glase V_{max} in this study was 0.24 units/mg protein (Fig.5.6

5.3.2.4 Survey of regulatory effectors of β-galactosidase

The results of a preliminary survey of the ability of several glycolytic and other metabolites to modulate the activity of the β-Glase preparation are shown in Table 5.7. Inhibition of enzyme activity was observed in the presence of fructose-1,6-bisphosphate. This catabolite may play a role in regulating β-Glase by a negative feedback mechanism (Fig 5.7). Since both fructose and galactose may be channelled via fructose-1,6-bisphosphate, it may also regulate other enzymes catalysing fermentation of intracellular monosaccharide.

5.3.3 Galactokinase

If lactulose uptake is assumed to occur by active transport, galactose released intracellularly following lactulose hydrolysis by β-Glase may be metabolised through the Leloir pathway.

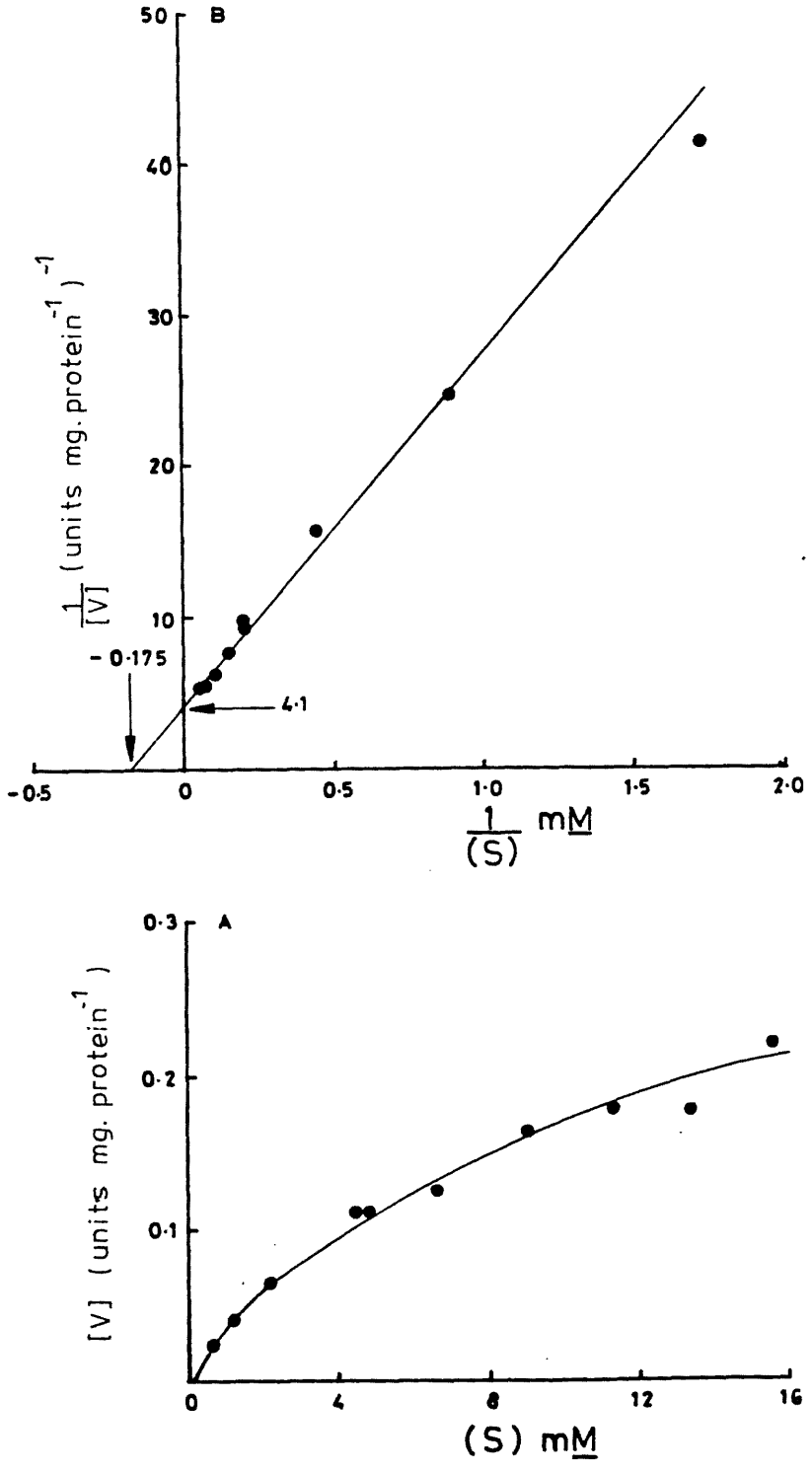


Fig. 5.6 (A) ONPG kinetics of β-Glase; enzyme activity was assayed as described in the text (Section 5.2.2.2) except that the concentration of ONPG was varied as indicated. Each reaction contained 110 µg of C2 (A) after dialysis (see Table 5.5). (B) Lineweaver-Burk plot of the same data. The apparent K_m was 5.71 mM.

Table 5.7 Effect of cellular metabolites on β-galactosidase activity

Metabolite[a]	% Inhibition[b]
Lactulose[c]	0
Lactose	0
Galactose	ND[d]
Glucose	0
Fructose	0
Glucose-1-phosphate	0
Glucose-6-phosphate	0
Fructose-1-phosphate	0
Fructose-6-phosphate	0
Fructose-1,6-bisphosphate	26
Galactose-1-phosphate	0
Galactose-6-phosphate	0
PEP	ND
Pyruvate	ND
3-phosphoglycerate	ND
ATP	0
ADP	0
AMP	0
cAMP	0
cGMP	0

[a] All additions of metabolite were to a final concn. of 5 mM. Data mean of two experiments.

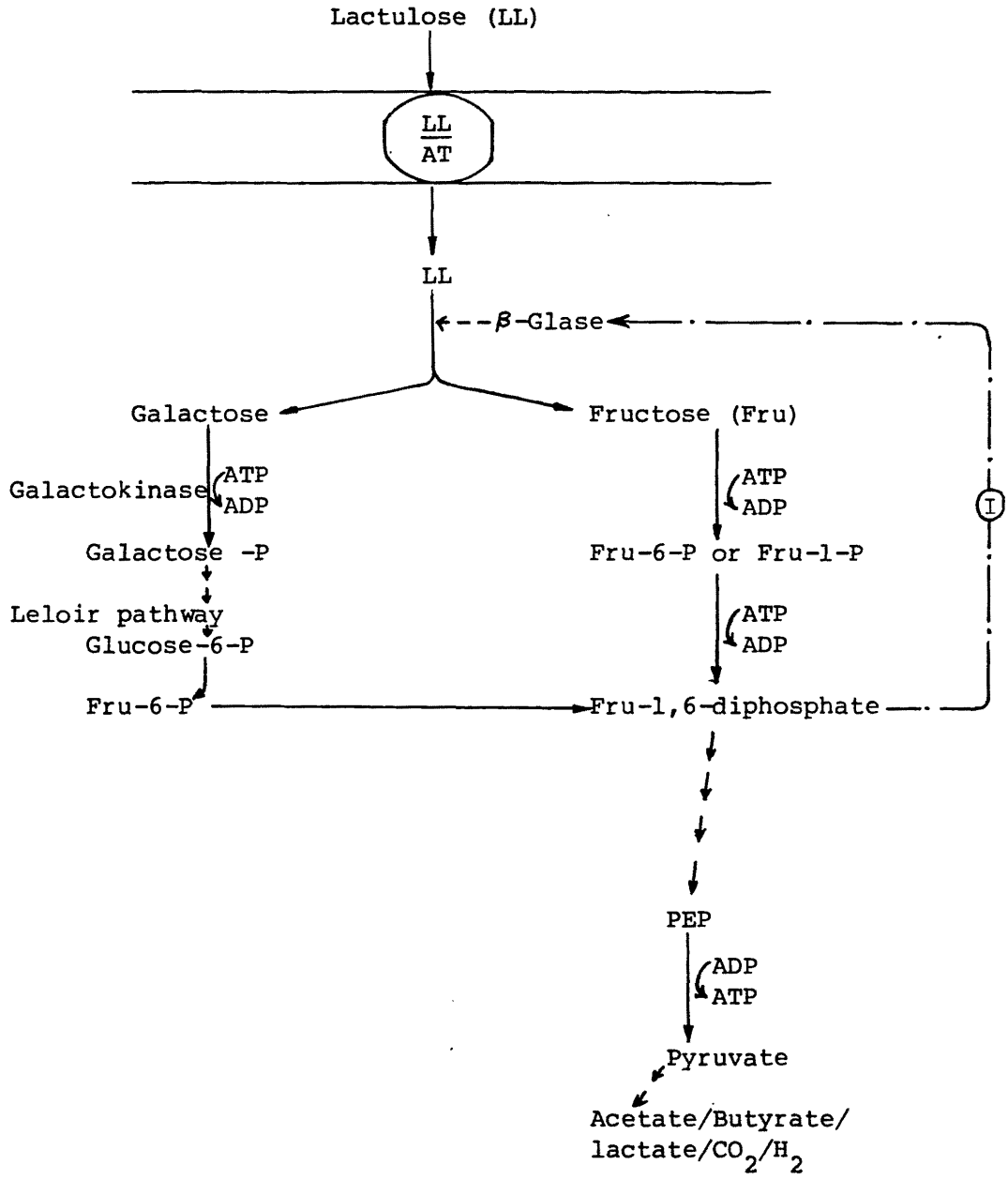
[b] β-Glase was assayed as described in the text; ONPG concn. was 4.75 mM; each assay was initiated by the addition of 11 ug of C2 (PG), Table 5.5, following ultrafiltration and dialysis (referred to as C2 (D)).

[c] ONPG controls, lactulose and lactose were assayed in 50 mM potassium phosphate buffer pH 7.3

[d] ND, not determined

[e] ONPG-P at a final concn. of 6.8 mM was not hydrolysed when substituted for ONPG.

Fig 5.7 Hypothesised pathway for lactulose catabolism in C.perfringens



Abbreviations: AT, active transport;
sugar -P, sugar-phosphate; I, inhibition;
details of Leloir pathway omitted

The first step in the latter scheme is catalysed by galactokinase (GK).

Cells of C.perfringens and E.coli grown on 5 mM lactose, respectively, exhibited significant GK levels, enzyme activity being linear up to 20 min. (Fig. 5.8). Phosphorylation can occur either at C1 or C6 of the galactose substrate. In the Staph.aureus control, GK was absent (since lactose uptake occurs via the PEP:PTS; Kennedy and Scarborough, 1967).

Growth of C.perfringens on a number of sugars showed that GK was inducible, notably by galactose lactulose lactose (Table 5.8). GK activity in glucose and fructose grown cell-free extracts may represent basal repressed levels of the enzyme or the specificity for galactose of the general hexokinase found in C.perfringens (Groves and Gronlund, 1969b).

5.3.4 Phosphofructokinases

Phosphofructokinases (PFKs) may be implicated in intracellular lactulose dissimilation. Fructose-1-phosphate kinase (EC 2.7.1.5.6; FIPK) has been detected in clostridia which transport fructose by the PEP:PTS; fructose-6-phosphate kinase (EC 2.7.1.11; F6PK) activities were also reported but at between two- to five-fold lower levels than FIPK (Hugo and Gottschalk, 1974). In K.aerogenes, the intracellular metabolism of the fructose moiety of sucrose is mediated by a specific fructokinase, distinct from FIPK (Kelker et al., 1970).

Crude cell-free extracts from fructose grown cells had variable endogenous levels of NADH oxidase (NOx; between 9.97 - 17.27 nmole NADH oxidised min^{-1} under assay conditions for PFKs). In the data on FIPK and F6PK in fructose grown cell-free

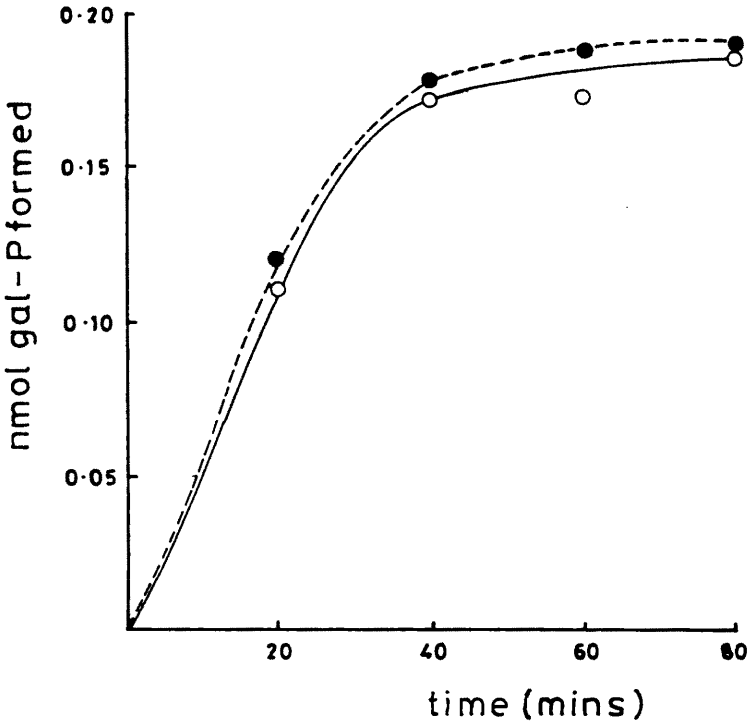


Fig. 5.8 The presence of galactokinase in selected organisms grown on lactose (5 mM); (●), E.coli, (○) C.perfringens; S.aurens cell-free extracts lacked GK activity. Growth and assay conditions are described in text. (Section 5.2.3).

Table 5.8 Galactokinase in C.perfringens grown on different carbohydrates

Sugar	nmole product min^{-1} in total extract
Glucose	0.20[a]
Fructose	0.03
Galactose	1.90
Lactulose	0.74
Lactose	0.39

[a] Activity corrected for total volume of cell-free extract from a cell suspension of 10^{10} viable cells; 10 µl aliquots used in assay as described in text

extracts (Table 5.9), NOx activity was low.

A single peak of FIPK activity was observed following chromatographic fractionation of the cell-free extract from fructose grown cells (Fig. 5.9). The cibacron blue dye covalently bound to cross-linked agarose gel sepharose (Blue Sepharose CL-6B) has some affinity for NAD^+ and ATP- requiring proteins; its use in the purification of PFK has been documented.

In a qualitative determination, mixing cell-free extracts with Blue Sepharose CL-6B material removed contaminating NOx activity in the supernatant (this effect requires further study). Table 5.10 indicates that a partial, five-fold purification of FIPK was achieved using the one-step affinity chromatography. FIPK activity in fructose-grown C.perfringens has not been previously reported. A succession of eluants applied as gradients failed to yield F6PK activity in subsequent fractions (Table 5.9). A clarification of this binding requires further work.

Similar procedures to assay for lactulose-specific PFKs awaits future work.

Table 5.9 Presence of two distinct phosphofructokinases in C.perfringens grown on fructose

Sample	Units ml^{-1}	
	FIPK	F6PK
Crude Extract	713.8[a]	934.9

[a] Units are nmole fructose phosphate consummed min^{-1} at 37°C; assay conditions described in text

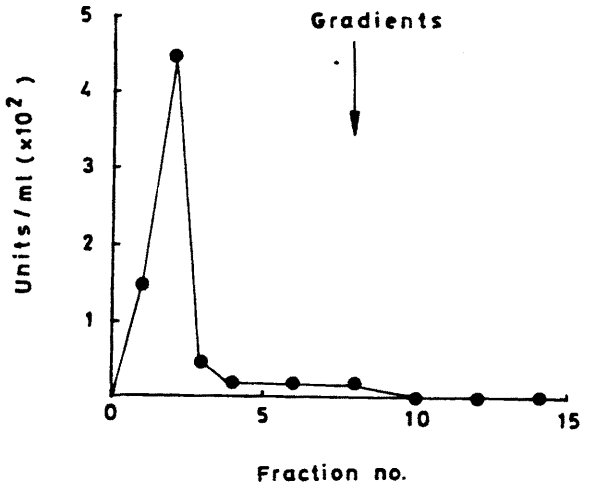


Fig. 5.9 Blue Sepharose CL-6B column chromatographic separation of FIPK from a crude cell-free extract of C.perfringens grown on 5 mM fructose. Conditions and assay procedures are described in Section 5.2.4. Successive NaCl, ATP, NAD^+ and $NADP^+$ gradients applied as indicated and described in the text failed to elute F6PK activity. Units are nmole FIP consummed min^{-1} at 37^oC.

Table 5.10 Partial purification of fructose-1-phosphate kinase FIPK from Cl.perfringens[a]

Sample	Total activity	Sp Act[b]	Purification
Crude Extract[c]	892.3	717.8	-
FIPK fractions	1466.4	3660.0	x 5

[a] Purification steps are described in the text

[b] Units of enzyme activity/mg protein

CHAPTER 6: PREFERENTIAL CARBOHYDRATE UTILISATION BY *CLOSTRIDIUM PERFRINGENS*

6.1 INTRODUCTION

Metabolic regulation is brought about at two levels, by changes in activity of preformed enzyme or other protein, and at the level of protein synthesis (Baumberg, 1981). The most familiar form of protein synthesis modulation is the catabolite repression phenomenon, now well characterised in E.coli (Ullman and Danchin, 1983; Ullman, 1985). For instance, enzymes of nitrogen catabolism, e.g. histidase (EC 4.3.1.3) show catabolite repression by glucose under conditions of nitrogen sufficiency (Niedhardt and Magasanik, 1957). The possibility of a similar substrate effect of lactulose in controlling nitrogen metabolism by colonic bacteria during PSE therapy (Vince et al., 1978) has as yet not been studied.

The aim of this section of the present study was to examine regulation at the level of protein synthesis or activity by evaluating susceptibility of the lactulose genome in C.perfringens to the glucose effect, and to other carbohydrates. Glucose may be available as a derivative of various dietary substrates, such as following bacterial metabolism of fibre (Vercelloti et al., 1978), and together with galactose and fructose has been detected in the gut following lactulose ingestion (Florent et al., 1985). Lactose is of interest as an isomer of lactulose.

6.2 MATERIALS AND METHODS

Materials and general methods were described previously (Chapter 2).

6.2.1 Use of permeabilised cells for β-galactosidase assay

Intracellular β-Glase activity was assayed by the cell-permeabilisation procedure of Putman and Koch (1975). A preliminary standardisation of the lytic technique was undertaken. A late-log culture (100 ml) grown on 5 mM lactulose was harvested, washed with 50 mM sodium phosphate buffer, pH 7.3 containing D-chloramphenicol (50 µg/ml) and resuspended to its original volume in the same buffer.

Losses of cell numbers during washing were found to be minimal, as judged by A_{612} values. A volume of washed cell suspension (1.0 ml) was treated with varied aliquots (0 - 500 µl) of the lysis mix (LM; 10% SDS : 0.02M manganous sulphate : toluene : 2-mercaptoethanol; 1:1:1:5, by vol.) in order to assess optimal permeabilisation and release of hydrolytic products.

The lysates (0.1 ml) were assayed for β-Glase activity as described previously (Section 2.2.5), and the results corrected for corresponding dilutions in total lysate volume (Fig. 6.1). The use of 25 µl of LM/ml cell suspension was chosen since 50 µl of LM created excessive turbidity from protein denaturation, which interfered with pipetting. However, the results (Fig. 6.1) showed that the plateau of enzyme activity allowed the treatment of samples of lower cell mass without the problem of excessive LM causing inhibition. Since up to 300 µl of LM could be tolerated, a significant increase in concentration of LM components indicates the remarkable stability of β-Glase, especially to SDS which tends to weaken hydrophobic bonds and thus denature proteins. The manganous cation may stabilise the enzyme, since its exclusion from the LM caused a 43% fall in observed enzyme activity; SDS was the main lytic agent, and its absence in the LM resulted in a 67% fall in enzyme activity (data not shown). When batches of washed cells harvested at various times were lysed and assayed routinely, the coefficient of variation was determined as ± 0.9% of the mean (data not shown), indicating a high degree of reproducibility. Assay time from cell harvest to recording ONPG hydrolysis was 20 - 25 min.

6.2.1.1 Hydrolysis of lactose and lactulose by permeabilised cells

In order to show that the lysis procedure did not alter the authentic properties of the enzyme, hydrolysis of lactose and lactulose was qualitatively tested in cell-lysates.

Cultures of C.perfringens (100 ml) grown on 5 mM lactose

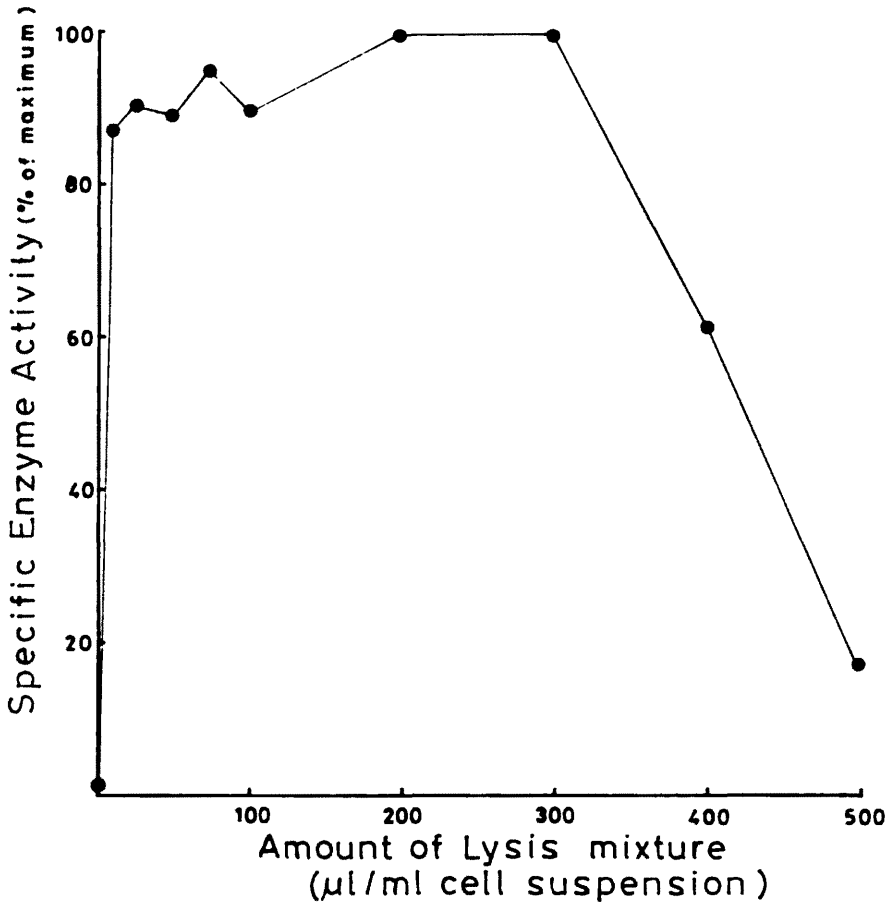
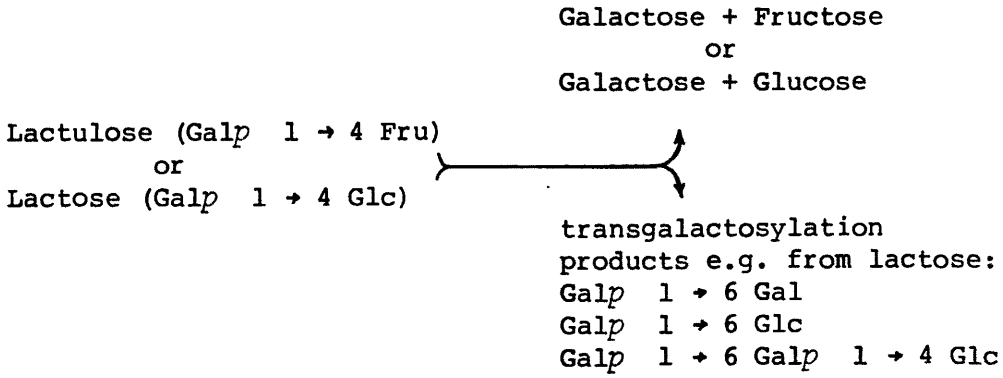


Fig 6.1

Release of cellular β -galactosidase activity as a function of the concentrations of lysis mixture. Known aliquots of a C.perfringens cell suspension were treated with varied amounts of lysis mixture, and the lysate assayed as described in Section 6.2.1.

or lactulose, were harvested after 8h, washed and resuspended in 60 mM potassium phosphate buffer, pH 7.3 containing 1 mM 2-mercaptoethanol and 50 µg/ml chloramphenicol (to 5 ml). Washed late log-phase cells of E.coli, grown aerobically in peptone-water broth, were similarly obtained to serve as standards. Hydrolysis of lactose by purified β-Glase from E.coli is well characterised (e.g. Wallenfels and Malhotra, 1961). Aliquots of bacterial suspensions and lysates (25 µl LM/ml) were used to determine disaccharide hydrolysis. The assay mixture contained (at 37°C): 0.03M $MgCl_2.6H_2O$ (0.2 ml), 3.36 M 2-mercaptoethanol (0.2 ml), whole cells or lysate (1.0 ml), and harvest buffer (2.6 ml). The reaction was initiated by the addition of 5% (w/v) lactose or lactulose (1.0 ml) in the same buffer. At various intervals, samples (2 µl) were removed, directly spotted on t.l.c. plates, and chromatographed as previously described (Section 2.2.4). The solvent used was ethyl acetate:pyridine:acetic acid:water (6:3:1:1; by vol.). The remaining cell suspensions were sonicated (Section 2.2.7; E.coli cells, 10 min; C.perfringens, 20 min), centrifuges and the supernatant assayed for disaccharidase activity following an overnight incubation.

A range of hydrolytic products were typically observed (Fig. 6.2). The unknowns (U1, U2 and U3) represent oligosaccharides generated by the transgalactosylation reactions of the β-Glase; U1 and U2 were common products generated by E.coli and C.perfringens extracts. The overall reaction may proceed in outline as follows:



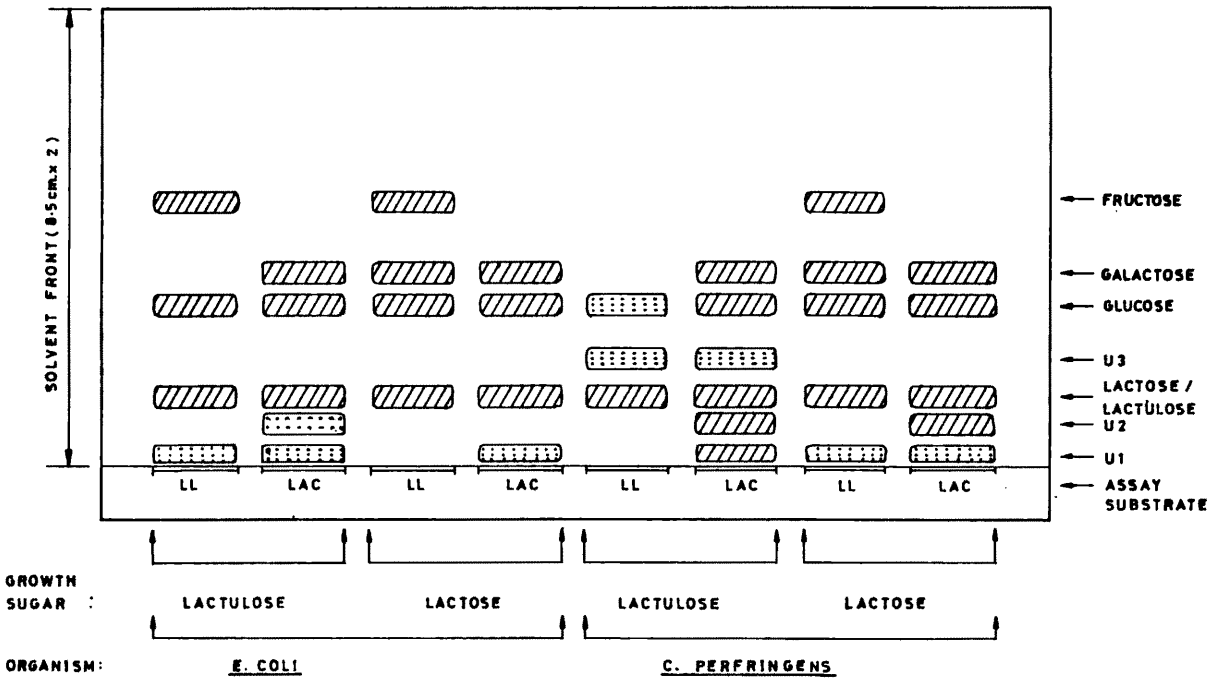


Fig. 6.2 Diagrammatic representation of a chromatogram showing hydrolysis of β-galactosides by cell-extracts of E.coli and C.perfringens grown on lactose and lactulose respectively. Assay details are described in Section 6.2.1.1. Hatched areas denote sugars giving a visual colour reaction, and dotted regions indicate identification under ultra-violet irradiation. U1, U2 and U3 are unidentified sugars.

Incubation of whole cells or lysates of either bacterium generally produced significant hydrolysis within 1 - 2h of incubation. Cell lysates appeared more effective than untreated cells, probably due to limitations in the rate of entry of substrates into intact cells. Hydrolysis of lactose or lactulose implied absence of a P-β-Glase in C.perfringens and E.coli; the latter enzyme has not been reported in E.coli cells grown on lactose.

Disaccharide hydrolysis was not observed when sodium phosphate buffer at various ionic strengths was used, whereas both Na^+ and K^+ ions permitted ONPG hydrolysis by permeabilised cells of both bacteria (data not shown).

6.2.2 cAMP assays

In two replicate experiments, late-log cultures (8 h) grown on 5 mM lactulose (250 ml), were harvested and the unwashed cells resuspended in 50 mM Tris-Hcl buffer, pH 7.5 containing 4 mM EDTA (to 5 ml). The cell suspension contained a mass of about 300 mg dry wt. Cell washes were avoided since Makman and Sutherland (1965) have reported decreased intracellular cAMP levels in stationary phase cells of E.coli as a result of washing. At this concentration, EDTA is a known phosphodiesterase inhibitor (cyclic AMP.Assay Kit, Product Information; 1977). An earlier report used 400-500 mg wet wt. of exponential phase cells of B.fragilis to assay intracellular cAMP levels (Hylemon and Phibbs, 1974).

The suspension was sonicated for 15 min in a water-cooled glass chamber at 4°C as previously described (Section 2.2.7), and the cell-free extract centrifuged at 10000 g for 10 min at 4°C. Aliquots of the supernatant were extracted as follows (see Cooper et al., 1972; Albano et al., 1974). Firstly, 10% trichloroacetic acid (w/v) (1.0 ml) was added to the cell-free extract (2.0 ml), and the ppt. centrifuged

at 10000 g for 15 min at 4°C. Trichloracetic acid was removed from the supernatant by four successive extractions with 10 vols of water-saturated diethyl ether. Traces of ether were removed by aspirating with a stream of N_2 as the extract was heated to about 60°C in a water bath. Samples of the aqueous extract (50 µl x 2) were assayed for cAMP as described below.

Extracellular cAMP was assayed with the following method. Culture fluid (20 ml), was mixed with ice-cold methanol (10 ml), the protein ppt. removed by brief centrifugation (approx. 2000 g for 10 min at 4°C) and the supernatant lyophilised to dryness. After resuspension in cold distilled water (4 ml), samples (50 µl x 2) were removed for routine assays.

A Cyclic AMP assay kit was used to determine cAMP concentrations. The assay method has a detection limit of 0.05 pmol. (Product Information, 1977) and is based upon the competition between unlabelled cAMP with a fixed quantity of the 3H-labelled compound for binding to a protein which has a high specificity for cAMP. The procedure is summarised as follows. All operations were conducted at 2 - 4°C in a cold room, 0.05 M Tris-Hcl buffer, pH 7.5 containing 4 mM EDTA (50 µl) was pipetted into a microcentrifuge tube and unknown (50 µl) or unlabelled cAMP standard (1 - 16 pmol; 50 µl) added, followed by (8 - ^{3}H) cAMP (5 µCi/180 pmol; 50 µl) and cAMP-binding protein (100 µl). After mixing, the assay tubes were left for about 100 min and a charcoal suspension (100 µl) then added. After a further 2 h, the samples were centrifuged and supernatant aliquots (200 µl) removed for radioassay. Radioactivity was determined following addition of distilled water (0.8 ml) and a scintillant cocktail (10 ml) containing Triton x-100: toluene:PPO (1 l; 2 l; 10 g).

6.3 RESULTS AND DISCUSSION

6.3.1 Growth on various carbohydrates: preliminary observations

Initially, utilisation of different sugars by C.perfringens was assessed by individual incubations (Table 6.1). All sugars supported growth in excess of basal medium, in contrast to previously reported data (Groves and Gronlund, 1969a). However, experimental and strain differences may account for this variation. High β-Glase activities were found in cells grown on lactose or lactulose, and basal enzyme activity noted in the absence of sugar and in the presence of glucose, fructose and galactose (Table 6.1).

Table 6.1 Utilisation of sugars by C.perfringens

Sugar[a]	Growth (A_{612})	pH	Sugar utilised (%)	β-Glase activity[c]
Lactose	1.50	4.7	52	2.07
Lactulose	0.95	5.7	17	1.05
Fructose	1.45	4.7	ND[b]	<0.14
Glucose	1.26	4.5	75	<0.14
Galactose	1.22	5.4	48	<0.14
None	0.73	6.0	-	<0.14

[a] Sugar concn. 0.5% (w/v); all incubations for 8h

[b] ND - not determined

[c] Units of enzyme activity are μmol o- itrophenyl released min^{-1} ml culture $^{-1}$

Based on growth yields, the following order of preference was indicated: lactose > fructose > glucose > galactose > lactulose. The preference for lactose over fructose has similarly been noted in S.lactis, (Thompson et al., 1978). The range of carbohydrates utilised enabled classical diauxic

studies with lactulose containing mixtures. A possible inhibitory effect of low pH and fermentation acids was minimised by reducing sugar concentrations in cultures to 5 mM (0.09%; $^{w}/v$).

6.3.2 Growth on carbohydrate mixtures

6.3.2.1 Glucose and lactulose

The preference of bacteria for glucose from mixtures containing other carbon sources is well known (Section 1.4.3). Originally termed the glucose effect (Monod, 1947), the phenomenon involves catabolite and transient repression and inducer exclusion (Magasanik, 1961; Saier, Jr. and Moczydlowski, 1978).

Growth of C.perfringens on a mixture of glucose and lactulose was found to result in classic diauxiae (Fig. 6.3). Glucose was preferentially utilised without an apparent lag phase and during its catabolism, β-Glase synthesis was repressed. Between 2 - 4 h, the exponentially growing cells (doubling time, T_D ~1.3 h) fermented glucose rapidly at a rate (R_s) calculated as ~0.27 µmol h^{-1} log. viable cells^{-1}. This *in-vitro* rate of growth is remarkably high when contrasted to *in-vivo* doubling times for intestinal anaerobes of between 12 - 24 h (Gibbons and Kapsemalis, 1967). Glucose was completely fermented by 4h, and stationary phase ensued by 6h, with an external pH of 5.6. This growth pattern closely resembled that observed with a glucose control culture (Fig. 6.4), with a biphasic disappearance of the sugar (0 - 2h, 8% of the original concn. utilised at R_s ~ 0.03; 2 - 4h, 92% utilised at R_s ~ 0.31).

The uptake of lactulose in the mixture induced β-Glase synthesis between 4 - 6h (Fig. 6.3). The increase in β-Glase activity after 4h was linear to maximal levels by 18h, with a specific activity (sp.act.) of 0.50. In comparison, growth on lactulose in a control (Fig. 6.4) indicated maximal sp. act. of 0.65 between 6 - 8h, with a

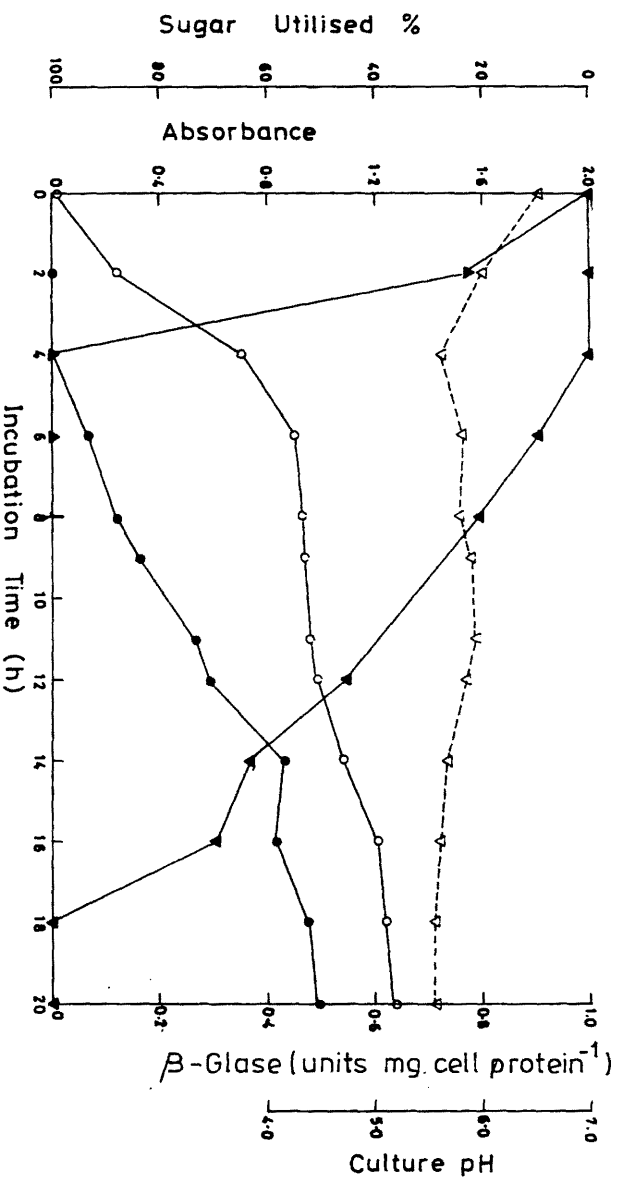


Fig. 6.3 Growth of C.perfringens on a mixture of glucose and lactulose (each at 5 mM). Symbols: (o) A_{612}; (●) β-Glase; (▽) pH; (▲) glucose; (▼) lactulose.

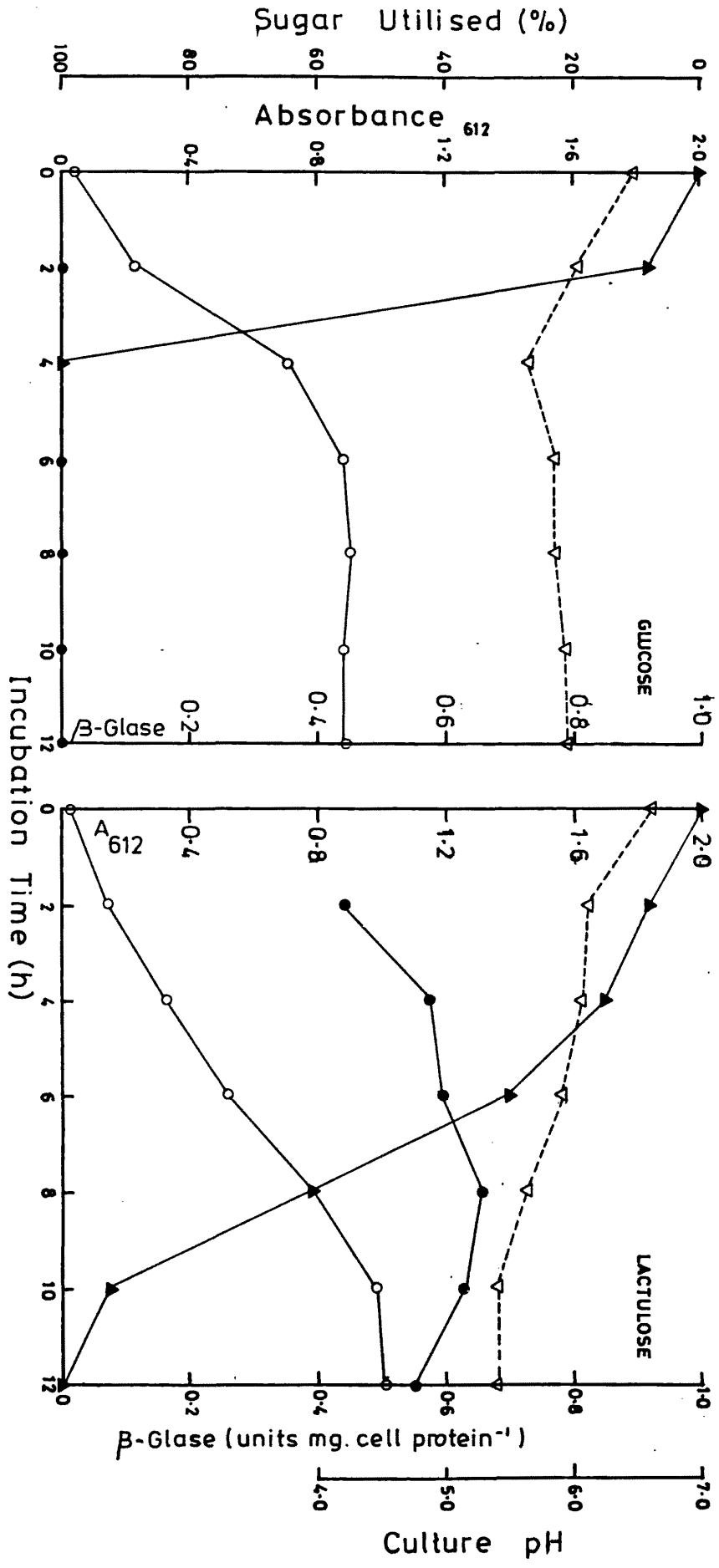


Fig. 6.4

Growth of C. perfringens on glucose or lactulose respectively (5 mM). Symbols (○) A612; (●) β-Glase; (▽) Culture pH; (▲) sugar utilised.

lag of < 2h for β-Glase induction. A protracted lag between 6 - 12 h in the mixed sugar incubation was paralleled by a slow rate of lactulose dissimilation (R_s ~0.03 between 4 - 16h). Disaccharide fermentation during this period probably meets energy requirements for renewed protein synthesis and homeostatic maintainance. During the exponential phase (12 - 16 h; T_D ~ 13.2 h) the rate of lactulose metabolism was unchanged (R_s ~ 0.03). The onset of stationary phase (16 - 20h) coincided with depletion of lactulose, and probably accumulation of toxins in the medium.

The maximal rate of lactulose utilisation (R_s ~ 0.13; 6 - 10h) was about half that of glucose degradation (R_s ~ 0.27) in respective control incubations (Fig. 6.4).

It was subsequently of interest to study the mechanism of the glucose-induced diauxiae of the lactulose regulon in C.perfringens. In previous reports, externally supplied cAMP has been shown to overcome the glucose effect in enterobacteria (Perlman et al., 1969; Saier, Jr. and Moczydlowski, 1978) and toluene-treated streptococci (Hamilton and Lo, 1978). Although there is a paucity of information on the presence of cAMP in clostridia, it was reported that addition of cAMP enhanced glucose consumption by sporing cells of C.botulinum (Emeruwa and Hawirko, 1975).

Consequently, in the present study, glucose and glucose with cAMP (at 1 mM and 5 mM concentrations) were added at 3h to respective lactulose-induced cultures (Fig. 6.5). The β-Glase activity was assayed 20 min after addition of glucose with and without cAMP. A significant inhibition of β-Glase synthesis was observed after this period from both additions. Decreased β-Glase levels (to about 60% of control total enzyme activity) were not due to dilution by growth as increases in cell mass (A_{612} values) were negligible during the

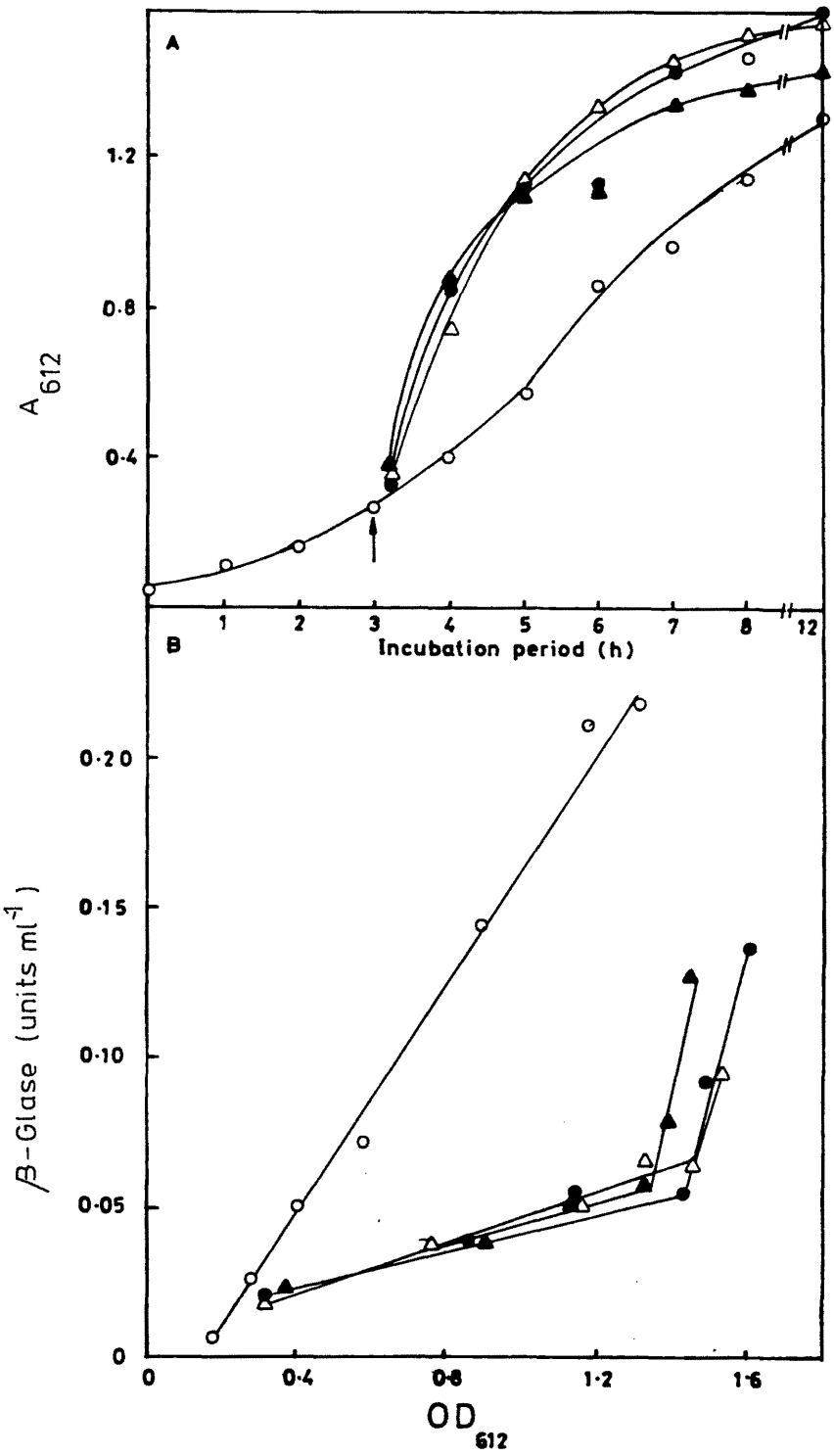


Fig. 6.5 Effect of glucose (± cAMP) on β-Glase synthesis in C.perfringens grown on 5 mM-lactulose. At the time indicated (arrow; A) the following additions were made to respective cultures: none, (○); 5 mM-glucose, (●); 5 mM-glucose + 1 mM-c-AMP, (△); 5 mM-glucose + 5 mM-cAMP. Turbidity and β-gal assays were performed 20 min after additions, as described in Section 5.2. Enzyme activity units are μmole ONPG hydrolysed min^{-1}.

20 min period. And, cAMP (Fig. 6.5) and dibutyryl cAMP (5 mM; data not shown) failed to aleviate the severe repression of β-Glase synthesis exerted by glucose.

The inability of exogenous cAMP to alleviate glucose-induced transient repression of the lactose operon in S.salivarius has been shown to be due to cell impermeability to the nucleotide, as assessed by (^{14}C) cAMP filtration assays (Hamilton and Lo, 1978). These authors used toluene (10 µl/ml) to permeabilise cells during growth, and addition of cAMP reversed transient repression caused by glucose. Permeability of C.perfringens cells to cAMP could not be measured in the present study due to methodological limitations of a filtration assay (see Section 5.3). Therefore, the effect of various amounts of toluene (2.5 - 10 µl/ml) on growth of C.perfringens was examined. In all cases, inhibition of growth occurred and consequently permeabilisation of cells to allow growth and enzyme induction was not feasible. Direct assays for cAMP in lactulose grown cells or extracellular fluid failed to detect the cyclic nucleotide with the assay procedures used (Section 6.2.2.).

Other reports have noted the absence of cAMP in Bacillus megaterium (Setlow, 1973), B.fragilis (Hylemon and Phibbs, 1974) and L.plantarum (Sayhoun and Durr, 1972). It is therefore possible that other modulators may be involved in the glucose effect on the lactulose genome in C.perfringens. It would be of interest to differentiate in further studies the two regulatory aspects, inducer exclusion and the kinetically differing transient and permanent repression, of this glucose effect. Some experimental approaches are discussed below.

The inducer exclusion phenomenon has been demonstrated in E.coli (Perlman et al., 1969). Following induction of the lac permease by isopropyl-β-thiogalactoside (IPTG), (^{14}C) thiomethyl-β-D-galactoside

(TMG) uptake was assayed in a control and in the presence of glucose (± cAMP). Glucose was found by the latter authors to inhibit (^{14}C) TMG uptake by 43%, which was not relieved by cAMP. The use of a gratuitous inducer at high external concentration thus can circumvent inducer exclusion because uptake occurs in the absence of a specific transport system (Paigen and Williams, 1970). The analogue can be maintained at internal concentrations sufficient to ensure induction even in the presence of an active exit process (Ullman and Danchin, 1983). A separate study is therefore required to locate suitable gratuitous inducers of the lactulose genome in C.perfringens prior to investigating catabolite/transient repression by glucose.

6.3.2.2 Fructose and lactulose

The provision of a fructose and lactulose mixture led to a preferential utilisation of the monosaccharide by C.perfringens (Fig. 6.6). This effect appeared to be dependant on the concentration of inhibitory sugar. The diauxic pattern of growth was not observed. However, such a pattern may be absent despite sequential sugar utilisation (Russell and Baldwin, 1978). Initial fructose uptake (0 - 2h) was utilised at R_s ~ 0.07 (compared to ~ 0.08 in a fructose control; Fig. 6.7). After 2h, β-Glase activity was higher than the basal activity in the fructose control, although lactulose was apparently not utilised. Lactulose uptake for β-Glase induction was observed only after exhaustion of glucose from the medium in the previous experiment (Fig. 6.3). A period of co-utilisation of fructose and lactulose may exist to allow cells to maintain their rapid rate of growth between 2 - 4h (with resultant T_D ~ 1.2h). About 80% of the extracellular lactulose was utilised by 8h following fructose depletion and maximal β-Glase levels were observed after 8h (Fig. 6.6).

The mechanism of the fructose effect is not clear. Regulation by transient/catabolite repression of enzyme synthesis

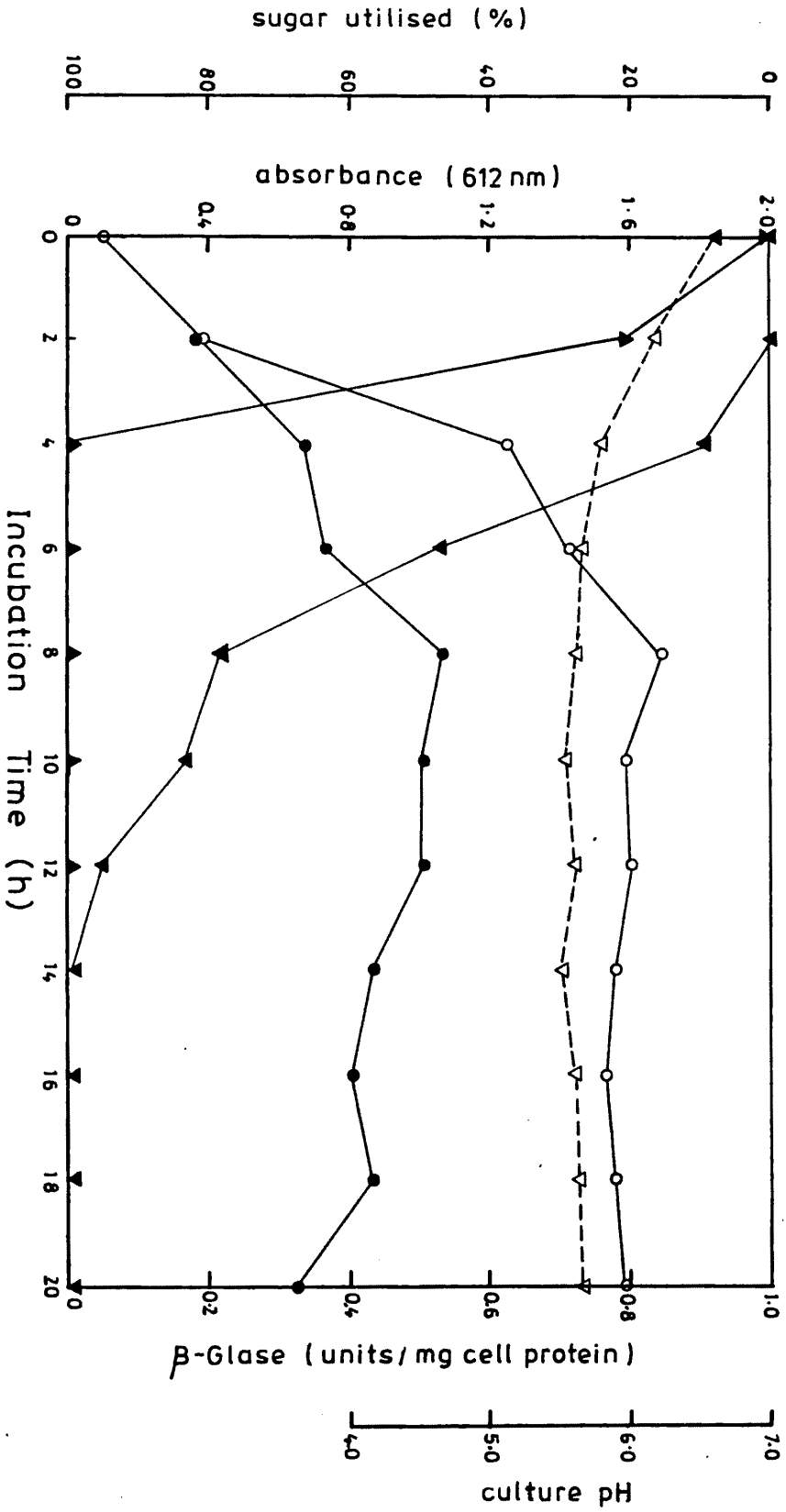


Fig 6.6

Growth of C. perfringens on a mixture of fructose and lactulose (each at 5mM); Symbols: (○) A612; (●) β-Glase activity; (▽) pH; (▲) fructose and (▼) lactulose utilisation.

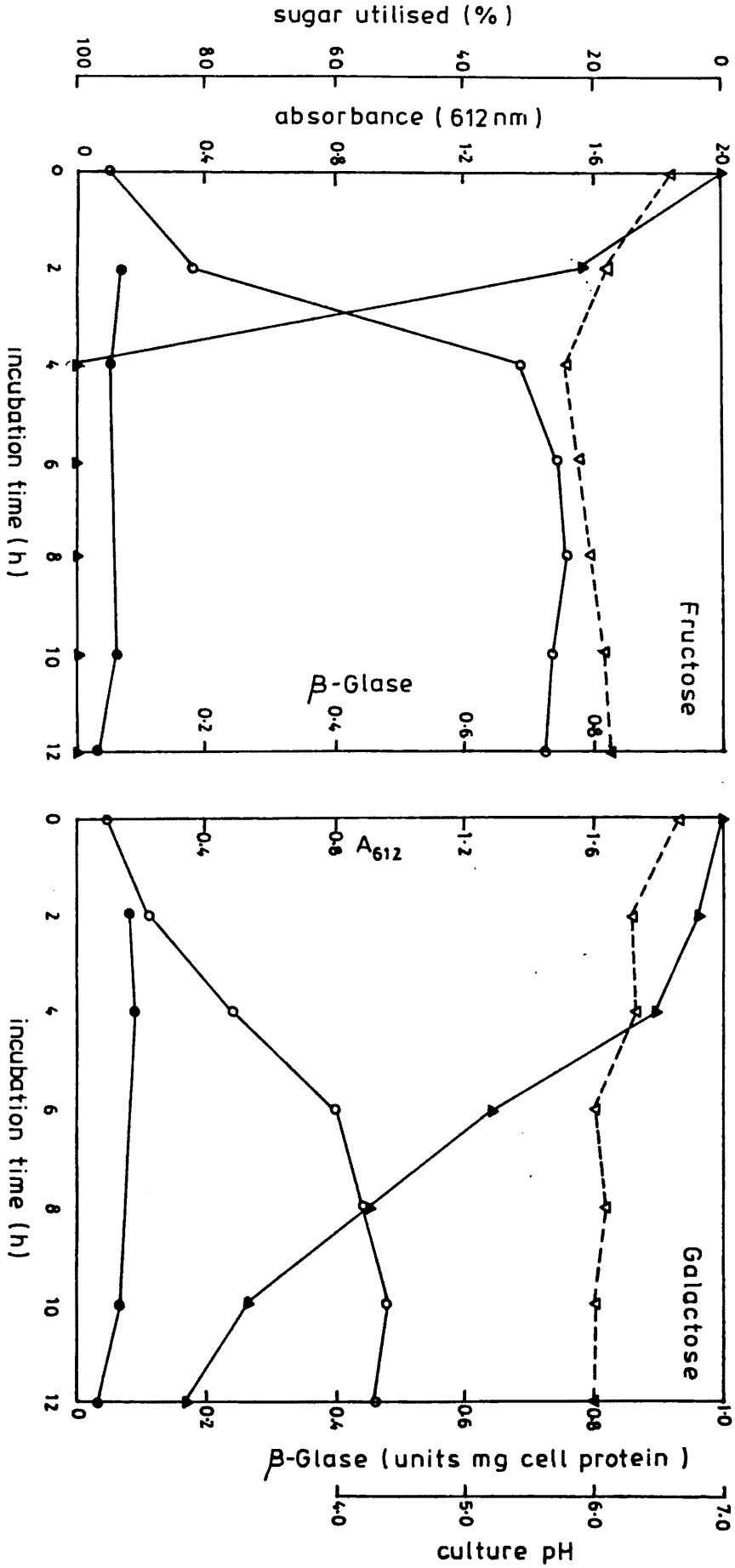


Fig 6.7

Growth of C. perfringens on fructose and galactose respectively (5 mM). Symbols: (○) A612; (●) β-Glase; (▽) pH; (▲) sugar utilised.

appears unlikely as β-Glase was induced by 2h despite the presence of a large amount of fructose in the medium (about 80% of original concn.; Fig 6.6). By preventing entry of inducer, a secondary effect of the inducer exclusion mechanism is to prevent enzyme synthesis. However, a complete inhibition of inducer uptake is necessary for this secondary phenomenon to be efficient. Perlman et al., (1969) found that inducer exclusion accounted for a 43% inhibition of (^{14}C) TMG uptake by the lac permease in E.coli. Consequently, it is feasible that fructose partially excludes lactulose entry in C.perfringens when external concentration of monosaccharide is sufficiently high. Energy-linked controls, mediated by cellular metabolites and regulating enzyme activity (Baumberg, 1981), may be involved in preventing lactulose uptake and catabolism. These possibilities require further study.

The rapid rate of fructose utilisation between 2-4h in the control (R_s ~ 0.22; Fig. 6.7) and mixed-sugar incubations (R_s ~ 0.23; Fig. 6.6) suggested maximal induction of the permease (s) responsible for fructose uptake. In E.coli, fructose translocation occurs by active transport or by the PEP:PTS depending on external sugar concentration (Cooper, 1978). Reported data on fructose uptake in various clostridia suggest a preponderance of the PTS (Hugo and Gottschalk, 1974). In an earlier aspect of the present study, both fructose 1-phosphate kinase and fructose 6-phosphate kinase were identified in cell-free extracts of C.perfringens grown on fructose (Section 5.3). However, as a non-specific hexokinase has been reported in glucose grown C.perfringens (Groves and Gronlund, 1969b), the identity of the fructose permease based on the presence of appropriate enzymes is as yet speculative. Further studies are required to examine (a) whether the fructose and lactulose transport systems respectively exhibit mutual affinity; and (b) the possibility that fructose released

from lactulose in the cell shares the initial catabolic sequence present in a fructose control.

6.3.2.3 Galactose and lactulose

A simultaneous uptake of galactose (R_s ~ 0.04) was observed after 2h. The log phase between 2 - 4h (T_D ~ 0.97) was paralleled by a rapid rate of β-Glase synthesis (Fig. 6.8) and high rates of sugar utilisation (R_s galactose ~ 0.08; R_s lactulose ~ 0.07). Galactose did not induce β-Glase in a control incubation (Fig. 6.7). The uptake of galactose between 4 - 6h appeared faster than the rate of lactulose uptake from the medium (Fig. 6.8). The catabolism of 2 mol of galactose, from the concurrent fermentation of galactose and lactulose in the cell, did not appear to affect fructose metabolism, as excretion of the ketohexose was not observed (Fig. 6.8). As expected, the control growth on galactose indicated relatively poor growth (Fig. 6.7), and a gradual assimilation of the sugar (R_s ~ 0.07) between 4 - 10h; after 12h, galactose was not depleted from the medium. Basal levels of β-Glase were observed throughout the latter incubation.

The fate of the combined intracellular accumulation of galactose and lactulose requires further study. It would be advantageous to the cell, in terms of conserving energy (e.g. for protein synthesis), if galactose and lactulose were translocated and fermented by the same pathway. Earlier experiments in the present study (Chapter 5) indicated that lactulose uptake is probably electrogenic. Galactose accumulation in C.pasteurianum has been shown to occur by active transport (Booth and Morris, 1975). The lac permease in E.coli is able to bind galactose, lactose and lactulose (Sandermann Jr., 1977). It is noteworthy that in S.lactis, galactose uptake may be energised by active transport and the PEP:PTS (Thompson, 1980).

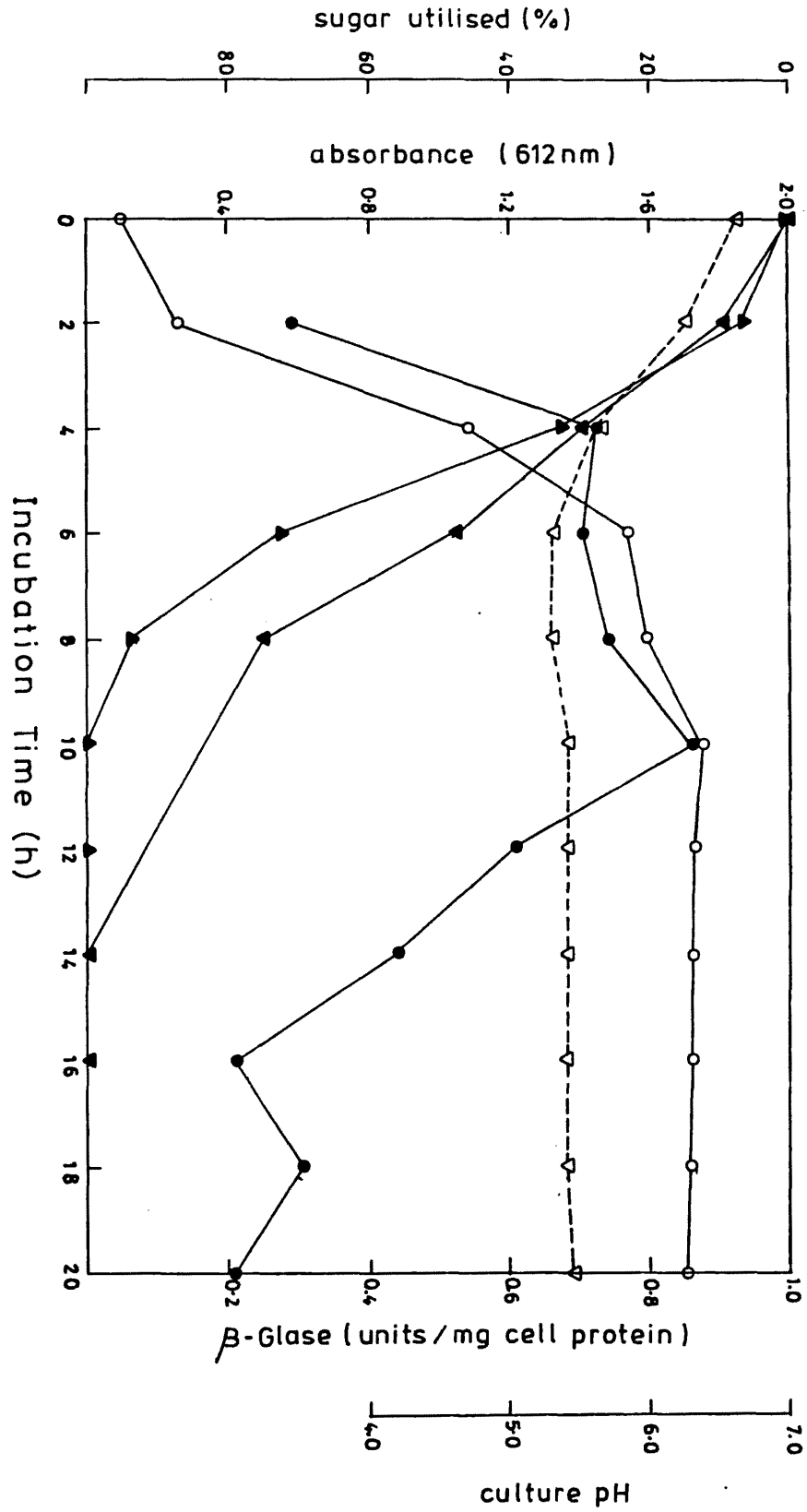


Fig 6.8

Growth of C. perfringens on a mixture of galactose and lactulose (each supplied at 5 mM concn.). Symbols: (○) A612; (●) β-Glase; (▽) pH; (▲) galactose and (▼) lactulose utilised.

6.3.2.4 Lactose and lactulose

A lag phase of about 2h was observed during growth of C.perfringens on an equimolar mixture of lactose and lactulose (Fig. 6.9), inducing near maximal levels of β-Glase (sp. act. ~ 0.82) after 2h. This level of β-Glase expression was significantly higher than observed in the lactulose control (sp. act. ~ 0.44; Fig. 6.4) and comparable to a lactose control (sp. act. ~ 0.73; Fig. 6.10).

During the 2 - 4h log-phase (T_D ~ 0.70), a sequential and subsequently, a simultaneous fermentation of the two disaccharides was observed (Fig. 6.9). During the first hour of this phase (2 - 3h), about 35% of the original lactose was preferentially utilised (R_s ~ 0.22), with the appearance of extracellular galactose (0.23 mM). Lactulose was then translocated between (R_s ~ 0.27) 3 - 4h to be co-utilised with lactose (R_s ~ 0.32). By 4h, over 90% of the lactose had been catabolised, whilst about 50% of the original lactulose quantity remained. The excreted galactose in the medium was also found to be 0.23 mM. A maximal peak of β-Glase activity in the cell was noted between 2 - 3h; enzyme synthesis was probably terminated between 3 - 4h, indicating an excess of cellular requirements. The remaining lactose was metabolised by 5h, whereas its keto-analogue was more gradually utilised to completion by 7h. A protracted late log-phase was observed, between 4 - 7h, during which period lactulose provided the main carbon and energy source.

Initial observations indicated that lactose is translocated at a rate comparable to monosaccharide uptake (Figs. 6.4, 6.6 and 6.10) but faster than lactulose uptake (Fig. 6.4).

Lactose appeared to induce β-Glase more efficiently than lactulose. This observation suggests that induction of β-Glase in the cell is more strongly repressed by lactulose than lactose. The

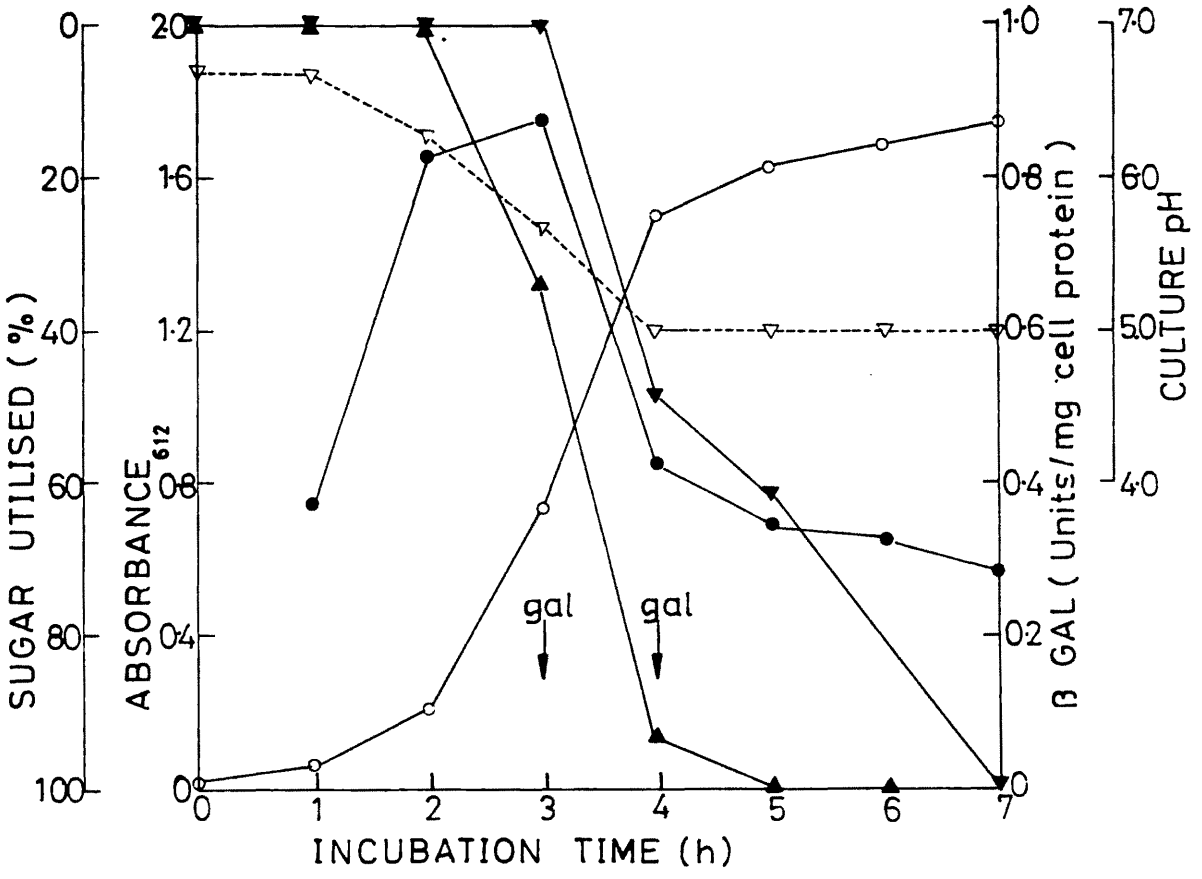


Fig. 6.9 Growth of C.perfringens on an equimolar (5 mM) mixture of lactose and lactulose. Symbols: as in legend to Fig. 6.3, except for (▲) lactose, (▼) lactulose, gal = galactose.

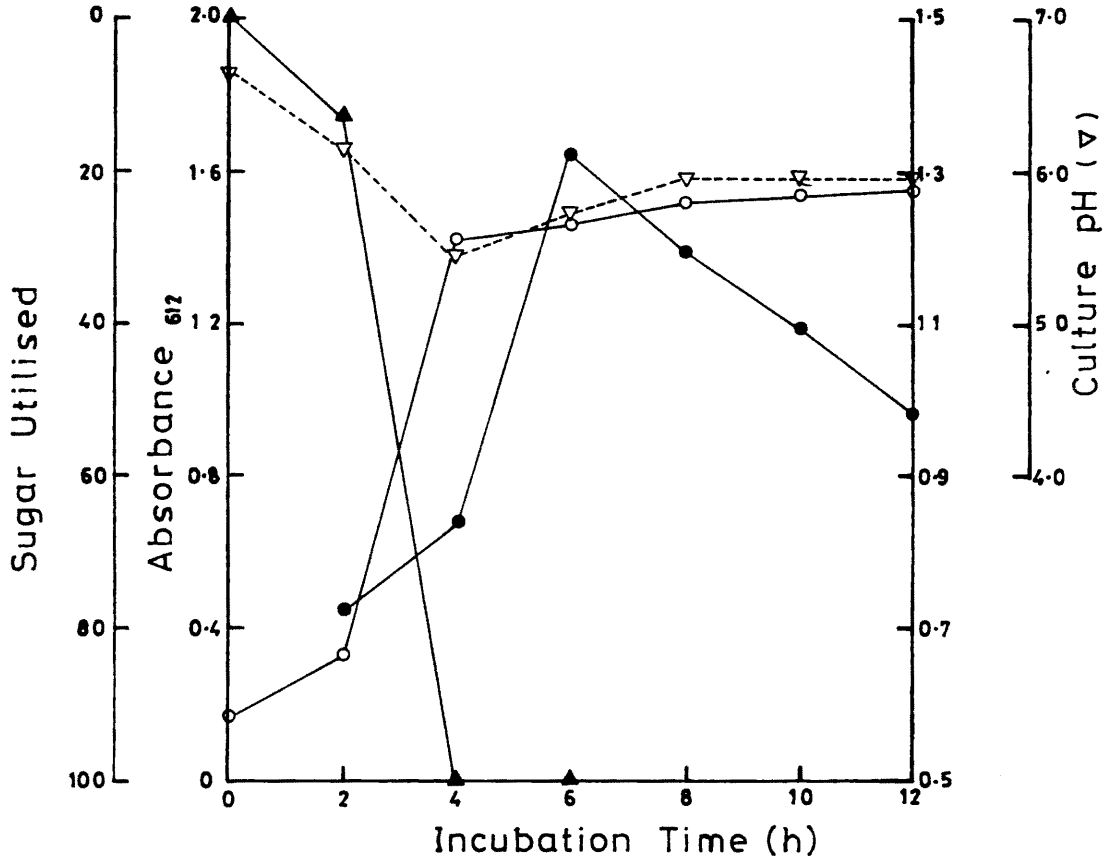


Fig. 6.10 Growth of C.perfringens on lactose (5 mM); symbols are as in legend to Fig. 6.3, except (▲) denotes lactose.

addition of IPTG to cells of S.salivarius has been found to induce higher levels of β-Glase than lactose, indicating a partial repression in the presence of the natural inducer (Hamilton and Lo, 1978). The *in-vivo* inducer of the lactulose genome is unknown. A range of trans-galactosylation products, as yet unidentified, were observed following β-Glase hydrolysis of lactulose (Section 6.2.1.1). Potential inducer activity of these oligosaccharide products, comparable to the allo-lactose product of the same reaction in E.coli, (Huber et al., 1976), requires further study. Iso-enzymic forms of β-Glase may be present for lactose and lactulose respectively; alternatively, there may be differing kinetic responses to the two substrates by a single enzyme species. Similarly, the specificity and identity of the permease system (s) is another aspect which requires a separate study.

The extrusion of galactose may provide a regulatory mechanism to remove excess carbon until required. The mechanism of exit of the monosaccharide is also of interest, requiring further examination, and may occur by proton-linked permeases, as in the case of efflux of β-Glase products from E.coli, (Huber et al., 1980). It is significant to note that, as expected, neither glucose nor fructose were excreted, suggesting that a preference for the three monosaccharides may mimic the order (i.e. fructose > glucose > galactose) observed when the organism was cultured on respective sugars (Table 6.1).

The absence of detectable monosaccharides in the control lactose incubation (Fig. 6.10) and previously with lactulose (Fig. 6.4) suggests that intracellularly released monomers may be fermented simultaneously. This requires verification by further studies.

CHAPTER 7: CONCLUSIONS

A review of the literature (see Chapter 1) indicates a general agreement on the effectiveness of lactulose in the clinical treatment of PSE, and suggests that, in the main, bacterial metabolism of the disaccharide is necessary to suppress formation of nitrogenous toxins implicated in the pathogenesis of PSE, especially ammonia. Studies on the mode of lactulose efficacy have not examined which specific bacteria are responsible for lactulose breakdown nor quantitated the amount and rate of its metabolism.

Consequently, the first aim of the present study was to identify the lactulolytic abilities of bacterial species representative of the major genera present in the gastro-intestinal tract. This study differed from previous investigations (Hoffman et al., 1964; Ruttloff et al., 1967) in several respects (discussed in Section 4.1). Sixty-four strains of bacteria were cultured under anaerobic conditions and quantitatively assessed for lactulose utilisation, pH change of media, and non-gaseous fermentation products, which were also estimated for selected species throughout their growth cycles; these fermentation products of pure cultures were compared with those from in vitro faecal homogenates incubated with lactulose (Sahota et al., 1982). Furthermore, cells from induced lactuloytic bacterial strains were also assayed for β -Glase activity.

The amount of lactulose fermented over a 48 h period varied significantly, not only from genus to genus, but also between species of the same genus. The main lactulolytic species were C.perfringens (utilising between 33-58% of sugar supplied; Table 4.7), Lactobacillus sp. (21-47%), L.casei sub casei (33%; Table 4.3), Bifid.bifidum (30.5%; Table 4.7) and two bacteroides species

B.oralis (15-21%) and B.vulgatus (< 5-21%; Table 4.10). The majority of the organisms, however, fermented an 'intermediate' (5-20%) amount of the disaccharide, including coliforms, bacteroides, streptococci and all but two species of clostridia. Some 14% of the 64 strains examined showed negligible (< 5%) utilisation of lactulose, including eubacteria which are quantitatively important in the gut (10^{10} per g faeces; Drasar and Hill, 1974).

The amount of lactulose utilised by a particular species in vivo will depend upon availability of the sugar in that region, the metabolic status of the bacterium, adaptability to inter-bacterial competition for the substrate, and tolerance to pH changes induced by lactulose fermentation as well as resistance to inhibition by end-products of fermentation. Monitoring growth of C.perfringens, B.vulgatus and Lactobacillus sp. over a 24 h period (Figure 4.1) indicated that in all three cases, the total quantities of lactulose utilised were similar to those at the 48 h stage in the earlier screening experiments. The rapid rate of lactulose fermentation by C.perfringens, calculated as ~0.13 umol sugar utilised h^{-1} log viable cells^{-1} during growth on a low concn. (5 mM; Fig. 6.4) coupled with a 2 h lag phase for induction of β-Glase, indicates an important role for this bacterium in vivo, in this respect. Both B.vulgatus and Lactobacillus sp. metabolised the majority of the disaccharide in the stationary phase, an example of deferred substrate utilisation (Figure 4.1). The constitutive β-Glase observed in all bacteroides isolates (Table 4.9) would suggest an immediate fermentation of lactulose on availability by bacteroides strains. Even those bacteroides isolates utilising an 'intermediate' (5-20%) amount of the sugar may be

important, as growth of bacteroides strains on monosaccharides in continuous culture indicates a maintainance coefficient of less than 0.1 g carbohydrate per g bacteria per h; this means that they have relatively high growth yields even at growth rates as slow as 35 h per generation (Salyers, 1984). A similar estimation of growth rates of selected bacteroides and other lactuloytic species in continuous culture may enable further extrapolations of *in vitro* results. The uptake of glucose by *B.thetaitaomicron* is partially chanelled into a polyglucose storage product (Hylemon *et al.*, 1977); the possibility of a similar fate for lactulose accumulation in bacteroides cells requires further study. This trait may contribute to their ability to compete successfully in the colonic ecosystem following lactulose ingestion. Consequently, a numerical predominance of a bacterial species which utilises lactulose is in itself not the sole determinant of its importance in metabolising the unabsorbed disaccharide. The concentration of lactulose used in this study for screening, 40 mM (approx. 13% $^{w}/v$) represents an estimated amount typically achieved in the colon during PSE therapy (Vince *et al.*, 1978). From the data of chronic lactulose ingestion in normal subjects, lactulose is available in ileal or caecal fluid for a period of about 8 h in respective regions, with peak concentrations of between 40 - 70 mM lactulose observed in caecal fluid (Florent *et al.*, 1985). The ileo-caecal region may be most important in lactulose dissimilation by the resident microflora (Bown *et al.*, 1974; Savage, 1977; Hill, 1982).

Lactulose utilisation generally increased turbidity (A_{612}) of cultures with a fall in pH of between 1.3-2.0 units in most strains of *C.perfringens*, bacteroides (except *B.thetaitaomicron*),

and P.vulgaris (Tables 4.6, 4.7, 4.10). Acidification of growth medium after 48 h was marked in lactobacilli cultures, ranging between 2.0-2.7 units (Table 4.3), with the most acidic culture pH being 4.0. Lactobacilli are able to maintain an intra-cellular pH of 6.5 even in the presence of an extracellular pH of 3.5 or less (Padan et al., 1981). Acidification of colonic contents to below pH 5.5 during lactulose therapy (Conn and Lieberthal, 1979) should favour growth of lactulolytic lactobacilli. Florent et al., (1985) observed a sharp decline in caecal fluid pH (to < 5.0) 3 h after ingestion of lactulose on day 8; this value returned to basal levels in all subjects. The tolerance of all bacterial strains demonstrating growth on this sugar to various pH conditions, as well as sensitivity to fermentation products described below, is a question for future investigations.

Assays for β-Glase activity in cells of bacteria grown on lactulose indicated an inducible enzyme in C.perfringens, some enterobacteria, but constitutive in C.butyricum and P.vulgaris (Tables 4.6 and 4.9). The lactobacilli and streptococci did not exhibit β-Glase activity (Table 4.6); a P-β-Glase may be involved, and this requires further study. Assays for β-Glase and P-β-Glase are necessary in all lactulose utilising strains, due to their co-expression in certain streptococci (see Section 5.1). These results in the present investigations also suggest that prokaryotes are relatively non-specific with respect to β-D-galactoside metabolism, in contrast to human intestinal β-Glase (Dahlqvist and Gryboski, 1965). In the study of chronic lactulose ingestion, faecal β-Glase concentration was markedly increased after 8 days (Florent et al., 1985).

A quantitation of the non-gaseous fermentation products of lactulose fermentation was undertaken by comparing types and amounts of products during growth on basal medium with or without lactulose. Both qualitative and quantitative differences were found on addition of lactulose to the medium. A similarity of these fermentation products to those reported when the same organisms are cultured on glucose (Holdeman et al., 1977) suggested that similar glycolytic pathways are operative for both carbohydrates. This is further evidence for the probable value of any rapidly fermentable carbohydrate in PSE treatment, provided it's use is clear of clinical side-effects. These data also enabled the type of fermentation carried out by lactulolytic bacteria to be identified. In the absence of (^{14}C) lactulose, the contribution of end-products from metabolism of non-carbohydrate substrates in basal medium could not be studied. A quantitation of gases released during fermentation is also necessary to fully assess carbon recovery, and requires future work.

Generally, large increases in acetic, lactic and butyric acids were found in lactulose-containing medium, with butyric acid being a characteristic product of clostridia. The products of clostridial fermentation of lactulose were butyric > acetic $\geqslant$ lactic acids (Table 4.8) and CO_2 and H_2, typifying the butyric type fermentation of hexose by clostridia (Wood, 1961). Lactic acid was only detected during the mid-log phase of C.perfringens growth on the disaccharide (Fig. 4.1); Pappenheimer and Shaskan (1944) observed the generation of lactic acid with depletion of iron in glucose-containing medium. A number of lactobacilli, Lactobacillus sp., L.casei sub.casei and L.acidophilus exhibited a homolactic fermentation of lactulose (Table 4.4), whereas L.brevis largely yielded

lactic acid and a small amount of acetate. Homofermentative lactobacilli usually employ the EMP pathway for hexose dissimilation (Section 1.4.2.1). All bacteroides species fermenting lactulose invariably yielded acetic, lactic and succinic acids (Table 4.11). Gas evolved during growth of B.fragilis on lactulose was identified as H_2; lactic acid was not observed during a 24 h growth experiment, possibly representing a strain variation or indicating its relevance between 24-48 h, whereas propionic acid, in addition to acetic and succinic acids were prominent (Fig. 4.1). Hexose fermentation by B.fragilis proceeds via the EMP scheme (Macy and Probst, 1979).

The products of fermentation of lactulose by mixed population of bacteria in a faecal homogenate system varied with incubation conditions such as pH, but correlated well with those produced by pure cultures. Untitrated incubations over a 48 h period with 90 mM lactulose showed large increases in acetic, butyric and lactic acids, but maintainance of pH $\geqslant$ 5.5 inhibited lactic acid formation while the production of fumaric, succinic and longer-chain fatty acids was elevated under these conditions (Table 4.13). In contrast, lactic acid was absent in the untitrated pH regimen in the presence of 30 mM lactulose, and levels of other fatty acids were similar irrespective of pH (Table 4.12). It is possible that in the lower concentration of lactulose, the pH of the faecal homogenate did not fall significantly. Lactulose therapy for PSE can acidify colonic contents to below pH 5.5 (Conn and Lieberthal, 1979), when the acid-tolerant lactobacilli, and to a lesser degree the streptococci, may predominate metabolically in utilising lactulose. With a pH $\geqslant$ 5.5, bacteroides and other acid-sensitive organisms would be favoured. Butyric acid in these observations is

probably largely of clostridial origin. Viable cell counts of streptococci and clostridia do not alter significantly over 48 h in untitrated incubations, but bacteroides decrease markedly over the same period (Vince et al., 1978). Ingestion of lactulose (20 g) for a period of 8 days significantly increased total VFA concentration and the molar ratio of acetic acid in caecal fluid after the test period, although levels of butyric, isobutyric, valeric and isovaleric acids were unchanged (Florent et al., 1985). Lactic acid (isomers D and L) concentration curves, assayed for 8 h, indicated an increase from day 1 to day 8. These observations are in agreement with the predominance of key fermentation metabolites estimated in the present study, and again suggest the importance of the ileo-caecal region in fermenting lactulose. The release of CO_2 and H_2 during lactulose fermentation forms the basis of pulmonary excretion tests (Bond and Levitt, 1975; Florent et al., 1985). In isolated human colon epithelial cells, absorbed butyric acid is metabolised in preference to glucose (Cummings, 1983); lactic acid is also known to induce peristalsis (Conn and Lieberthal, 1979). Therefore, the products of lactulose fermentation by bacteria are implicated in gut function in a number of ways.

Although the mode of action of lactulose in the alleviation of PSE is unclear, the role of the substrate effect of lactulose in increasing the rate of incorporation of colonic ammonia into bacterial proteins is an important factor (Vince et al., 1978; Vince and Burridge, 1980). Such investigations would be assisted by more detailed knowledge of bacterial interactions occurring in the colon, and also of the selective toxicity to bacterial species of fermentation products (Bergeim, 1940; Lee and Gemmell, 1971). The present study provides the basis for future work on this complex

subject. A system to facilitate this is the continuous culture of individual species, or systematically mixed cultures, and faecal homogenates in media based on the composition of ileostomy effluent to serve as a model of the proximal colon (Edwards et al., 1985). This system may allow investigation of: a) lactulose transport, fermentation and regulation in selected species; b) emergence of a dominant strain during mixed bacterial experiments; c) the presence of catabolite repression of lactulose catalysing enzymes, and of polysaccharidase activity; d) fermentation of polysaccharides and oligosaccharides (e.g. raffinose) or sorbitol to mimic the lactulose effect; e) changes in nitrogen metabolism, e.g. glutamine metabolism, resulting from lactulose fermentation by bacteria in faecal homogenates from subjects fed chronic levels of lactulose, lactose, fibre and control substances; and f) the influence of pH and fatty acids (lactic, acetic, butyric) on such metabolism.

The rapid and extensive utilisation of lactulose by C.perfringens suggested this organism for studies aimed at identifying the lactulose permease and assaying key catabolic enzymes, in the second part of the present investigation.

To characterise the transport step, uptake of (^{14}C) lactose by a filtration assay was undertaken as (^{14}C) lactulose was not available; direct enzymatic procedures to assay a PEP-dependant lactulose PTS, and ONPG entry into lactulose induced cells were also carried out. In the filtration assay, lactulose or lactose grown cells were able to accumulate (^{14}C) lactose, but the pattern of intracellular concentration was found to be variable and

not reproducible (Fig. 5.1). Similar results were obtained with the uptake of (^{14}C) galactose by lactulose grown cells, and in a control assay with the uptake of (^{14}C) glucose by C.perfringens, previously cultured on glucose. A number of methodological difficulties did not permit use of this assay procedure to identify the lactulose transport mechanism. Subsequently, a variety of solvent mixtures used to permeabilise cells for PEP:PTS assays indicated high endogenous levels of NADH oxidase (despite use of NADPH) which interferred with transport assays. However, ONPG hydrolysis by lactulose induced cells indicated high levels of a specific permease during late-log phase of growth (Table 5.3). The same cells, when lysed using standardised procedures showed that transport was a rate-limiting step, as lysed cells markedly increased the rate of ONPG hydrolysis. Thio-β-D-digalactoside (TDG) had no effect on ONPG uptake, suggesting a facilitated diffusion mode of entry, assuming TDG specificity for the lactulose permease. These data on rates of uptake were significantly higher than reported values for ONPG uptake in S.faecalis (Heller and Roschenthaler, 1979). The ONPG-based method for assaying lactulose uptake in future work may be amenable to modification in differentiating between energy-dependant and energy-independent uptake by use of specific inhibitors, under anaerobic conditions; specific inhibitors of proton flux in C.perfringens reported by Daltrey and Hugo (1974) are noteworthy in this respect. The substrate-binding specificity of the permease which translocates ONPG requires further study, using the procedures described for the E.coli lac permease where ONPG entry was estimated in the presence of metabolic inhibitors (Sandermann, 1977). The absence of a P-β-Glase in lysed C.perfringens cells

(Table 5.4) strongly indicated lactulose accumulation to be mediated by active transport. Whilst this mode of uptake corresponds to β-galactoside entry in enteric bacteria, and a limited number of streptococci strains (Hamilton and Lo, 1978), other Gram-positive isolates are known to transport lactulose and β-galactoside analogues mainly by means of a PEP-dependant:PTS, as in Staph.aurens (Bissett and Anderson, 1973), S.lactis (Bissett and Anderson, 1974) and L.casei (Chassy and Thompson, 1983a). The methodology reported in quantifying (^{14}C) lactose uptake by lactulose grown L.casei (Chassy and Thompson, 1983a) is of use in future work to examine PTS-mediated uptake of lactulose in this organism and other lactobacilli important in lactulose metabolism in the gut (Table 4.3). A comparison of rates of lactulose uptake by active transport or via the PEP:PTS in different (perhaps the same) species would be of interest. The ability of lactulose to induce a lactose-PTS (Chassy and Thompson, 1983a) as well as a galactose-PTS (Chassy and Thompson, 1983b) in L.casei, suggests a non-specific metabolism of β-galactosides in lactobacilli and possibly in most intestinal bacteria generally. The specifity of the transport systems for galactose and fructose in recognising lactulose also requires further work. In order to overcome the problem of separating substrate transport from subsequent metabolism, isolation of mutants blocked in the initial stages of lactulose metabolism require investigation. Preparation of (^{3}H) lactulose would also be of use in transport studies. The role of lactic acid, a major fermentation product of lactulolytic bacteria, in generating a proton motive force by efflux may also be important in overall cellular energy balance (see Konings, 1985).

The β-Glase induced by lactulose in C.perfringens (Table 4.9) was purified several-fold by a simple, two-step procedure using DEAE-cellulose and Bio-gel A column chromatography (Table 5.5). The β-Glase had a pH optimum between 7.0-7.4 (Fig. 5.5), and was activated by Mg^{2+} ions (Table 5.6); substrate kinetics of the enzyme indicated an apparent K_m for ONPG of 5.71 mM, and V_{max} 0.24 units mg protein^{-1} (Fig. 5.6). A survey of 15 possible regulatory effectors of β-Glase indicated a 26% inhibition of ONPG hydrolysis by fructose-1,6-bisphosphate (Table 5.7), which consequently may be a key regulatory metabolite in vivo, affecting β-Glase activity by a negative feedback mechanism. These studies require future repetition with a homogenous β-Glase preparation, and comparison with properties of a purified P-β-Glase induced by lactulose in other bacteria, possibly the lactobacilli (Table 4.6). A P-β-Glase in lactulose grown L.casei has been reported (Chassy and Thompson, 1983a). In a qualitative assay, the β-Glase from C.perfringens in the present study hydrolysed both lactulose and lactose in the presence of K^+ ions; Na^+ ions were inhibitory (Section 6.2.1.1). The constitutively expressed β-Glase in Klebsiella sp. (Hall, 1980) has similar affinities for lactulose (K_m, 39 mM) and lactose (K_m, 35 mM). It would appear therefore, that bacterial β-Glase and P-β-Glase are capable of hydrolysing lactose and its keto-isomer in two distinct metabolic systems. Preparation of affinity columns (Baues & Gray, 1977) with linked lactulose or phospholactulose (although phospholactose would also be effective) in future work may enable a characterisation of the specific transport linked metabolic pathway to be identified by chromatography of cell-free extracts of lactulose utilising bacteria.

The *in vivo* inducer of the β-Glase in *C.perfringens* is as yet not known; galactose failed to induce this enzyme (Fig. 6.7), but lactose grown cells exhibited significantly higher levels of β-Glase (Fig. 6.4). Allo-lactose, a product of the transglycosylation reaction of β-Glase during lactose hydrolysis, is the *in vivo* inducer of *lac* enzymes in *E.coli* (e.g. Glass, 1982).

Galactokinase was found to be inducible in cells grown on galactose, lactulose or lactose, but not in glucose or fructose grown cells (Table 5.8). The site of phosphorylation of the product of this reaction, whether galactose 1-phosphate or galactose 6-phosphate requires an extension of these studies (see Chassy and Thompson, 1983b, for methods). The Leloir pathway may be a possible route for the breakdown of galactose when released in the cell during lactulose hydrolysis by β-Glase. Other enzymes in this pathway (e.g. see Cooper, 1978) require further study. In *L.casei* grown on galactose, uptake of the sugar occurred via a PEP:PTS, yielding galactose 6-phosphate in the cell, but significant levels of galactokinase were also detected (Chassy and Thompson, 1983b).

Phosphofructokinase (PFK) may also be implicated in intracellular lactulose dissimilation. Two distinct PFKs were detected in fructose grown cell-free extracts, fructose 1-phosphate kinase (FIPK) and fructose 6-phosphate kinase. Affinity chromatography of the extract on Blue Sepharose CL-6B, gave a partial 5-fold purification of FIPK (Table 5.10). This affinity chromatography procedure may also be of use in removing contaminating NADH oxidase activity; both this and the role of PFKs in lactulose and lactose grown cells requires further work.

In the third part of the present study, the glucose effect on lactulose utilisation by C.perfringens, and metabolism of other carbohydrate mixtures of lactulose with fructose, galactose and lactose respectively was investigated.

Growth on a mixture of glucose and lactulose, each at 5 mM, was found to result in classic diauxiae (Fig. 6.3). Glucose was preferentially utilised, at a rate (R_s) during log-phase of $\sim$ 0.27 umol h^{-1} log. viable $cells^{-1}$. Only with the uptake of lactulose between 4-6 h was β-Glase induced, maximal levels achieved after 18 h (sp.act.~0.50). In a lactulose-supplemented control incubation, maximum β-Glase activity was noted after 6-8 h (sp.act.~ 0.65). The role of cAMP in this glucose-mediated diauxiae of the lactulose regulon was subsequently examined. Glucose $\pm$ cAMP (at 1 mM and 5 mM concentrations) were added to lactulose-induced cultures (Fig. 6.5); in all cases, a marked cessation of β-Glase synthesis occurred. Both cAMP (Fig. 6.5) and dibutyryl cAMP (5 mM; data not shown) failed to eleviate the severe repression exerted by glucose. One methodological problem is cAMP accessibility; attempts to culture C.perfringens in the presence of small amounts of toluene to permit sufficient permeabilisation for cAMP entry were not successful. Furthermore, direct assays for cAMP in lactulose grown cells or extracellular fluid failed to detect the cyclic nucleotide with a specific cAMP assay procedure. Further studies to look for alternative regulatory mediators, for instance the catabolite modulating factor described by Ullman and Danchin (1983), are required. The fact that cAMP may not play a major role in regulating anaerobic sugar fermentation was discussed in Section 1.4.2.

The provision of a fructose and lactulose mixture led

to the preferential utilisation of fructose (Fig. 6.6). A diauxic effect was not observed, and inhibition appeared not to be as severe as the glucose effect (Fig. 6.5), as β-Glase induction was noted despite presence of fructose in the medium. A period of co-utilisation of fructose and lactulose between 2-4 h to maintain the rapid growth rate was inferred. The fructose effect may partially be an inducer exclusion phenomenon (discussed in Section 6.3.2.2).

A simultaneous uptake of galactose and lactulose was paralleled by high levels of β-Glase activity and high rates of sugar utilisation during early log-phase between 2-4 h (Fig. 6.8). It is noteworthy that galactose did not induce β-Glase in a control incubation (Fig. 6.7). It would be advantageous to the cell, in terms of conserving energy (e.g. for protein synthesis), if galactose and lactulose were translocated and fermented by the same pathway. Specificity of permease therefore necessitates further work; earlier experiments in the present study (Chapter 5) indicated that lactulose uptake is probably electrogenic. Galactose uptake in C.pasteurianum has been shown to occur by active transport (Booth and Morris, 1975).

An equimolar mixture of lactose and lactulose induced near maximal levels of β-Glase (sp.act. ~ 0.82), detected at 2 h. This level of β-Glase expression was significantly higher than during lactulose growth (sp.act. ~ 0.44; Fig. 6.4) and comparable to a lactose control (sp.act. ~ 0.73; Fig. 6.10). However, this assumes an absence of β-Glase isoenzymes. Excretion of galactose to an extracellular concentration of 0.23 mM was observed during the late-log period of co-utilisation, possibly a regulatory mechanism to remove excess carbon until required (why is fructose preferred intracellularly?). The mechanism of galactose exit is of interest,

and requires another study. Lactose appeared to induce β-Glase more efficiently than lactulose: the in vivo inducer is as yet unidentified. The specificity of the β-galactoside permease, β-Glase isoenzymic forms from lactose grown cells (only one enzyme was observed under the chromatographic conditions used in Chapter 5 to fractionnate the lactulose induced β-Glase), and fate of intracellularly released monosaccharides requires more detailed studies.

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The Fermentation of Lactulose by Colonic Bacteria

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Sixty-four strains of intestinal bacteria were cultured under anaerobic conditions in lactulose-containing media to assess their ability to ferment lactulose. Some organisms were unable to metabolize the disaccharide, while others, e.g. clostridia and lactobacilli, metabolized lactulose extensively. Quantitative analyses of the fermentation products indicated that the major non-gaseous metabolites were acetic, lactic and butyric acids. Hydrogen and carbon dioxide were the only gases detected. Fermentation products were estimated for selected species throughout their growth cycles. The products of fermentation of lactulose by stool cultures varied with incubation conditions such as pH, but correlated well with those produced by pure cultures. These results are discussed in relation to the therapeutic uses of lactulose.

INTRODUCTION

The synthetic disaccharide lactulose (4-*O*-*β*-D-galactopyranosyl D-fructofuranose) cannot be hydrolysed by human intestinal *β*-galactosidases (EC 3.2.1.23; Dahlquist & Gryboski, 1965; Udupihille, 1974) and, except for slow permeation of the intact disaccharide, is not absorbed from the small intestine (Müller *et al.*, 1969; Carulli *et al.*, 1972). Consequently, following ingestion it passes to the colon, where it undergoes rapid fermentation by the colonic flora (Hoffmann *et al.*, 1964) to products which cause acidification of the stool contents and flatulence (Agostini *et al.*, 1972). Ingestion of sufficient amounts of lactulose produces a diarrhoea that is both osmotic and fermentative.

Because of this resistance to intestinal hydrolases, lactulose is used clinically for the treatment of acute constipation (Wesselius-De Casparis *et al.*, 1968) and portal systemic encephalopathy (e.g. Bircher *et al.*, 1966; Simmons *et al.*, 1970), and for the assessment of small bowel transit time (Bond & Levitt, 1975).

Although the therapeutic uses of lactulose involve, or may be modified by, bacterial degradation within the intestine, there is little detailed knowledge of this process. The few reported investigations indicate that some Gram-positive cocci and bacilli metabolize lactulose (Hoffmann *et al.*, 1964) as do several bacteroides (Ruttloff *et al.*, 1967). The former workers showed that lactic acid is the principal end-product, but reported no quantitative data.

The present investigation was undertaken to extend knowledge of the fermentation of lactulose by individual types of intestinal micro-organisms. Utilization of lactulose and formation of fermentation products have been estimated quantitatively for 64 strains of bacteria, cultured anaerobically, the behaviour of selected species being assessed throughout their growth cycles. These data have been compared with the results of anaerobic fermentations of lactulose by cultures of mixed organisms from faecal homogenates.

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METHODS

Organisms. Strains of lactobacillus were purchased from the N.C.I.B., Torry Research Station, Aberdeen. All other organisms were kindly provided by Dr A. Vince, The Rayne Institute, University College Hospital Medical School, London; Dr M. Hill, Bacterial Metabolism Research Laboratory, Central Public Health Laboratory, London, and Professor I. Phillips, Department of Microbiology, St Thomas's Hospital Medical School, London.

Media and growth conditions. All organisms were maintained on Oxoid 'Cooked Meat' medium (CM349) and stored at −70 °C, after the addition of glycerol to a final concentration of 15% (v/v). The purity of the strains was verified at regular intervals after culturing on blood agar. A standard sugar-free basal liquid medium (Holdeman *et al.*, 1977) was used, and when necessary, lactulose was added via a 0·22 μm bacteriological filter, to a final concentration of 40 mM.

Routine screening of the bacterial strains for their ability to utilize lactulose was carried out by inoculating (5%, v/v) liquid media (15 ml, with and without lactulose) with cultures which had been grown overnight in basal medium. They were then incubated at 37 °C for 48 h in an atmosphere of $N_2/H_2/CO_2$ (80:10:10, by vol.). Time-course experiments over a 24 h period were conducted similarly, with sampling every 4 h. Bacterial growth was measured turbidimetrically at 612 nm, and pH was measured by a microelectrode. Culture supernatants were obtained by centrifugation at 2000 *g* for 15 min.

Determination of carbohydrates. The carbohydrates in culture supernatants were separated by thin-layer chromatography on silica gel G and subsequently quantified by locating and scanning the appropriate bands (Menzies *et al.*, 1978). Alternatively, when lactulose was the only sugar present it was estimated by the method of Vachek (1971).

Determination of non-gaseous fermentation products. These were separated by gas chromatography (Pye-Unicam model 204, fitted with a flame ionization detector) on glass columns (2 m × 4·5 mm, internal diameter) packed with Diatomite C (acid washed, 60–80 mesh) coated with 10% polyethylene glycol 20M. The following conditions were employed: gas flow rates of 50, 750 and 45 ml min^{-1} for H_2, air and N_2, respectively; injection port temperature 120 °C, detector temperature 180 °C and a temperature programme of 8 °C min^{-1} from 60 °C to 160 °C, the final temperature being maintained for 15 min.

Quantification of non-gaseous products was achieved by comparing the integrated peak areas of standard compounds with those of experimental samples, using internal markers of butanol and malonic acid for volatile and non-volatile fatty acids, respectively.

In order to avoid losses due to evaporation, acetic and formic acids were determined directly on culture supernatants, using a standard test kit for acetic acid (Boehringer Corporation) and formate dehydrogenase (EC 1.2.1.2) for formic acid (Schutte *et al.*, 1976).

The other non-gaseous products were extracted from acidified culture supernatants (2·4 ml) containing the appropriate internal marker (0·1 ml). Volatile fatty acids were obtained directly by partition with diethyl ether (1·0 ml), while non-volatile fatty acids were methylated with 14% (v/v) boron trifluoride in methanol (2·5 ml) at room temperature, overnight, and then partitioned with chloroform (1·25 ml). This methylation procedure gave recoveries of 93 ± 2% for lactic acid.

Determination of gaseous fermentation products. These were initially detected and their total volumes estimated after collection in inverted sample tubes which had been placed in the media prior to inoculation. Qualitative analyses were then carried out by gas chromatography on glass columns (1·5 m × 4·5 mm i.d.) packed with silica gel (80–100 mesh; Phase Separations, Queensferry, Clywd). The following conditions were used: carrier gas (N_2) flow rate 50 ml min^{-1}; injection port temperature 50 °C; column temperature 50 °C; detector temperature 50 °C and a bridge current of the catharometer of 95 mA.

Selected bacteria were cultured, as described earlier, in gas-tight vials (Hypo-vials, Pierce and Warriner, Chester) under N_2. Samples (1 ml) were removed from the head space with a gas-tight syringe and injected on to the column. Retention times were compared with those of a standard gas mixture containing methane, ethane, propane and butane (Phase Separations) and also with hydrogen and carbon dioxide (British Oxygen, Wembley, Middlesex).

Incubation with mixed faecal organisms. These samples were kindly provided by Dr A. Vince. They were incubated anaerobically at 37 °C for 48 h in the presence of 90 mM-lactulose, as described elsewhere (Vince *et al.*, 1978). After centrifugation at 47500 *g* for 90 min at 4 °C the supernatants were removed and analysed for fermentation products as described above.

RESULTS

Utilization of lactulose

The amount of lactulose fermented over a 48 h period varied significantly, not only from genus to genus but also, in some cases, between different strains of the same species (Table 1).

Table 1. *Utilization of lactulose (40 mM) by colonic bacteria*

	Organism*	Percentage utilization after 48 h cultivation	Gas production†
Organisms utilizing >20% lactulose	*Bacteroides oralis*‡	21·0	–
	Bacteroides vulgatus‡	21·0	+
	Bifidobacterium bifidum	30·5	–
	Clostridium perfringens (8)	33·6–43·1	3+
	Lactobacillus casei sub. *casei*	33·5	–
	Lactobacillus spp. (4)	21·1–47·3	–
Organisms utilizing 5–20% lactulose	*Bacteroides distasonis*‡ (3)	11·5–19·5	–
	Bacteroides fragilis (6)	9·0–13·5	+
	Bacteroides ovatus (2)	5·0–8·1	+
	Bacteroides thetaiotaomicron (3)	5·0–8·0	+
	Bacteroides uniformis (3)	9·4–13·5	+
	Bacteroides vulgatus‡	9·8	+
	Clostridium butyricum (3)	7·5–19·0	2+
	Clostridium cadaveris (2)	5·0–17·5	2+
	Clostridium difficile (2)	5·0–13·8	2+
	Enterobacter aerogenes	12·0	+
	Escherichia coli	5·0	+
	Klebsiella aerogenes	9·6	–
	Lactobacillus acidophilus (2)	10·0–13·1	–
	Lactobacillus brevis	13·2	–
	Peptostreptococcus anaerobius	10·0	2+
	Proteus mirabilis	11·6	2+
	Proteus vulgaris	16·1	2+
	Staphylococcus aureus	7·0	–
	Streptococcus faecalis	6·0	+
	Streptococcus intermedius	6·6	+
	Streptococcus sp.	8·8	+
	Streptococcus viridans	6·0	+

The following organisms were found to utilize <5% of the added lactulose and no gaseous products were formed: *Bacteroides mel assacharolyticus, Clostridium paraputrificum, Eubacterium lentum, Eubacterium limosum, Fusobacterium nucleatum, Lactobacillus buchneri, Neisseria catarrhalis, Propionibacterium acnes, Veillonella parvula.*

* Number of strains shown in parentheses if more than one used.
† Scored on a scale + to 3+.
‡ Galactose and/or fructose found in the culture medium after growth.

Some 14% of the 64 strains examined showed negligible (<5%) utilization of lactulose, while the majority (61%) of the organisms fermented an 'intermediate' (5–20%) amount of the disaccharide. The latter group consisted of coliforms, bacteroides, streptococci and all but two species of clostridia. The culture supernatants of *Bacteroides distasonis, B. oralis* and *B. vulgatus* contained small quantities of galactose and fructose (Table 1).

Of the three organisms selected from those exhibiting over 20% utilization of lactulose, only *Clostridium perfringens* exhibited a typical growth pattern when cultured on 40 mM-lactulose (Fig. 1). Both *Bacteroides vulgatus* and the *Lactobacillus* sp. metabolized the majority of the disaccharide in the stationary phase, an example of deferred substrate utilization. In all three, the total quantities of lactulose utilized after 24 h were similar to those at the 48 h stage in the earlier screening experiment (Table 1). A fall in pH of 2·0–2·5 units was found over the 24 h fermentation (data not shown).

Fermentation products

In order to accurately quantify the products of lactulose fermentation, it was necessary to compare the types and amounts of the products after growth on basal medium and on lactulose-supplemented medium.

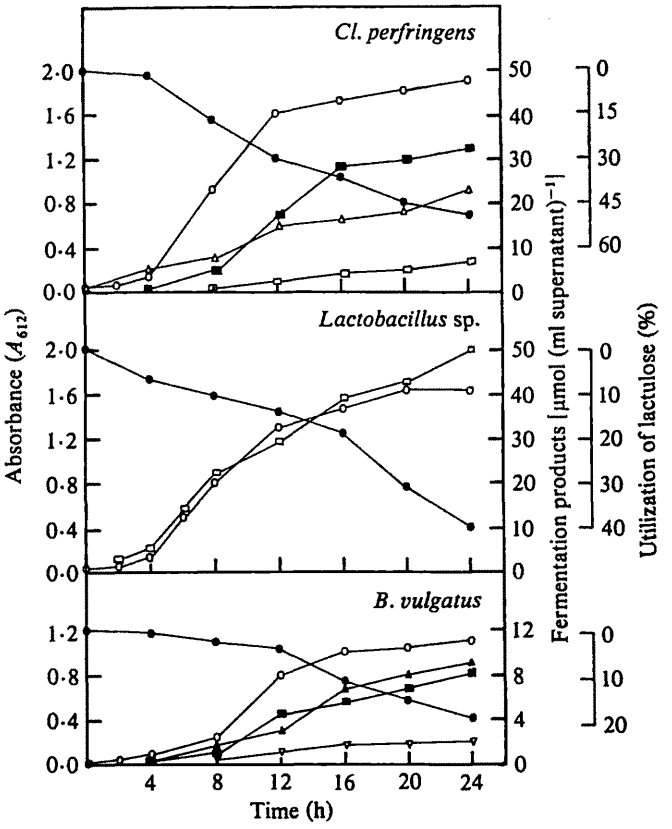

Fig. 1. Growth of selected organisms on 40 mM-lactulose. Absorbance (○), utilization of lactulose (●), concentrations of fermentation products – lactic acid (□), acetic acid (■), butyric acid (△), succinic acid (▲), propionic acid (▽).

Table 2. *Quantitative analysis of significant non-gaseous products of lactulose fermentation*

The values quoted show the increment in the fermentation product observed with the strains employed.

	Product [μmol (ml culture supernatant)$^{-1}$]				
Organism*	Acetic acid	Butyric acid	Isovaleric acid	Lactic acid	Succinic acid
B. fragilis (6)	3·2– 8·9	–	–	3·9– 7·25	2·1–4·1
Cl. perfringens (8)	7·3–11·3	14·05–25·4	–	3·2–15·4	–
E. coli	3·8	–	–	2·75	–
L. acidophilus	–	–	–	21·2	–
P. anaerobius	1·9	–	1·00	–	–
St. faecalis	4·5	–	0·7	3·0	–

* Number of strains shown in parentheses if more than one used.

Both qualitative and quantitative differences were found on addition of 40 mM-lactulose to the medium. Generally, large increases in acetic, lactic and butyric acids were found, with butyric acid being a characteristic product of clostridia. Bacteroides predominantly metabolized lactulose to acetic and succinic acids, but produced smaller quantities of higher fatty acids during lactulose fermentation than with basal medium alone. The lactobacilli exhibited a homolactic fermentation pattern. Formic acid was not detected by either enzymic or gas–liquid chromatographic analyses. Quantitative analyses of the non-gaseous fermentation products of representative species are shown in Table 2.

Table 3. *Non-gaseous products of in vitro incubation of 90 mM-lactulose with faecal homogenates*

Each value is the mean of duplicate incubations.

Incubation condition	Product* [μmol (ml culture supernatant)$^{-1}$]										
	Ac	Prop	iBut	But	iVal	Val	Cap	iCap	Lac	Fum	Succ
+ Lactulose, 0 h	19·9	2·4	0·3	2·8	0·4	0·4	0·4	0·4	0·0	0·0	0·0
+ Lactulose, 48 h	116·7	9·5	0·3	29·8	0·4	0·5	0·6	0·2	52·6	0·0	0·0
+ Lactulose, 48 h, titrated to pH $\geqslant$ 5·5	99·1	17·2	1·7	65·5	1·7	3·4	9·3	0·4	0·0	2·3	3·6

* Ac, acetic acid; Prop, propionic acid; iBut, isobutyric acid; But, butyric acid; iVal, isovaleric acid; Val, valeric acid; Cap, caproic acid; iCap, isocaproic acid; Lac, lactic acid; Fum, fumaric acid; Succ, succinic acid.

Of those organisms utilizing more than 5% of the available lactulose, some 63% produced gases during fermentation, with strains of *Cl. perfringens* evolving the largest quantities (Table 1). Carbon dioxide and hydrogen were the only gases detected in these cases, methane being absent.

Analyses of fermentation products throughout growth of the three selected bacteria showed that all the metabolites were excreted during each phase of growth (Fig. 1).

Incubations with faecal homogenates

The fermentation products of the mixed cultures from faecal homogenates, incubated with lactulose, differed depending on the condition of the incubations (Table 3). Untitrated incubations over a 48 h period showed large increases in acetic, butyric and lactic acids, but maintenance of pH $\geqslant$ 5·5 inhibited lactic acid formation while the production of butyric, succinic and longer-chain fatty acids was elevated under these conditions.

DISCUSSION

The amounts of lactulose fermented by the 64 strains of gut bacteria (Table 1) indicate that of the three quantitatively predominant gut genera (all approx. 10^{10} per g faeces; Drasar & Hill, 1974), eubacteria do not play a significant role in colonic lactulose metabolism, whereas strains of both bacteroides and bifidobacteria show high amounts of lactulose utilization *in vitro* (Table 1). Other quantitatively important colonic organisms such as streptococci and enterobacteria (both approx. 10^8 per g faeces) exhibit a rather low ability to use lactulose (5–10%, Table 1). Although both lactobacilli and clostridia do not occur in such high numbers in the lower intestine (10^5–10^6 per g faeces), the large amount of lactulose utilized by certain strains (Table 1), the extent of which was confirmed by their comparatively high rates of uptake during exponential and stationary phases of growth (Fig. 1), suggests that such organisms are important in the metabolism of lactulose in the colon.

The non-gaseous fermentation products of lactulose (Table 2) were qualitatively the same as those obtained when these organisms were cultured on glucose (Holdeman *et al.*, 1977), suggesting that similar glycolytic pathways are operative for both carbohydrates. This is the first report of butyric acid formation from lactulose and indicates that the mixture of fermentation products present in the colon during lactulose therapy is more complex than has been claimed previously (Conn & Lieberthal, 1979). These organisms are also able to utilize the aldose isomer lactose (Holdeman *et al.*, 1977), suggesting that prokaryotes are relatively non-specific with respect to β-D-galactoside metabolism, in contrast to human intestinal β-galactosidases (Dahlquist & Gryboski, 1965).

The formation of carbon dioxide and hydrogen during growth on lactulose (Table 1) correlates with the characteristic flatulence experienced after ingesting therapeutic doses of lactulose (Elkington, 1970). The evolution of hydrogen forms the rationale of tests for sugar absorption and intestinal transit time based on the appearance of this gas in expired air when a non-absorbed sugar, such as lactulose, enters the lower intestine (Bond & Levitt, 1975; Metz *et al.*, 1975, 1976).

Mixed populations of bacteria, derived from faecal homogenates and incubated with lactulose, generated increased amounts of acetic, lactic and butyric acids in the same way that occurred when individual organisms were cultured (Table 3) and is probably due to the activity of acid-tolerant lactobacilli, streptococci and clostridia. Viable cell counts of streptococci and clostridia do not alter significantly over 48 h in untitrated incubations, but bacteroides decrease markedly over the same period (Vince *et al.*, 1978). It is significant, therefore, that on maintenance of a pH $\geqslant$ 5·5 during incubation, the balance of fermentation products is altered in favour of succinic and higher fatty acids, presumably because bacteroides and other acid-sensitive organisms were metabolically more active at such pH values. Since high oral doses of lactulose, as used in the treatment of hepatic coma, produce acidification of colonic contents to below pH 5·5 (Conn & Lieberthal, 1979), acetic, butyric and lactic acids may become the predominant metabolic products in the colon.

The mode of action of lactulose in the alleviation of hepatic coma is still unclear (Conn & Lieberthal, 1979). However, blood ammonia levels, a major aetiological factor in this condition, do fall significantly during lactulose therapy (Conn *et al.*, 1977). Since significant quantitative changes in the gut flora of adults do not appear to be induced by oral lactulose administration (Vince *et al.*, 1974), a current hypothesis to explain this phenomenon is that lactulose fermentation in the colon enables certain unspecified organisms to increase the rate of incorporation of colonic ammonia into bacterial proteins (Vince *et al.*, 1978). Such investigations would be assisted by more detailed knowledge of bacterial interactions occurring in the colon, and also of the selective toxicity to bacterial species of fermentation products (Bergeim, 1940; Lee & Gemmell, 1971). Our present studies, which detail the products of lactulose metabolism and show which colonic organisms are capable of growth on lactulose, should facilitate future work on this complex subject.

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