THE ROLE OF GUT BACTERIA IN THE METABOLISM

OF

DIETARY XYLITOL

by

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"Reading maketh a full man;
conference a ready man;
and writing an exact man."

Francis Bacon,
from "Essays-Civil and Moral,
sub-heading- Of Studies"

In dedication of:

my grandfather who was the motivating influence in all my academic endeavours;

my mother and father for their warmth and support;

my sister and brothers for raising my morale;

my wife for her love.

CONTENTS

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university. In addition, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except when due reference is made in the text of this thesis.

Ravi Krishnan

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ABSTRACT

THE ROLE OF GUT BACTERIA IN THE METABOLISM OF DIETARY XYLITOL
Ravi KRISHNAN, University of Adelaide.
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Xylitol, a sugar polyol, will be increasingly used as a nutritive, anti-cariogenic, sugar substitute. In both man and animals, however, the ingestion of relatively large amounts of xylitol is often accompanied by an osmotic diarrhoea. Persistence with xylitol diets results in an adaptation (i.e. cessation of diarrhoea) and the ability to tolerate much greater amounts of dietary xylitol. It is argued in Chapter One that the mechanism of adaptation occurs either by increasing the xylitol-metabolising ability of the microflora in the gut lumen; the gut wall mucosa or the liver.

In Chapter Two it is shown that the plasma metabolite and electrolyte concentrations, hepatic enzyme activities and liver homogenate metabolism of $|^{14}C|$-labelled carbohydrates are similar in both xylitol-adapted and non-adapted rats. On the other hand, xylitol-adapted rats have enlarged caeca with increased amounts of gas, acidity and thiamin. With the use of a sensitive radioisotopic assay, the caecal microflora revealed a xylitol-metabolising ability and, in the presence of xylitol diets, this could increase 40-fold. In further studies reported in Chapter Three, K.pneumoniae, S.liquefaciens and Micrococcus sp. were identified as caecal bacteria capable of growth on xylitol. The in vitro studies reported in Chapter Four indicated that the induction of a xylitol transport protein, a xylitol dehydrogenase and a xylulokinase are essential for the increased production of $^{14}CO_2$ from $|U\text{-}^{14}C|$ xylitol. Bacterial growth on arabitol, ribitol or sorbitol demonstrated neither induction of all these enzyme activities nor enhancement of xylitol metabolism.

The caeca of xylitol-fed rats also contains an insoluble polysaccharide-like material which, in Chapter Five, is shown to be a product of bacterial metabolism rather than host secretion. Evidence supportive of this conclusion includes the production of a similar polysaccharide by K.pneumoniae in culture and electronmicrographs showing that the polysaccharide is attached directly to the bacteria.

It is concluded in Chapter Six that the mechanism of adaptation to xylitol ingestion occurs through increased bacterial metabolism of xylitol in the rat caecum and concomitant reduction in caecal osmotic load. Neither the gut wall mucosa nor the liver play a major role in the host adaptation, at least, not in the short-term.

ENZYME NONENCLATURE

Name used in Text	E.C. Number	Systematic Name
alanine aminotransferase	2.6.1.2	L-alanine:2-oxoglutarate aminotransferase
alcohol dehydrogenase	1.1.1.1	alcohol:NAD^+ oxidoreductase
alkaline phosphatase	3.1.3.1	orthophosphoric-monoester phosphohydrolase
arabitol (arabinitol) dehydrogenase	1.1.1.11	D-arabinitol:NAD^+ 4-oxidoreductase
aspartate aminotransferase	2.6.1.1	L-aspartate:2-oxoglutarate aminotransferase
glucose 6-phosphate dehydrogenase	1.1.1.49	D-glucose 6-phosphate: $NADP^+$ 1-oxidoreductase
glutamate dehydrogenase	1.4.1.2	L-glutamate:NAD^+ oxidoreductase (deaminating)
γ-glutamyltranspeptidase	2.3.2.2	(5-glutamyl)-peptide:amino acid 5-glutamyltransferase
α-ketoglutarate dehydrogenase	1.2.4.2	2-oxoglutarate:lipoamide oxidoreductase (decarboxylating and acceptor succinylating)
lactate dehydrogenase (lactate to pyruvate)	1.1.1.27	L-lactate:NAD^+ oxidoreductase
phosphoenol pyruvate:sorbitol 6-phosphotransferase	(not listed)	Reaction catalysed: the transfer of phosphate from phosphoenol pyruvate to sorbitol at C6 (Kelker and Anderson, 1971)

ENZYME NOMENCLATURE (Continued)

Name used in Text	E.C. Number	Systematic Name
6-phosphogluconate dehydrogenase	1.1.1.44	6-phospho-D-gluconate:$NADP^+$ 2-oxidoreductase (decarboxylating)
pyruvate kinase	2.7.1.40	ATP: pyruvate 2-O-phosphotransferase
ribitol dehydrogenase	1.1.1.56	ribitol:NAD^+ 2-oxidoreductase
ribulokinase	2.7.1.16	ATP: L(or D)-ribulose 5-phosphotransferase
ribulose phosphate 3-epimerase	5.1.3.1	D-ribulose-5-phosphate 3-epimerase
sorbitol dehydrogenase	1.1.1.14	L-iditol:NAD^+ 5-oxidoreductase
sorbitol-phosphate dehydrogenase	1.1.1.140	D-sorbitol-6-phosphate:NAD^+ 2-oxidoreductase
transketolase	2.2.1.1	sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycolaldehyde transferase
xylitol dehydrogenase (D-xylulose reductase)	1.1.1.9	xylitol:NAD^+ 2-oxidoreductase (D-xylulose-forming)
xylulokinase	2.7.1.17	ATP: D-xylulose 5-phosphotransferase
xylose isomerase	5.3.1.5	D-xylose ketol isomerase
L-xylulose reductase	1.1.1.10	xylitol:$NADP^+$ 4-oxidoreductase (L-xylulose-forming)

CHAPTER ONE

DIETARY XYLITOL AND OSMOTIC DIARRHOEA: MECHANISMS OF HOST ADAPTATION

INTRODUCTION

The plight of sucrose and most other dietary sweeteners can be summarised as follows:

> "Nothing mortal is enduring, and there is nothing sweet which does not presently end in bitterness"
>
> Petrach (1367-1372),
> Letter to posterity.

Xylitol, a new sucrose substitute has been described as being both safe and toxic (Brin et al, 1974; Thomas et al, 1974). Its real value as a nutritive sweetener, however, can only be assessed in relation to other dietary sweeteners and their potential to cause side-effects.

SUCROSE SUBSTITUTES

Nowadays, most health workers acknowledge the existence of a definite relationship between the increased consumption of sucrose and the incidence of dental caries (Nikiforuk, 1970; Cleave, 1974; Russell, 1974). This has led these workers to stress, both to governments and commercial food

interests, the need to replace sucrose with a sweetener that can minimise dental caries (Muhlemann, 1966; Newbrun, 1973; Scheinin, 1978; Aminoff, 1974; Scheinin and Mäkinen, 1975). The non-nutritive sweeteners, however, such as cyclamates and saccharin have fallen into disrepute due to their reputed carcinogenic properties (Batzinger, Ou and Bueding, 1977; Brunzell, 1978). Furthermore, the protein sweeteners such as monellin (Morris et al, 1973) and thaumatin (van der Wel, 1980), although several thousand-fold sweeter than sucrose, are unstable and therefore may not be acceptable for commercial purposes.

Naturally occurring carbohydrates which have been suggested as replacements for sucrose include the non-glucose sugars such as fructose (Brunzell, 1978), and the sugar polyols which include maltitol (Edwardsson, Birkhed and Mejare, 1977), sorbitol (Moller, 1977) and xylitol (Mäkinen, 1976). Chemically-modified carbohydrates, such as Lycasin^{R} (hydrogenated starch hydrolysate containing a mixture of sorbitol, maltitol and higher hydrogenated saccharides and dextrines) (Frostell, 1971) and chlorine-substituted sucrose (Hough and Khan, 1978) have also been considered.

For a substance to be suitable as a sweetener of commercial utility, it must possess properties such as sweetness, non-toxicity, inexpensive synthesis and thermostability (Newbrun, 1977). Although a large variety of sweeteners are available, either carbohydrate or non-carbohydrate, nutritive or non-nutritive, most of them have been excluded as commercial sweetening agents because they do not possess all of the above properties (Newbrun, 1973). On the other hand, the sugar polyol, xylitol, is commercially acceptable for it has a sweetness equal to sucrose (Kracher, 1977); several trials have shown it to be relatively non-toxic (FDA, 1978); it can be efficiently prepared in commercial quantities (Aminoff, Vanninen and Doty, 1978); and it has been shown that the total or partial substitution of dietary sucrose by xylitol considerably reduced the incidence of dental caries in volunteers who participated in a two-year trial in Turku, Finland (Scheinin and Mäkinen, 1975).

OCCURRENCE, MANUFACTURE AND USE OF XYLITOL

Xylitol is a five carbon, acyclic sugar polyol. The term sugar polyol will henceforth be used in this text to indicate both the chemical

and biochemical properties of sugar alcohols (polyhydric alcohols). Like citrate, xylitol is optically inactive but biologically asymmetrical. It is commonly found in plants, fruits and vegetables. The richest edible sources of xylitol consumed by man are in plums, strawberries, raspberries, cauliflowers and endives where the concentration ranges from 0.3 to 0.9 g per 100 g of dry material (Mäkinen, 1978). Although present in only small quantities in our diet, xylitol is ubiquitous in foods (Washuttl, Reidener and Bancher, 1973).

The commercial production of xylitol is based on the hydrogenation of D-xylose which is obtained from the hydrolysis of various xylan-containing materials such as birchwood, cotton seed hulls and coconut shells (Mäkinen, 1978; Aminoff, 1974). The abundance of xylan-containing material (Emodi, 1978; Moore, 1977) provides an unlimited supply of the raw material for xylitol production. In addition, the microbial production of xylitol from glucose via D-arabitol and D-xylulose is also possible (Onishi and Suzuki, 1969) but expensive.

The physico-chemical properties of xylitol, such as its high melting point (93-95^{o}C), boiling point (216^{o}C), solubility, resistance to caramelisation and its inability to function in the Maillard reaction (Mäkinen, 1978), enable it to be used in a variety of foodstuffs like sauces, condensed milk, candy, toffee, chocolates and chewing gums (Voirol, 1977; Kracher, 1977). Since xylitol and sucrose are equal in sweetness, on a weight-for-weight basis (Moskowitz, 1971; Kracher, 1977), and have the same calorific value (Aminoff, Vanninen and Doty, 1978) similar quantities of these carbohydrates can be used to sweeten foodstuffs (Voirol, 1977).

In contrast, other sugar polyols such as sorbitol and mannitol, which have a sweetness scale of 0.5 compared to 1.0 for xylitol (Kracher, 1977), need to be used in larger quantities to achieve the required sweetness and this results in a higher calorific intake. Another important advantage in the commercial use of xylitol is that it is not fermented by most microorganisms which spoil food (Mortlock, 1976).

METABOLISM OF XYLITOL

In mammals, xylitol is an intermediate in the glucuronate-xylulose pathway (Fig. 1.1) which occurs in the liver and kidneys (Touster and Shaw,

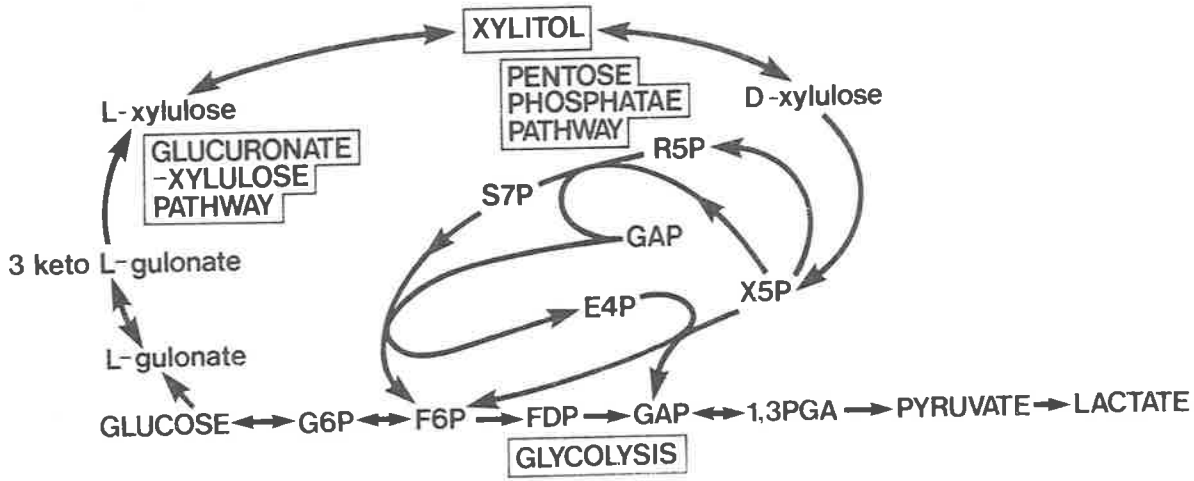


Fig. 1.1 The Hepatic Metabolism of Xylitol. The intermediary metabolites are as follows: R5P, ribose 5-phosphate; X5P, D-xyulose 5-phosphate; S7P, sedoheptulose 7-phosphate; GAP, glyceraldehyde 3-phosphate; E4P, erythrose 4-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose di-phosphate; 1,3PGA, 1,3-di-phosphoglycerate; 3PGA, 3-phosphoglycerate.

1962; Horecker, 1969). Touster (1974) has estimated the physiological rate of formation of xylitol from the glucuronate-xylulose pathway in a healthy individual to be between 5 and 15 g per day. While the enzymes of the glucuronate-xylulose pathway may exist in other tissues, the liver is the major site of metabolism of exogenously introduced xylitol (Froesch and Jakob, 1974).

The significance of xylitol as a metabolite was first appreciated in studies of the disease, essential pentosuria. Patients suffering from this genetic abnormality have L-xylulose present in their urine, whereas in normal people the L-xylulose is metabolised to xylitol by the NADPH-linked enzyme, L-xylulose reductase (Touster, 1974). Using erythrocyte preparations the genetic defect was shown to be related to the presence of a mutant L-xylulose reductase with a lowered affinity for NADP and hence a lowered catalytic activity for L-xylulose (Wang and van Eys, 1970).

In the normal hepatic metabolism of xylitol the initial reaction is an NAD-dependent dehydrogenation to D-xylulose catalysed by xylitol dehydrogenase (D-xylulose reductase) followed by the phosphorylation of D-xylulose to D-xylulose-5-phosphate by D-xylulokinase (Touster, 1974). The D-xylulose-5-phosphate which is formed enters the pentose phosphate pathway where it can form D-fructose-6-phosphate and D-glyceraldehyde-3-phosphate (Horecker, 1969) both of which are intermediates in glycolysis and gluconeogenesis (Fig. 1.1). In the liver, the initial metabolism of xylitol is insulin-independent, because the transport of xylitol into cells is not facilitated by insulin (Bässler and Prellwitz, 1964; Lang, 1971); although the subsequent peripheral metabolism of the glucose formed by the liver metabolism of xylitol is insulin-dependent (Froesch and Jakob, 1974). Furthermore, its relatively slow intestinal absorption (Asano et al, 1973), its antiketogenic effect (Bässler, 1966; Kumagi, 1969), and the delay and reduction of the insulin response, have led some investigators to claim that xylitol is a suitable dietary sweetener for diabetics when insulin deficiency is not extreme (Mehnert and Förster, 1977). The use of xylitol in diabetic foods in Canada and Europe reflects the increasing commercial interest in the development of new products with xylitol as a substitute sweetener (FDA, 1978). The consumption of xylitol is likely to increase and it will therefore be necessary to assess any adverse effects that might result from these increases in the consumption of xylitol. Some evidence for adverse effects of xylitol is already available in the literature and is now discussed.

THE USE OF XYLITOL AS A PARENTERAL NUTRIENT

In disease, injury and other metabolically stressful states, excessive loss of body protein can often be avoided by the parenteral administration of carbohydrates as nutrients (Meng, 1974). Carbohydrates can minimise gluconeogenesis from amino-acids, supply energy for protein synthesis and, therefore, reduce protein breakdown (Ahnefeld et al, 1975). However, when disturbances in glucose utilisation occur in diabetic patients or in trauma-related glucose intolerance, hyperglycaemia can result and the constant and inconvenient monitoring of blood glucose concentration becomes essential (Ahnefeld et al, 1975). In these clinical conditions xylitol has been promoted as a more appropriate parenteral nutrient, since it was considered that it can be infused intravenously in relatively larger amounts than glucose (Korttilla and Mattila, 1979) to provide calories by a metabolic pathway independent of insulin (see above), but which is anti-ketogenic (Yamagata et al, 1965; Schumer, 1971). Furthermore, unlike glucose, xylitol can be stored and sterilised in the same container as amino acids without undergoing the Maillard reaction (Mäkinen, 1978).

Xylitol as well as fructose and sorbitol have been used in parenteral nutrition in Europe and Japan for several years without apparent adverse effects (FDA, 1978). For instance, Lang et al (1969) reported that 5 to 10% (w/v) xylitol solutions were infused intravenously in 1135 surgical patients who showed no side-effects. Halmagyi and Israng (1968) also reported favourable effects with parenteral xylitol in 1189 patients. Even a high concentration of 50% (w/v) xylitol was reported to be safe and utilised efficiently when compared with fructose and sorbitol (Coats, 1969).

On the other hand, certain adverse and sometimes fatal reactions were observed by our laboratory in patients receiving large intravenous infusions of xylitol (Thomas et al, 1970; 1972a; 1972b; Evans et al, 1973; Conyers et al, 1984). These adverse reactions included a metabolic acidosis, hyperbilirubinaemia, elevation of plasma levels of liver enzymes, hyper-uricaemia and the deposition of calcium oxalate crystals in the kidney and brain. Other groups have also independently observed metabolic acidosis, hyperuricaemia, hyperbilirubinaemia and oxalate crystal deposition in patients infused with xylitol (Schumer, 1971; Donahoe and Powers, 1970; 1974; and Shröder et al, 1974; for reviews see Shröder, 1980; Wang and van Eys, 1981; Conyers et al, 1984). While extensive studies have since

shown that low dosages and slow rates of infusion of xylitol do not cause clinically significant side-effects (Mehnert and Förster, 1977), other investigations with high concentrations of xylitol administered parenterally in rabbits showed marked increases in the plasma activities of liver enzymes such as lactate dehydrogenase and amino-aspartate transferase. The degree of toxicity correlated with the concentration of xylitol and the rate of infusion (Wang et al, 1973).

Among the most serious of the adverse reactions reported to result from the parenteral administration of xylitol are oxalosis and the tissue deposition of calcium oxalate. The unexplained nature of this effect led to investigations at both the animal and cellular level (Hannet et al, 1977; Rofe et al, 1977; 1979; 1980; James et al, 1982). The use of $|U\text{-}^{14}C|$ xylitol in in vitro experiments with rat hepatocytes and in infusions in rats was associated with the production of $|^{14}\text{-}C|$ oxalate (Rofe et al, 1977). Vitamin B_6 deficiency enhanced the production of oxalate from xylitol in rats (Hannet et al, 1977). The infusion of xylitol in these vitamin B_6 deficient rats produced greater urinary excretions of oxalate and oxalate precursors such as glyoxylate and glycine when compared to vitamin B_6 deficient rats receiving similar infusions of glucose, fructose and sorbitol (Hannet et al, 1977). Although there is some suggestion that thiamine deficiency may have contributed to oxalate crystal deposition in some patients infused with xylitol (Hannet, et al, 1977; Rofe et al, 1977) other factors such as the nature of the underlying disease and previous drug history cannot be excluded (Rofe et al, 1979; Conyers et al, 1984). Recently a pathway for the conversion of xylitol to glycolaldehyde by fructokinase and aldolase has been defined (Barngrover et al, 1981; James et al, 1982). The toxicity associated with parenteral xylitol, especially when it is infused in large quantities, has shed doubts on the safety of orally consumed xylitol. The alleged toxicity of dietary xylitol has been examined and reported in several animal studies.

SIDE-EFFECTS AND TOXIC REACTIONS OF DIETARY XYLITOL IN ANIMAL STUDIES

Orally-consumed xylitol has been shown to be safe in mice and rats which received 10% (w/w) dietary xylitol over 12 weeks or 30% (w/w) over 5 weeks. These animals tolerated xylitol without any pathological changes being observed in their livers, kidneys or hearts. Furthermore, there was no cataract formation, weight gain, fertility problems or disturbances

in blood chemistry (Kieckebach et al, 1961). Even a "curative effect" of xylitol in diseases of the hepatobiliary system was suggested by Sheleketina (1969) since, in xylitol-fed rats, increases in liver glycogen, vitamin C and B_1 and niacin were observed. On the other hand, a diet consisting of 50% (w/w) xylitol was lethal in 1 to 2 weeks for some rats (Kieckebach et al, 1961). A feature common to animals fed a moderately high dose of xylitol (20% (w/w) to 50% (w/w)) is the presence of a usually transient, dose-dependent diarrhoea (Tudisco, 1960; Banziger, 1970).

The effect of the long term exposure to dietary xylitol was investigated at the Huntingdon Research Centre, Huntingdon, England (FDA, 1978). In these chronic toxicity studies a two year feeding experiment with mice, a one year feeding and multigeneration feeding study with rats (Hunter et al, 1978), a two year feeding trial with dogs (Heywood et al, 1978) and teratogenicity experiments in rabbits (Hummler, 1978) were performed. In addition, the potential mutagencity of xylitol was investigated. A diet consisting of 0, 2, 10 or 20% (w/w) xylitol or 20% (w/w) sucrose was fed to 100 male and 100 female CFLP mice for 102 to 106 weeks (Hunter et al, 1978). During the initial period of 4 to 11 weeks, mice on the 20% (w/w) xylitol diet exhibited diarrhoea which subsided from week 22 onwards. In the group of male mice, there was an increased mortality rate in the first year of treatment. Autopsies of male mice fed 10% (w/w) and 20% (w/w) xylitol diets showed that there were more bladder calculi than in male mice fed control, 2% (w/w) xylitol or 20% (w/w) sucrose diets. Although histological investigations on male mice fed 10-20% (w/w) xylitol exhibited a reduction in hepatocellular tumours, they revealed an increase in hyperplasia, metaplasia and malignant neoplasis of the transitional epithelium of the bladder associated with the calculi when compared to control mice. While neither calculi nor neoplasms of the bladders were observed in rats and dogs (Hunter et al, 1978; Heywood, 1978), the rats fed 5, 10 or 20% (w/w) xylitol or 20% (w/w) sorbitol exhibited an increased incidence of adrenal medullary hyperplasia and, in the 20% (w/w) xylitol fed rats, phaeochromocytomas were noted (Hunter et al, 1978; Salsburg, 1980). No adverse effects were observed in the dogs other than a slight hepatomegaly associated with hepatocyte enlargement in five out of the 12 dogs fed 20% (w/w) xylitol and 3 out of 12 dogs fed the 10% (w/w) xylitol diet (Heywood, 1978).

Teratogenicity studies with female rabbits fed 2, 5, 10 or 20% (w/w) xylitol or 20% (w/w) sucrose or sorbitol in their diet from the 7th and 19th day of gestation showed no effects on reproductive behaviour, litter size, foetal resorption rates or deformation of foetuses (Hummler, 1978). Also in the multigeneration feeding experiments with rats receiving up to 20% (w/w) dietary xylitol, it was found that there was some variation in litter size but no evidence of teratogenic effects was reported (Palmer and Bottomley, 1978; Palmer et al, 1978).

Xylitol and its metabolites were shown to be non-mutagenic by microbial assay systems at a level equivalent to 2.5 g/kg body weight (Batzinger et al, 1977). These mutagenicity tests were carried out directly on histidine-dependent strains of Salmonella typhimurium or indirectly with the mouse host-mediated assay system (Batzinger et al, 1977). Other host-mediated assay systems and chromosome analyses showed no mutagenic or genetic aberrations for xylitol concentrations up to 5.3 g/kg body weight (FDA, 1978). According to Scheinin (1978), in some of the animal studies the intake levels as well as the achieved dosage levels were much higher than the recommended safe dosage of 6 g/kg/d. Hence the validity of extrapolating data from experimental animals which were grossly overloaded with xylitol to the human situation, in which the dosage is less than 1 g/kg/d, is questionable (Scheinin, 1978).

DIETARY XYLITOL IN HUMAN CLINICAL TRIALS

A study of the medical and dental effects of xylitol-, fructose- and sucrose-containing foodstuffs was carried out in a selected population of volunteers in Turku, Finland (Scheinin and Mäkinen, 1975; Mäkinen and Scheinin, 1975a, b). The results from both the Turku studies and a concurrent one-year chewing gum test (Larmas et al, 1975) suggested that xylitol but not sucrose or fructose decreased the incidence of dental caries. The only side-effects experienced by the volunteers who consumed xylitol were diarrhoea and flatulence. These disturbances showed inter-individual variation in that some volunteers had mild or severe attacks of diarrhoea. Furthermore these xylitol-associated gastrointestinal disorders which occurred more frequently in the initial period of the trial tended to become less frequent in occurrence and intensity in the latter part of the two year study. Mäkinen (1976) suggested that the subsidence of the

gastrointestinal effects produced by xylitol in the volunteers was due to the adaptation of the gastrointestinal flora to xylitol although adaptation by the host physiological mechanism was not discounted. The safety of orally consumed xylitol was confirmed by analyses of serum and saliva which indicated no major differences among the xylitol-, fructose- or sucrose- trial groups (Mäkinen, 1976; Mäkinen, 1978). More conclusive evidence of the safety of orally consumed xylitol was obtained when the subjects of the Turku studies, as well as an additional small group of individuals, who regularly consumed xylitol over a period of 4 to 5 years, were reinvestigated with respect to all possible side-effects (Mäkinen, Ylikahri, Söderling et al, 1982; Mäkinen, Ylikahri, Mäkinen et al, 1982). The investigations comprised of blood plasma values, blood pressure measurements, changes in body weight, anamnesis of illnesses during participation in the dietary study, eye cataracts and urine analyses (for example, volume, pH, specific-gravity, calcium, magnesium, sodium, potassium phosphate, oxalate, citrate, urate, urea, creatinine, xylitol, sediment, catecholamines and their metabolites (metanephrine and 3-methoxy-4 hydroxy-mandelic acid)). Based on these tests none of the subjects showed any metabolic disturbance in urinary, renal or adrenal function. In addition, during the course of the Turku Sugar Studies there occurred eight pregnancies in three of which both parents consumed xylitol. The pregnancies, deliveries and infants were all normal (Scheinin, 1979).

SUMMARY OF XYLITOL DIETARY STUDIES IN HUMANS AND ANIMALS

The results of human dietary studies indicate that the oral consumption of high quantities of xylitol (<220 g/d) does not cause changes in the blood and urine biochemical values (Mäkinen, 1976) in contrast to the adverse metabolic effects observed with the intravenous administrations of hypertonic solutions of xylitol (0.25 g/kg/h). The oral consumption of xylitol by the volunteers in the Turku Sugar Studies was self-limiting due to the occurrence of gastrointestinal side-effects (flatulence and diarrhoea) when consumed in doses greater than 50 g/d. The self-limiting dosage of xylitol and the slow rate of its intestinal absorption appear to prevent high plasma xylitol concentrations from being attained. The enteral route of xylitol administration would thus seem to be safer than the parenteral route.

The results of long-term animal toxicity experiments described in the Huntington Studies, (see above), are difficult to evaluate. Any interpretation is complicated by the different toxic effects observed in the different species of animals fed xylitol. For instance, mice receiving high concentrations of xylitol in the diet had urinary calculi and malignant neoplasms in the bladder whereas, in rats, hyperplasia and phaeochromocytomas of the adrenal medulla were noted. In contrast, dogs did not show any adverse effects of dietary xylitol. However, all the species of animals investigated suffered from diarrhoea. The long-term human studies with dietary xylitol conducted in Turku, Finland, showed no adverse effects other than the inconvenience caused by diarrhoea and flatulence. In all species, diarrhoea is the most common side-effect of the oral consumption of xylitol. This contrasts with many of the other side-effects which are described above and which appear to be highly species-specific and markedly dose-related. Furthermore, both humans and animals eventually adapt to xylitol feeding and recover from their diarrhoea. The likely mechanisms of host adaptation to dietary xylitol are now discussed.

MECHANISMS OF ADAPTATION TO XYLITOL-INDUCED DIARRHOEA

In association with the consumption of large amounts of xylitol, the relatively slow rate of xylitol absorption from the gastrointestinal tract can result in the accumulation of unabsorbed xylitol in the caecum and colon (Bässler, 1969). The osmotically active xylitol will then increase the retention of water in the gastrointestinal lumen. While the diarrhoea that ensues is therefore osmotically-induced (Förster, 1978) it, nevertheless, disappears after prolonged xylitol intake (Bässler, 1969). The cessation of xylitol-induced diarrhoea implies that some adaptation has taken place to remove xylitol rapidly from the intestines. The liver, the gut wall and the gut lumen (i.e. gut microflora) are three possible sites where xylitol can be removed or metabolised. A model indicating these sites of adaptation in the host and the likely interactions between these sites, is shown in Fig. 1.2. Possible mechanisms of adaptation to xylitol-induced diarrhoea at these sites are now discussed.

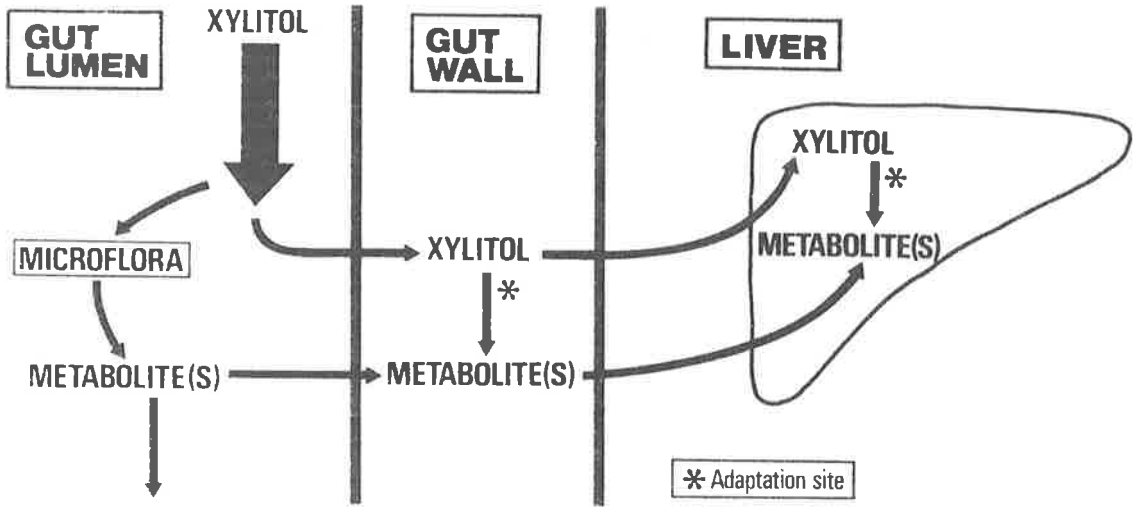


Fig. 1.2 A model representing sites of adaptation to dietary xylitol.

Liver

The liver is the major organ for xylitol metabolism (Lang, 1969). It has also been claimed by a number of workers that the liver is the site of adaptation in the host who receives either enteral or parenteral xylitol (Bässler, 1969; van Eys et al, 1974; Förster, 1978). The induction of hepatic xylitol dehydrogenases has been implicated in this adaptation. Hosoya and Iitoyo (1969) found that the liver NAD-linked xylitol dehydrogenase doubled in activity in rats fed up to 10% (w/w) xylitol. Furthermore, Fujisawa et al (1969) found that rats fed a 10% (w/w) xylitol diet had significant changes in the activities of liver enzymes within three to seven days of commencing the diet. These enzymes included those in the glucuronate-xylulose, gluconeogenic and pentose phosphate pathways. Bässler (1969) also demonstrated the induction of the liver xylitol dehydrogenases in association with xylitol feeding and suggested that these inductions were responsible for the increased rates of disappearance of xylitol from plasma in rats.

Gut Wall

In a histological study of the rat gastrointestinal tract, Müller (1964) showed that xylitol is primarily absorbed at the apex of the intestinal villi, distributed into the epithelial layer and then transported into the connective tissue and blood vessels of the villi. Nevertheless, the absorption of xylitol into the cells lining the gut lumen is very poor (Förster, 1978). Generally, the rapid intestinal absorption of sugars is due to the presence of active and or specific transport processes (Wilson, 1962; Förster and Menzel, 1972). Evidence for an active transport system for xylitol has not been forthcoming and, therefore, absorption of xylitol across the gut lumen appears to occur by passive diffusion or by a transport system with a low affinity for xylitol (Bässler, 1969; Lang, 1971; Förster, 1978). While the absorption of xylitol increases with corresponding increases in oral xylitol intake the proportion absorbed is decreased. This was illustrated in an absorption study in which aspirates were taken from the distal ileum of five male subjects (Asano, 1973). When 5, 15 and 30 g of xylitol were orally consumed by the subjects the amount absorbed increased with higher oral doses, but the percentage absorbed decreased from 90% to 76% and 66%, respectively. Thus,

this experiment clearly demonstrates that there is a threshold concentration for the absorption of xylitol. In addition, it was reported that the percentage of xylitol absorbed from the intestines was not affected by continuous oral consumption of xylitol.

Adaptation at the level of the gut wall has been shown after the dietary administration of sugars, by the elevation of glycolytic enzymes of the jejunum (Herman, 1974; Rosenwig, 1974; Greene et al, 1972), but similar studies with xylitol have not been performed. Although the role of the gut wall enzymes in the adaptation to dietary xylitol is unknown, such a possibility cannot be excluded, since xylitol dehydrogenase activities have been detected in the intestinal mucosa (Müller et al, 1967). Hence a possible induction of this enzyme in the intestines, by the dietary exposure to xylitol could contribute to the adaptative processes.

Gut Lumen

Any contribution of the gut microflora in the host adaptation to xylitol-induced diarrhoea has generally been considered to be negligible. This is because it has been believed that xylitol is not a substrate for microbial metabolism and growth. This belief has been confirmed by the inability of oral bacteria to produce acid, grow or adapt in the presence of xylitol (Bässler, 1969; Hosoya and Iiotoyo, 1969; Scheinin, 1978). Nevertheless, evidence of xylitol-induced changes in the gut microflora have been provided, but such studies are scarce. For example, in one study it was shown that when the microflora of the rumens of cows were incubated in the presence of xylitol or other sugar polyols, increased bacterial metabolism of these polyols were observed within 24 h. In that period, 20% of xylitol was fermented (Poutiainen, Touri and Sirvio, 1976). This evidence suggests that although it is rare for oral microflora to metabolise xylitol, the prevalence of xylitol-metabolising bacteria elsewhere in the gastrointestinal tract cannot be excluded.

MICROBIAL METABOLISM OF XYLITOL

According to Mäkinen (1976) the cariogenic flora of man can neither grow on nor metabolise xylitol. Mortlock (1976) has reported in his

review, however, that bacteria, yeasts and fungi, which are not necessarily gut commensals, can utilise xylitol. Indeed, a variety of pentitols including ribitol, D- and L-arabitol and xylitol, have been used as sole sources of carbon and energy for growth and metabolism by Aerobacter aerogenes PRL-R3 (Anderson and Wood, 1962; Mortlock and Wood, 1964). Other bacteria that can utilise xylitol as a major substrate include Azotobacter agilis (Marcus and Marr, 1961) and Acetobacter suboxydans (Arcus and and Edson, 1956). With Pseudomonas flurosens, Pseudomonas coronafaciens, Sarcina marginata and Sarcina aurantiaca, metabolism of xylitol was only possible after growth on other polyols such as mannitol or sorbitol (Yamanaka and Sakai, 1968). Horowitz and Kaplan (1964) have shown that Bacillus subtilis can grow on xylitol as a substrate provided a trace amount of sorbitol is present in the growth medium to cause an induction of the polyol dehydrogenase. Enzymic pathways have also been described for the oxidation of xylitol in fungi such as Penicillin chrysogenum, Geotrichum candidum, Candida utilis and Sachromyces rouxii (Chiang and Knight, 1961; Moret and Sperti, 1962; Scher and Horecker, 1966; Ingram and Wood, 1965). Growth and metabolism of xylitol has also been reported in strains of yeasts such as Kluyveromyces, Torulopsis, Trigonopsis and Debaryomyces (Barnett, 1968). Only few microorganisms have the ability to metabolise xylitol compared with the large number of microorganisms that can metabolise glucose. A possible limitation in finding xylitol-metabolising organisms in general, can be attributed to the traditional and insensitive microbiological methods of assessing substrate utilisation by growth, pH changes or the production of non-volatile metabolites. For example, pH measurements may be underestimated if the production of acid is low and alkaline high or if the growth medium buffers any acid produced (Hayes and Roberts, 1978). A rapid and sensitive radioisotopic assay to assess substrate utilisation was employed in the experiments discussed in Chapter Two in order to overcome these problems.

Although extensive investigations to the effect of dietary xylitol on the oral microflora were carried out in the Turku Sugar Studies, there was no evidence of acid production or adaptation to xylitol in the major bacterial categories. On the other hand, in a New Zealand study the presence of xylitol-fermenting lactobacilli and propionibacteria were isolated from the saliva of subjects participating in the test and, furthermore, the pure isolates fermented xylitol to a pH less than 5, which is an indication of cariogenic potential (Gallagher and Fussell, 1979). Isolations

of other bacterial commensals which possess xylitol-metabolising activities include streptococci of unknown serological groups and gram-negative bacilli from the oral cavity of the hamster (Gehring, 1974), Streptococcus avium (Bergey, 1974), Lactobacillus, Salivarius subsp. salivarium (Bergey, 1974) and Staphylococcus saprophyticus (Schleifer and Kloos, 1975).

It is therefore possible that microorganisms, capable of metabolising xylitol, could undergo selective growth in the caecum or the colon where high concentrations of xylitol would provide the nutrient source for both metabolism and growth. The increased metabolism of xylitol in the gut by bacterial adaptation or selection would lead to a decrease in the xylitol concentration in the gut and thus cessation of the osmotically induced diarrhoea. That is, "host adaptation" to xylitol-induced diarrhoea could, in reality, involve microbial mechanisms in the gut lumen as well as mechanisms within the gut wall and the liver of the host (Fig. 1.2).

AIMS OF THESIS

Major developments in the food industry over the past few decades indicate that a variety of commercially refined carbohydrate sweeteners will be included in our foods in larger and larger amounts over the next few decades. Xylitol, because of its food properties and its anti-cariogenic effects, is likely to be such a sugar. While a variety of side-effects and toxic complications have been associated with the enteral and parenteral use of this substance in the past, it would appear that the only non-species-specific and universal side-effect of moderate, oral doses of xylitol is diarrhoea. This osmotically-induced diarrhoea disappears on continued feeding and strongly suggests that some form of adaptation takes place. This adaptation process could occur in the gut microflora, in the gut wall or in the liver. A study of the mechanism of this adaptation to xylitol-feeding is important in the understanding of xylitol-induced diarrhoea. Thus the investigations carried out in this thesis were as follows:

To examine either directly or indirectly the role of the gut microflora, the gut wall and the liver in the host adaptation to oral xylitol, using

a rat model. These studies are reported in Chapter Two where the gut microflora are shown to play a major role in this process.

To isolate and identify, from the rat gastrointestinal tract, bacteria that are capable of metabolising and growing on xylitol (Chapter Three).

To attempt to characterise the biochemical changes associated with these bacteria in their adaptation to xylitol and further to examine possible biochemical mechanisms of adaptation (Chapters Four and Five).

CHAPTER TWO

STUDIES ON THE ADAPTATION ASSOCIATED WITH XYLITOL INGESTION IN RATS: A ROLE FOR GUT MICROFLORA

INTRODUCTION

While the oral consumption of xylitol is often accompanied in the initial period by flatulence and osmotic diarrhoea, persistence with such diets leads to the cessation of the diarrhoea (Chapter One). The sites of this adaptation to dietary xylitol could be located within the gut lumen, at the gut wall or within the liver. Experimental details involving the gut wall are ambiguous since some studies suggest that prolonged dietary intake of xylitol causes an increase in its absorption from the gastrointestinal tract (Bässler, 1969) while other studies indicate that there is no effect (Asano, 1973). In fact, Bässler (1969) suggested that the increase in absorption of xylitol from the gut lumen was a function of adaptation in the liver, since higher rates of xylitol clearance from the blood in adapted rats were associated with increased activity of xylitol dehydrogenase in the liver. The role of the microflora in the adaptation to xylitol has not been seriously considered (Chapter One), owing to the rarity of pentitol- and especially xylitol-metabolising commensals in the gut; on the other hand, hexose- and hexitol-metabolising bacteria are prevalent (evidence reviewed by Mortlock,

1976 and Mäkinen, 1978). In addition, there is little evidence to indicate any alteration in the microfloral pattern of the gut in animals fed xylitol. Due to the inconclusive results from these studies, it was decided to examine the various aspects of the adaptation process to xylitol in more detail.

The significance of these sites of adaptation was studied using the rat as an experimental model. Rats were fed various quantities of xylitol for periods up to two weeks and observed for diarrhoea and its subsequent cessation during the course of the dietary regimen. Liver homogenates from xylitol- and various other carbohydrate-fed rats were examined for adaptative changes in enzyme activities while the overall metabolic activities were assessed by the use of radio-labelled substrates. Histological examination of the gut wall and measurements of xylitol concentrations attained in the gut lumen and plasma were used to assess whether any adaptation had occurred at this site. In order to clarify the involvement of the gut microflora in this adaptation, the caecal contents of xylitol-fed rats were examined closely for alterations in metabolic activity, as determined by pH measurements, and observations of the absence or presence of caecal gas production. The detection of xylitol metabolism in the caecal contents of xylitol-fed and other carbohydrate-fed rats was achieved by a radioisotopic assay. In addition, direct microscopical examination of the caecal contents was used for the assessment of any gross changes in the population of the caecal microflora. The results of the above investigations are discussed in the following sections.

MATERIALS AND METHODS

Laboratory Supplies

Xylitol, D-sorbitol and D-arabitol were supplied by Roche Products, Australia. Sucrose, D-glucose, D-fructose, D-xylose and all other chemicals were obtained from either Calbiochem, Sydney, or Ajax Chemicals, Sydney. Labelled $|U\text{-}^{14}C|$ D-glucose, D-fructose, D-xylose, xylitol and D-sorbitol were obtained from Amersham, Australia, $|1\text{-}^{14}C|$ and $|6\text{-}^{14}C|$ labelled D-glucoses were obtained from New England Nuclear, U.S.A. Sorbitol dehydrogenase was purchased from Sigma, U.S.A.

Animals and Diet

Male Porton rats, which were bred at the I.M.V.S. field station, were used in these experiments. Rats weighing between 250g and 300g each were housed in groups of 8 or less on sawdust in metal cages. The animals had free access to water and food (mouse M & V cubes, Charlicks, Adelaide). Two different diet regimens were used in these studies. In one set of feeding experiments (type A) each rat also received, by daily gavage, 4 ml of 50% (w/v) carbohydrate in water for 7 days. Control rats received only water in their gavage. In the other set of feeding experiments (type B), the food was first prepared by grinding the mouse cubes to a powder and mixing in the appropriate carbohydrate to give a final concentration of 2.5, 5, 10 or 20% (w/w) carbohydrate. The mixtures were immediately reformed into rough cubes by the addition of a little water and then dried in a 50^{o}C oven overnight. The diet for control animals was prepared in a similar manner but without the added carbohydrate. Animals were maintained on these diets for periods ranging from 1 to 14 days. Body weights and water and food consumption were recorded daily. The presence of diarrhoea was also noted.

Tissue Preparations and Assay Conditions

Designated animals were killed on the appropriate day by cervical dislocation. Blood was collected into heparinized plastic tubes for the determination of a variety of plasma biochemical values by a Sequential Multiple Analyser with Computer (SMAC, Technicon Instruments Corporation, U.S.A.). Plasma xylitol concentrations were determined by the method of Bässler (1974). This assay is based on the oxidation of xylitol by sorbitol dehydrogenase in the presence of NAD^{+} and was carried out on an Abbott Bichromatic Analyzer 100 (Abbott Laboratories, U.S.A.).

The abdominal cavity of each rat was opened and, when required, the liver was removed and placed on ice. The bowel was then divided 10 mm above and below the caecum. The caecum and its contents were removed together _in toto_ and their wet weights determined. The caecal contents were than gently extruded into a test tube and diluted 1 in 5 with phosphate-buffered saline (PBS; 10 mmol/l sodium phosphate buffer, pH 7.0, containing 140 mmol/l NaCl). The caecal xylitol concentration was measured on the supernatant obtained by centrifuging this suspension at

10,000 x g for 10 min at 4°C. The pH of the caecal contents, however, was determined on a 3% (w/v) suspension of caecal contents in water.

Liver homogenates (25% w/v) were prepared at 0°C in an oxygenated, 10 mmol/l sodium phosphate buffer, pH 7.4, containing 0.2 mol/l KCl and 1.0 mmol/l MgCl, using a Potter-Elvehjem homogeniser. The production of $^{14}CO_2$ from labelled carbohydrate substrates was determined in standard glass scintillation vials. The reaction mixture contained 10 µmoles of labelled substrate (0.1 µCi/µmol) in a volume of 0.1 ml to which was added 1.0 ml of liver homogenate. A small inner vial (10 x 45 mm) containing 0.2 ml of 1 mol/l hyamine hydroxide in methanol was inserted into the incubation vial (see Fig. 2.1) at the beginning of the reaction, the large vial was capped and the whole reaction mixture was then shaken at 80 oscillations/min for 90 min at 37°C. At the end of the incubation the inner vial was removed, the exterior surface was washed with distilled water and the $^{14}CO_2$ was measured after the addition of 2.6 ml of toluene scintillant (0.4% 2,5-diphenyloazole, 0.02% 1,4-bis{2- (5-phenyloxazolyl)}-benzene), on a Model 3375 Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., U.S.A.). The efficiency of trapping $^{14}CO_2$ in hyamine hydroxide in this system is 99% (Duncombe and Rising, 1969).

Enzyme activities were determined on supernatants prepared from liver homogenates by centrifugation at 9,000 x g for 10 min at 0°C with the exception of glutamate dehydrogenase activity which was determined on a 2,000 x g supernatant (mitochondrial fraction and supernatant). The activities of aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase (lactate to pyruvate) and γ-glutamyltranspeptidase were all assayed by methods available on the SMAC. Transketolase activity was determined by the method of Smeets, Muller and De Wael (1971) on a Gilford 2400-S spectrophotometer (Gilford Instrument Laboratories Inc., U.S.A.). The activities of other liver enzymes were determined on a CentrifiChem System 400 centrifugal analyser (Union Carbide, U.S.A.) by the following methods: alanine aminotransferase (Committee on Enzymes of the Scandinavian Society, 1974); alcohol dehydrogenase (Blair and Vallee, 1966); glutamate dehydrogenase (Recommendations of the German Society, 1972); xylitol dehydrogenase (substrate: 0.1 mol/l xylitol) (Bässler, 1974); and glucose 6-phosphogluconate dehydrogenase (Rudack, Chisholm and Holten, 1971). All enzyme activities were assayed at 37°C.

Liver homogenates and supernatant fractions were prepared and the performance of those fractions in the Salmonella (TA/100) mammalian microsome mutagenicity test was carried out according to the method of Ames, McCann and Yamasaki (1975) using 2-aminofluorene as the mutagen. Protein was determined by the method of Lowry et al, (1951).

Preparation of Caecal Flora Samples

The 20% (w/v) suspension of caecal contents in PBS was centrifuged at 100 x g for 1 min at 4°C to remove food debris. The supernatant fraction from this centrifugation provided the caecal flora preparation.

Radioisotopic Metabolic Assay

The production of $^{14}CO_2$ from |U-^{14}C| carbohydrates by caecal flora preparations was also determined. A volume (0.1 ml), containing 10 µmoles and 5 x 10^5 c.p.m. of labelled carbohydrate, was added to 0.9 ml of caecal flora preparation in a standard plastic scintillation vial. Cultures of bacterial isolates were resuspended in PBS and then used in the same way. The procedures for incubation of the vials, trapping of $^{14}CO_2$ and the counting of the radioactivity are the same as those described for the liver homogenate assays (see Fig. 2.1). The $^{14}CO_2$ produced in the 90 min incubation period is expressed as c.p.m./mg of protein.

Results are reported as the mean with its standard error and the number of determinations is given in parentheses. Statistical significance has been calculated by the t-test for unpaired means. The term carbohydrate is used in the biochemical sense and includes sugars and sugar polyols.

RESULTS

The Effects of Dietary Carbohydrates on Liver Function

A comparative study of the effects of various carbohydrates on liver function was carried out, particularly to investigate the effects of xylitol in relation to the conventional sugars and other related sugar polyols under similar experimental conditions. These effects were first determined on the enzymatic activities in livers taken from rats fed approximately 10%

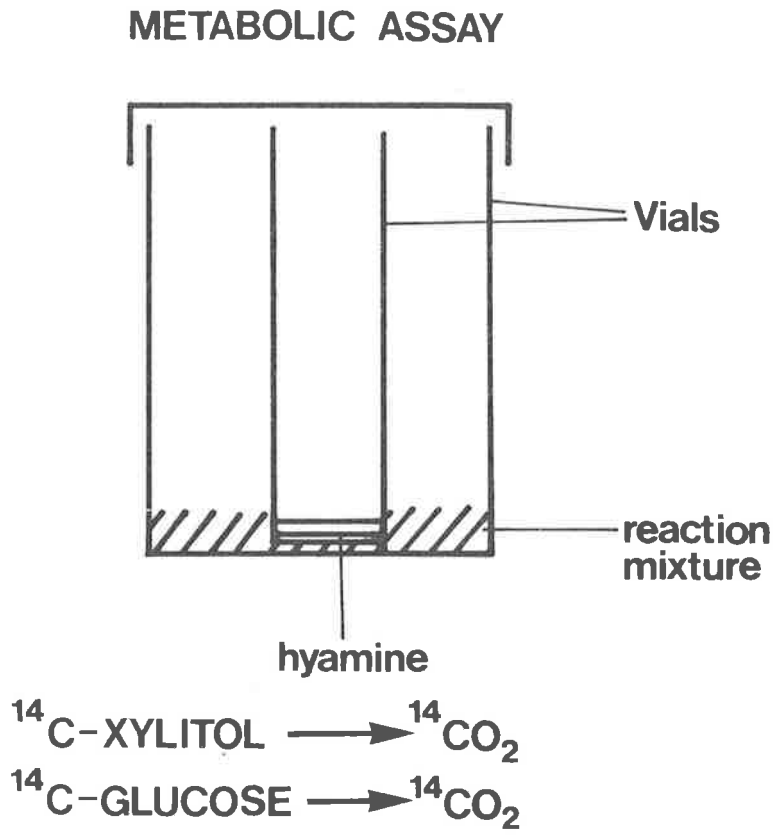


Fig. 2.1 Apparatus for collecting $^{14}CO_2$

(w/w) of their dietary intake as carbohydrate for 7 days (type A feeding regimen). In essence, none of the carbohydrates induced any major changes in the activities of a variety of enzymes associated with mitochondria, microsomes, pentose phosphate pathway, amino acid metabolism and clinical liver disease (Table 2.1). Fructose significantly increased microsomal activity in the mutagenicity test but had no effect on the γ-glutamyltranspeptidase activity. Minor, but statistically significant, increases in activity were observed for transketolase with glucose and fructose feeding and for glutamate dehydrogenase with sorbitol and xylitol feeding. Although the weights and protein concentrations of the livers were generally unaffected by these diets (Table 2.1) only xylitol and sorbitol appeared to cause increased liver weights. This is probably a reflection of loss of body weight due to the effects of diarrhoea (Karimzadegan et al, 1979).

In the same study a more direct measure of hepatic pentose phosphate pathway activity was obtained by comparing the $^{14}CO_2$ produced from the metabolism of $|1\text{-}^{14}C|$ glucose with that from $|6\text{-}^{14}C|$ glucose (Landau et al, 1964). Under the experimental conditions used, the various carbohydrates had no effect (Table 2.2) thus complementing the enzyme studies. Rats were also fed 20% (w/w) carbohydrates in their diets for 7 days using a type B feeding regimen. Rat livers were then assessed for their ability, as homogenates, to metabolise a range of $|U\text{-}^{14}C|$ labelled carbohydrates to $^{14}CO_2$ (Table 2.2). In general terms, the feeding of carbohydrate did not cause the liver to metabolise that or other carbohydrates more or less than the livers of control animals. The production of $^{14}CO_2$ from $|U\text{-}^{14}C|$ fructose by liver homogenates is much higher than other labelled carbohydrates (Table 2.2). This substrate is able to enter the glycolytic pathway readily and be metabolised more rapidly than other sugars (Hue, 1974). In the studies reported in Tables 2.1 and 2.2 diarrhoea was only observed in those rats receiving sugar polyols in their diets. Subsequent studies were carried out using only xylitol as the dietary sweetener.

The Effect of Dietary Xylitol on Certain Biochemical Values of Plasma

In these experiments one rat from a control group (0% xylitol in the diet) and two rats from each of four xylitol-fed groups (2.5, 5, 10 and 20% (w/w) dietary xylitol) were killed on days 1, 2, 3, 4, 5, 7, 10 and 14 (type B feeding regimen). When the concentrations for each plasma constituent

TABLE 2.1 Values of liver constituents in rats fed various carbohydrates in their diets.

Liver constituents	Values of constituents (n=3 except for food intake where n=7)					
Carbohydrate added to diet	none	glucose	fructose	sucrose	sorbitol	xylitol
Daily intake of carbohydrate (g)	-	2	2	2	2	2
Daily intake of other food (g) (Type A feeding regimen)	21 ± 1	23 ± 1	21 ± 1	17 ± 2	18 ± 1	20 ± 1
Weight of liver (% body weight)	4.9 ± 0.1	4.8 ± 0.1	5.0 ± 0.1	4.6 ± 0.1	4.1 ± 0.1†	4.4 ± 0.1†
Liver protein concentration (mg/g fresh wt. of tissue)	85 ± 5	91 ± 3	89 ± 5	87 ± 3	95 ± 4	93 ± 3
Enzyme activities (μmol/min/g fresh wt of tissue)						
alkaline phosphatase ($X10^3$)	184 ± 21	179 ± 15	151 ± 13	168 ± 7	216 ± 24	179 ± 9
aspartate aminotransferase	67 ± 17	48 ± 2	50 ± 5	54 ± 10	70 ± 4	51 ± 3
alanine aminotransferase	52 ± 6	46 ± 7	51 ± 5	49 ± 7	66 ± 7	50 ± 8
glutamate dehydrogenase ($X10^2$)	30 ± 3	33 ± 3	37 ± 6	30 ± 4	40 ± 1*	41 ± 1*
lactate dehydrogenase	467 ± 9	500 ± 19	493 ± 6	480 ± 21	485 ± 18	486 ± 17
alcohol dehydrogenase (X10)	24 ± 3	23 ± 2	26 ± 2	23 ± 2	29 ± 2	24 ± 1
glucose 6-phosphate dehydrogenase (X10)	20 ± 4	23 ± 1	25 ± 2	25 ± 1	19 ± 2	14 ± 1
6-phosphogluconate dehydrogenase (X10)	20 ± 2	21 ± 1	22 ± 1	23 ± 1	20 ± 1	18 ± 1
transketolase ($X10^2$)	44 ± 1	51 ± 2*	51 ± 2*	48 ± 2	48 ± 3	45 ± 3
γ-glutamyltranspeptidase	35 ± 7	35 ± 14	32 ± 15	34 ± 10	26 ± 15	28 ± 8
Salmonella/mamallian-microsome mutagenicity test$^{\pi}$ (revertant colonies /mg S9 protein)	103 ± 30	179 ± 39	261 ± 40*	117 ± 11	176 ± 8	151 ± 2

Values significantly different from control (no added dietary carbohydrate) value, $p < 0.05$(*); $p < 0.01$(†).

π values corrected for blank activity

Results are reported as mean ± SEM

TABLE 2.2 The production of $^{14}CO_2$ from $|^{14}C|$ - labelled carbohydrates by homogenates of livers taken from rats fed various carbohydrates in their diets.

Experimental Conditions	Measured values (n=3 for type A and n=5 for type B feeding regimens)							
Carbohydrate in diet (A ∿10% w/w) (B 20% w/w)	none	glucose	fructose	sucrose	xylose	sorbitol	xylitol	arabitol
$^{14}CO_2$ production from labelled substrate (c.p.m. x 10^{-2})								
A. $\frac{\|1\text{-}^{14}C\|\ \text{glucose}}{\|6\text{-}^{14}C\|\ \text{glucose}}$	10.1 ± 1.3	8.4 ± 0.9	8.9 ± 0.3	8.5 ± 0.4	-	9.9 ± 0.6	9.0 ± 1.1	-
B. $\|U\text{-}^{14}C\|$ glucose	22 ± 4	37 ± 4*	36 ± 11	19 ± 2	29 ± 5	24 ± 5	23 ± 3	25 ± 6
$\|U\text{-}^{14}C\|$ fructose	249 ± 53	326 ± 33	296 ± 25	288 ± 27	320 ± 69	282 ± 49	329 ± 49	420 ± 119
$\|U\text{-}^{14}C\|$ xylose	19 ± 1	22 ± 1	21 ± 2	18 ± 1	20 ± 1	20 ± 1	18 ± 2	19 ± 3
$\|U\text{-}^{14}C\|$ sorbitol	13 ± 2	15 ± 2	16 ± 2	11 ± 1	14 ± 2	12 ± 1	11 ± 1	14 ± 2
$\|U\text{-}^{14}C\|$ xylitol	16 ± 2	22 ± 2	26 ± 6	17 ± 2	16 ± 4	13 ± 2	12 ± 2	16 ± 3

* significantly different from control (no added dietary carbohydrate) value, $p < 0.05$

obtained for these rats were examined graphically, no obvious trends could be discerned with respect to time and so the results were pooled for each constituent and diet. Table 2.3 shows that xylitol feeding, in general, as assessed by clinical chemistry determinations on blood, caused little or no disturbance in fluid and electrolyte balance (sodium, chloride), acid-base balance (bicarbonate), renal function (creatinine), liver function (albumin, bilirubin, γ-glutamyltranspeptidase), lipid metabolism (cholesteral, purine metabolism (urate), and carbohydrate metabolism (glucose), findings similar to the observations of Huttunen, Mäkinen and Scheinin (1975) and Mäkinen and Scheinin (1975b) in man. The glucose values, however, are higher than the published reference range for rats (Mitruka and Rawnsley, 1977; Table 2.3), but this is probably a result of the free access the rats had to food. Certainly, at the time of death (0900 h) all rats had distended stomachs full of recently ingested food. Plasma urea concentration decreased with increasing dietary xylitol content, but this is more than likely due to the inverse relationship between the xylitol and protein contents of the diets (Table 2.4). While dietary xylitol increased plasma xylitol concentration in rats (Table 2.3) and man (Förster, 1978), the changes are not significant.

The Effect of Dietary Xylitol on Other Biological Parameters in the Rat

Both the incidence and degree of diarrhoea in these rats increased with the content of xylitol in the diet (Table 2.4). While the control, 2.5 and 5% xylitol diet groups of rats were essentially free of diarrhoea, the 10 and 20% xylitol diet groups had obvious diarrhoea in most animals for the first 7 days of the diets. However, unlike the 10% (w/w) xylitol diet group rats which passed mainly soft stools and were essentially free of diarrhoea after the first 7 days, the 20% (w/w) xylitol diet group rats had frank diarrhoea in all animals for the first 7 days of the diet with further sporadic attacks in some animals up to 14 days of the diet. The incidence of diarrhoea in these animals was roughly paralleled by the intake of water and the volume of caecal contents. On the other hand, the intake of food was reduced by approximately 20% in all groups of animals ingesting xylitol, irrespective of the amount of xylitol in the diet.

TABLE 2.3 Values of plasma constituents in rats fed varying amounts of xylitol in their diets.

Plasma constituents		Values of plasma constituents				
Concentration of xylitol in the diet (% w/w) (type B feeding regimen)		0	2.5	5	10	20
Plasma constituent	Published reference ranges for laboratory rats$^{\pi}$	(n = 4)	(n = 9)	(n = 10)	(n = 11)	(n = 16)
sodium	143 - 156 mmol/l	139 ± 3	137 ± 1	137 ± 1	138 ± 1	138 ± 1
chloride	100 - 110 mmol/l	100 ± 2	100 ± 1	102 ± 1	101 ± 1	102 ± 1
bicarbonate	12.6 - 32.0 mmol/l	22 ± 2	20 ± 1	20 ± 1	20 ± 1	20 ± 1
calcium	1.8 - 3.5 mmol/l	2.55 ± 0.10	2.38 ± 0.04	2.36 ± 0.04	2.35 ± 0.04	2.37 ± 0.05
phosphate	1.0 - 3.6 mmol/l	3.01 ± 0.05	2.80 ± 0.05*	2.82 ± 0.08	2.97 ± 0.05	2.78 ± 0.08*
urea	1.8 - 10.4 mmol/l	9.0 ± 0.4	8.1 ± 0.2	7.8 ± 0.2*	6.9 ± 0.2†	6.3 ± 0.3†
creatinine	0.02 - 0.07 mmol/l	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
urate	0.07 - 0.45 mmol/l	0.13 ± 0.03	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
total protein	47 - 82 g/l	60 ± 1	59 ± 1	59 ± 1	57 ± 1	58 ± 1
albumin	27 - 51 g/l	34 ± 1	35 ± 1	34 ± 1	33 ± 1	34 ± 1
cholesterol	0.26 - 1.40 mmol/l	2.12 ± 0.08	2.15 ± 0.07	2.23 ± 0.09	2.03 ± 0.06	2.02 ± 0.05
total bilirubin	0 - 9 µmol/l	1 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 1
γ-glutamyl transpetidase	-	5 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1
alkaline phosphatase	56.8 - 128 µmol/min/l	360 ± 46	373 ± 21	311 ± 9	327 ± 23	324 ± 17
glucose	2.8 - 7.5 mmol/l	8.4 ± 0.2	8.5 ± 0.2	8.4 ± 0.2	8.3 ± 0.2	8.0 ± 0.2
xylitol	-	0.20 ± 0.06	0.37 ± 0.12	0.38 ± 0.08	0.42 ± 0.09	0.31 ± 0.05

Values significantly different from control (0% dietary xylitol) values, $p < 0.05$(*); $p < 0.01$(†).

$^{\pi}$ Mitruka and Rawnsley (1977)

TABLE 2.4 Observations made on rats fed varying amounts of xylitol in their diets.

Observation	Values of observation				
Concentration of xylitol in diet (% w/w) (type B feeding regimen)	0	2.5	5	10	20
Observations made on live rats:					
(i) Daily food intake (g)	27.5 ± 0.7(14)	22.7 ± 0.9(14)†	20.7 ± 0.8(14)†	22.5 ± 0.7(14)†	22.4 ± 1.1(14)†
(ii) Daily water intake (ml)	32.3 ± 1.2(14)	28.4 ± 1.0(14)*	26.4 ± 0.7(14)†	42.9 ± 2.4(14)†	46.1 ± 2.9(14)†
Ratio: water/food	1.17	1.25	1.28	1.91	2.06
Observations made at death of rats:					
(i) Incidence of diarrhoea:					
soft to squelchy stools	1/8	1/16	0/16	10/16*	2/16
watery stools	0/8	0/16	0/16	2/16	11/16†
(ii) Caecum and contents:					
weight (g)	6.4 ± 0.3(8)	6.9 ± 0.3(16)	7.2 ± 0.2(16)*	10.1 ± 0.5(16)†	15.3 ± 0.5(16)†
% body weight	2.1 ± 0.1(8)	2.4 ± 0.1(16)*	2.5 ± 0.1(16)†	3.3 ± 0.1(16)†	4.9 ± 0.1(16)†
pH of contents	7.2 ± 0.1(8)	7.1 ± 0.1(16)	6.7 ± 0.1(16)†	6.1 ± 0.1(16)†	6.5 ± 0.1(16)†
presence of gas	0/8	0/16	0/16	5/16*	12/16†
presence of fluffy layer	0/8	1/16	9/16*	16/16†	16/16†

Values significantly different from control (0% dietary xylitol) value, $p < 0.05$(*); $p < 0.01$(†).

The Effect of Xylitol on the Caecal Contents of Rats

Since the presence of 20% (w/w) xylitol in the diet caused the caecum and its contents to more than double in weight (Table 2.4), the contents were analysed further. The pH of the contents decreased with increasing xylitol in the diet and this finding, taken in conjunction with the associated increase in caecal gas content (Table 2.4), suggested that xylitol feeding caused changes in the metabolic activity of either the gut luminal contents or the gut wall. More evidence for an altered metabolic activity of the whole gut in the presence of dietary xylitol was obtained with the observation that, in some rats, centrifugation of the suspension of caecal contents at 10,000 x g resulted in the appearance of an insoluble material between the pellet and the supernatant. This insoluble material was easily disturbed and was of low density. Some preliminary experiments suggest that this insoluble material could be either a bacterial polysaccharide or a rat glycoprotein. However, histological examination of haematoxylin and eosin-stained sections of the caecal walls revealed no evidence of inflammation or other pathological change (personal communication; Dr. J. McClure, Division of Tissue Pathology, Institute of Medical and Veterinary Science). (A more detailed account of this material is given in Chapter Five).

The concentration of xylitol in the caecal contents was also measured (Fig. 2.2) and, in the first few days of the feeding study, found to be in proportion to the amount of xylitol in the diet. The lower the xylitol content of the diet, however, the more rapidly the xylitol concentration of the caecal contents fell to almost undetectable levels. This period was 1, 2, 5 and 10 days for the rats on 2.5, 5, 10 and 20% (w/w) dietary xylitol, respectively. The presence of diarrhoea in the rats was closely related to the concentration of xylitol in the caecal contents. The caecal concentrations were in turn related to dietary intake.

Dietary Xylitol and Microbial Composition of Caecal Flora

Further investigations were conducted to see whether xylitol had any effect on the spectrum of microbial morphology or on the numbers of organisms in the major microbial categories present in the caecum. Samples of caecal flora preparations of rats fed xylitol diets for 10 days were examined by Gram stain. It was found that 2.5 and 5% (w/w) xylitol diets

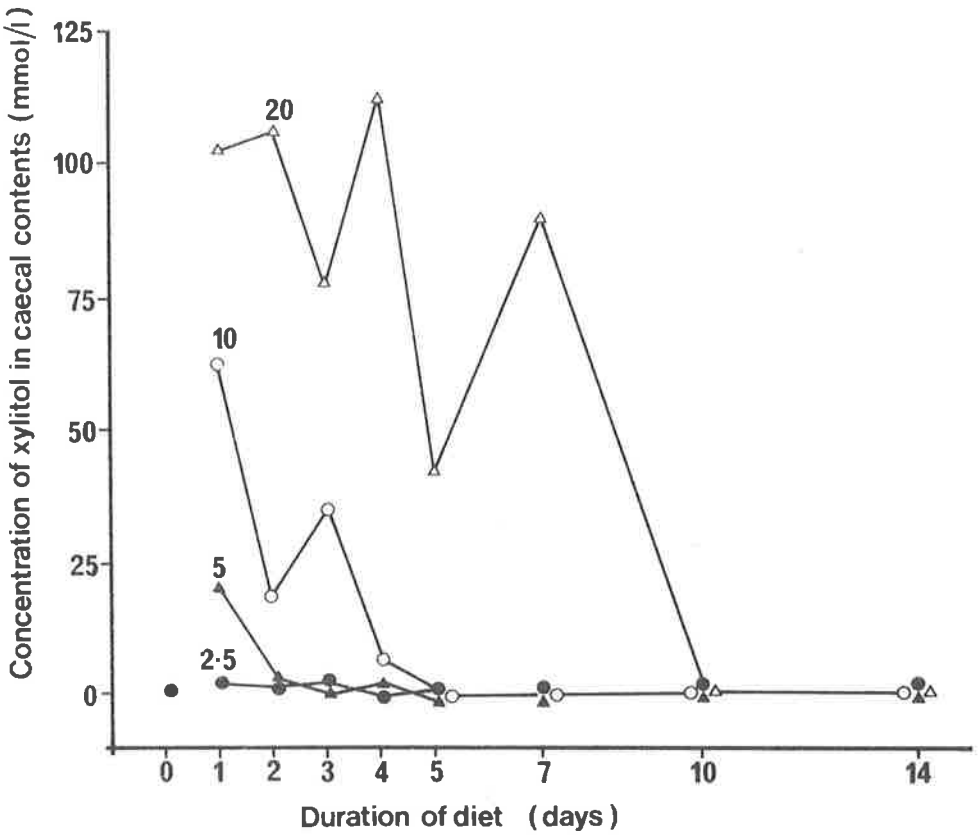


Fig. 2.2 The effect of the continuous feeding of xylitol on the concentration of xylitol in the caecal contents of rats. The curve labels 2.5, 5, 10 and 20 indicate the xylitol content (% w/w) of each diet. Each point represents the mean of the determinations for two individual animals except for the day 0 value which represents the mean of all the control animal determinations (0.8 $\pm$ 0.2(8) mmol/l).

caused an increase in gram-positive cocci. The 10% (w/w) xylitol diet caused similar changes but the gram-positive cocci had a more bizarre morphological appearance (Fig. 2.3). There were only scant bacteria in the caecal flora preparations from rats fed 20% (w/w) xylitol.

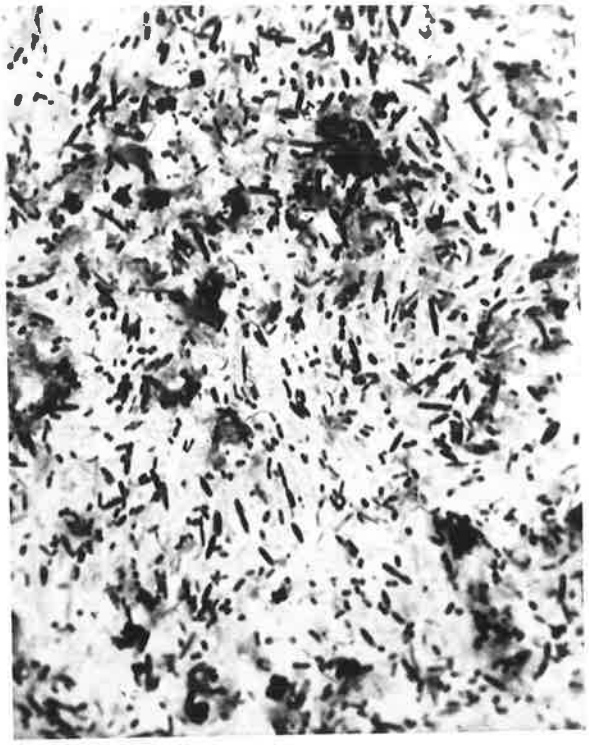
The rats fed 10% (w/w) xylitol in their diets showed the most consistent changes, and a semi-quantitative study was therefore performed on these caecal flora preparations as shown in Table 2.5. Gram stains were prepared from at least 2 individual rats for each day of the study. There was an increase, within the first 24 h of xylitol feeding, of both gram-positive bacilli and gram-positive cocci. While the gram-positive cocci continued to increase in numbers up until the fifth day, the gram-positive bacilli only did so until the fourth day and then on the fifth day decreased in numbers to a population similar to that seen in control fed rats. There were no further changes after day 5. On the other hand, no quantitative changes were observed in the gram-negative bacilli which formed the bulk of the caecal flora. The caecal flora of control rats showed only slight variation over the 14-day dietary study.

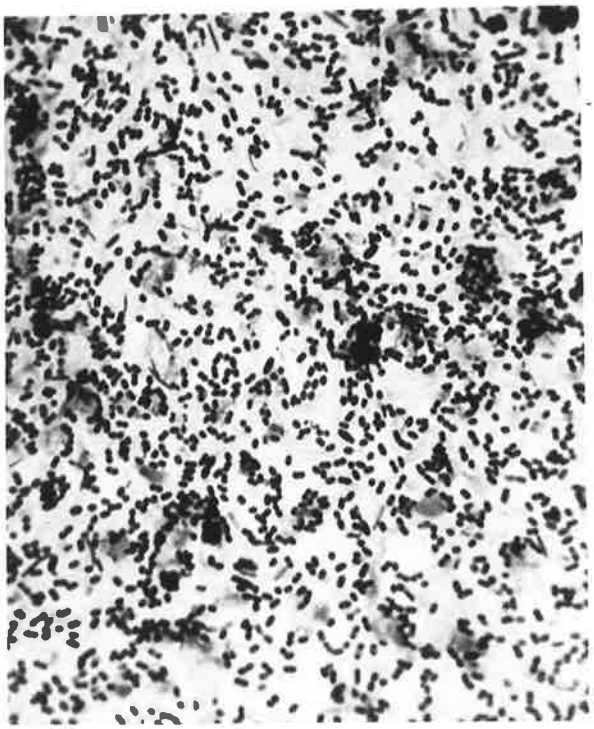
Because of these observations it was decided to investigate more closely whether gut commensals from the rat could metabolise xylitol, and if so, whether this activity could be increased in the presence of xylitol and therefore provide a possible mechanism for the host adaptations seen in xylitol-feeding. It was first necessary, however, to develop a sensitive and specific method for detecting xylitol metabolism. This method involved the measurement of $^{14}CO_2$ production from $|U\text{-}^{14}C|$ xylitol by gut commensals.

Gut Commensal Production of $^{14}CO_2$ from Labelled Carbohydrates

The optimal conditions for the metabolic radioisotopic assay were determined in preliminary experiments. In order to determine the ability of caecal flora to metabolise xylitol with minimal cell growth, the assay was performed in PBS. A 20% (w/v) suspension of caecal contents in PBS was used because suspensions of 10% (w/v) or less exhibited less than maximal metabolic activity with respect to glucose and fructose. The 20% (w/v) caecal flora samples prepared from normal rats gave a protein content per incubation flask of 11.0 $\pm$ 0.5 mg (n = 20). The

Fig. 2.3 The effect of feeding 10% (w/w) xylitol in the diet on the morphological and population characteristics of caecal flora in rats (Magnification x 250). The duration of feeding xylitol to the rats was 0 days (control, upper left); 4 days (upper right); 7 days (lower left); and 14 days (lower right).





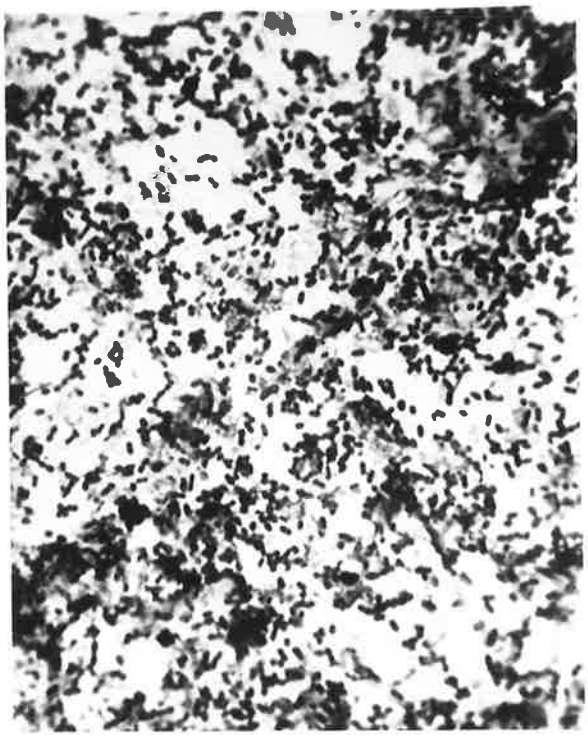


TABLE 2.5 The effect of feeding rats xylitol in their diet on the microbial population of their caeca

Duration of diet (days)	Concentration of gram stained organisms in rat caecal flora (organisms/high power microscopic field*)		
	Gram-positive cocci	Gram-positive bacilli	Gram-negative bacilli
0% (w/w) xylitol diet	+	+	+ + +
10% (w/w) xylitol diet			
1	+ +	+ +	+ + +
2	+ +	+ + +	+ + +
3	+ +	+ +	+ + +
4	+ +	+ +	+ + +
5	+ + +	±	+ + +
7	+ + +	+	+ + +
10	+ + +	±	+ + +
14	+ + +	±	+ + +

* Legend: ±, 1-10; +, 11-50; + +, 51-500; + + +, > 500.

removal of food debris by centrifugation at 100 x g for 1 min only reduced the metabolic activity of the suspension by 10% or less. More stringent centrifugal preparation and washing procedures resulted in large decreases in the metabolic activity of the suspensions. The 90 min incubation period was chosen as a balance between maximal rates of $^{14}CO_2$ production from most carbohydrates on the one hand and increased assay sensitivity on the other. The measurement of $^{14}CO_2$ production in this assay was reproducible with an intrabatch variation between duplicates of 7.1 $\pm$ 1.2% (n = 30).

The assay was used to assess the ability of caecal flora preparations to utilise various carbohydrates (Fig. 2.4). The production of $^{14}CO_2$ from labelled substrates by caecal flora was in the order glucose>fructose>xylose>sorbitol$\geq$xylitol. Where xylitol was the substrate, 3640 $\pm$ 196 c.p.m. (n = 4) per incubation flask appeared as $^{14}CO_2$ in the first 90 min of incubation. This value is significantly different ($p < 0.01$) from the value for the 0 time controls and the incubation blanks (47 $\pm$ 3, n = 40), and represents a 0.73% conversion of U-^{14}C xylitol to $^{14}CO_2$. Supernatant fractions prepared from caecal flora samples by centrifugation at 10,000 x g for 30 min showed no xylitol metabolising ability.

Effect of Dietary Xylitol on the Metabolism of Xylitol by Caecal Flora

The inclusion of xylitol in the diet of rats increased the ability of caecal flora to metabolise xylitol (Fig. 2.5). This dietary regimen has been described in Materials and Methods (type B feeding regimen). Caecal flora preparations in PBS were obtained by killing rats which had had the diets for periods of 1, 2, 3, 4, 5, 7, 10 and 14 days. The protein concentration of the caecal contents was 48.0 $\pm$ 2.9 (8), 49.6 $\pm$ 1.2 (16), 49.1 $\pm$ 1.5 (16), 33.4 $\pm$ 1.7 (16) and 19.4 $\pm$ 2.3 (16) mg/ml for rats on diets containing 0, 2.5, 5, 10 and 20% (w/w) xylitol, respectively. The specific activity of the production of $^{14}CO_2$ from |U-^{14}C| xylitol was 416 $\pm$ 26 (8) c.p.m./mg protein for caecal flora from normal rats. This activity was increased up to 10, 15, 30 and 40-fold in caecal flora taken from rats fed diets containing 2.5, 5, 10 and 20% (w/w) xylitol, respectively for a period of 14 days. Despite the decrease in protein concentration of the caecal contents with increasing dietary xylitol concentration, the

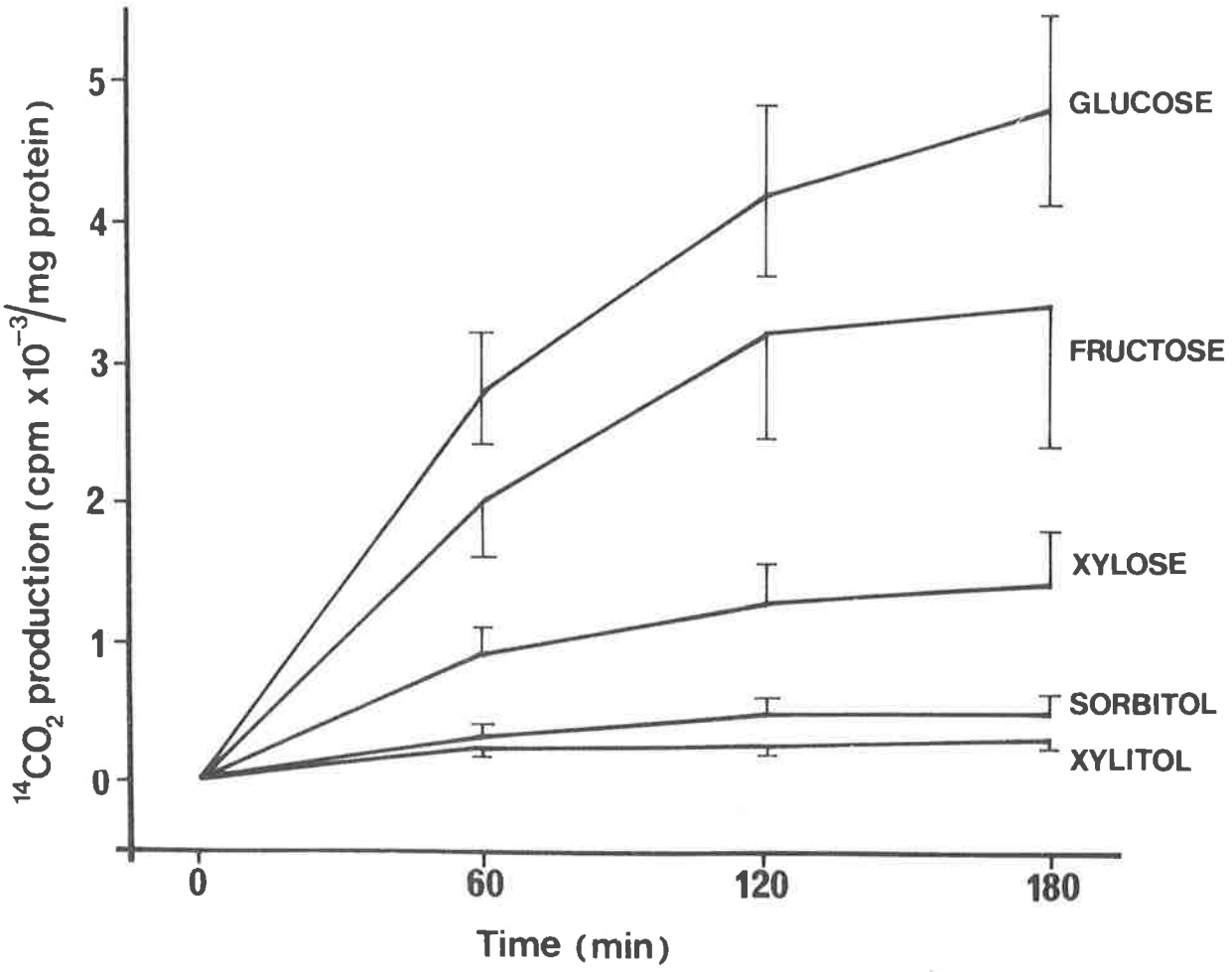


Fig. 2.4 The production of $^{14}CO_2$ from |U-^{14}C| carbohydrate substrates by preparations of rat caecal flora. Each point represents the mean ± S.E.M. for 4 determinations.

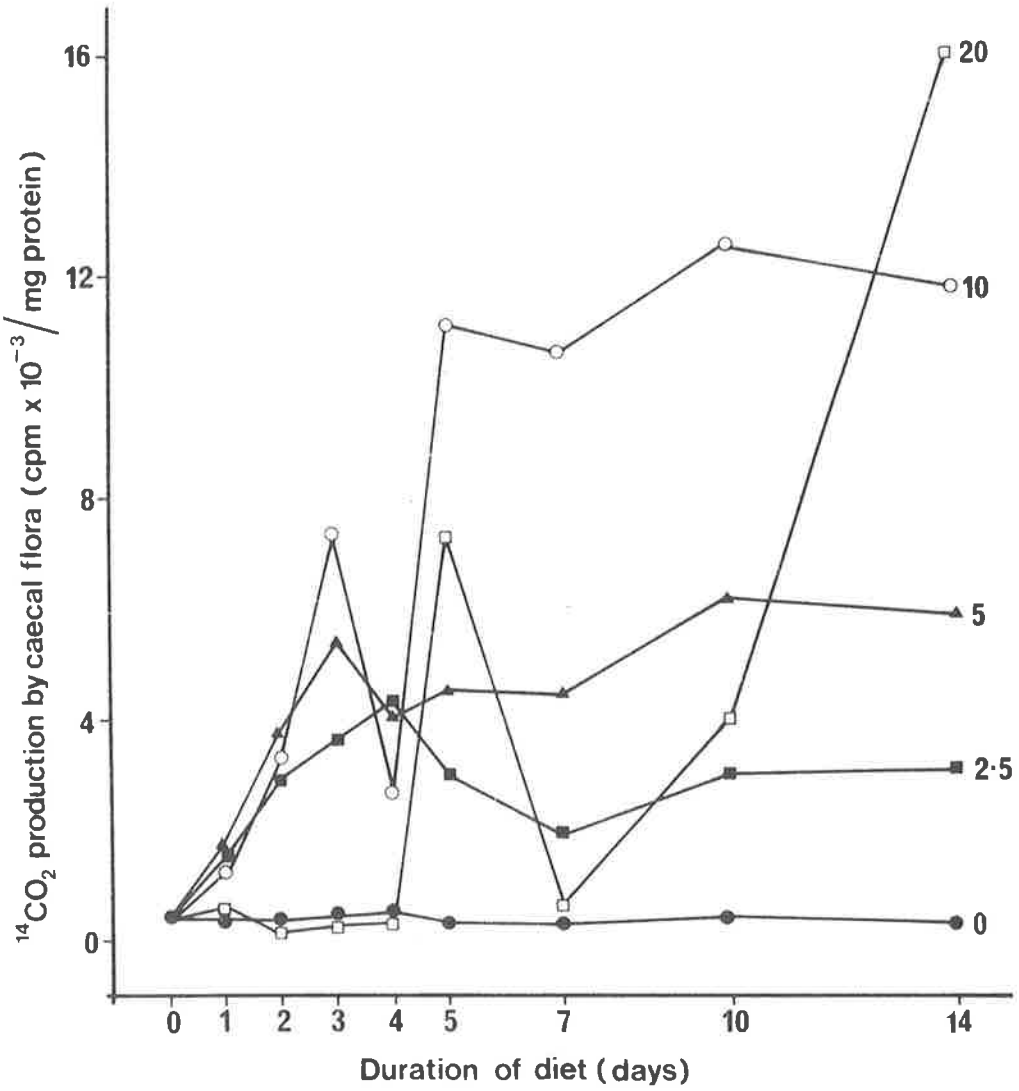


Fig. 2.5 The production of $^{14}CO_2$ from |U-^{14}C| xylitol by preparation of caecal flora taken from rats fed xylitol in their diets. The curve labels, 0, 2.5, 5, 10 and 20 indicate the amount of xylitol (% w/w) in the diet.

xylitol-metabolising activity of caecal flora also increased up to 18-fold per unit volume of caecal contents. Furthermore, the total volume of the caecal contents increased with increasing concentrations of xylitol in the diet.

The caecal flora in the rats on 2.5, 5 and 10% (w/w) xylitol diets showed increased xylitol metabolism after 24 h, reached maximal xylitol-metabolising activity in 3 to 5 days and then maintained that activity over the remaining 10 days of the experiment (Fig. 2.5). The caecal flora taken from rats on the 20% (w/w) xylitol diet, however, only showed consistently increased activity after 7 days and then continued to increase up to 14 days. On the other hand, the specific activity of the production of $^{14}CO_2$ from |U-^{14}C| glucose by caecal flora remained constant during the period of xylitol feeding (10,640 $\pm$ 640) (8), 11,410 $\pm$ 860 (16), 10,770 $\pm$ 680 (16), 13,360 $\pm$ 2,380 (16), and 9,510 $\pm$ 1,320 (16) c.p.m./mg protein for caecal flora from rats fed diets containing 0, 2.5, 5, 10 and 20% xylitol, respectively.

There was an inverse relation between the incidence of diarrhoea and the caecal xylitol concentration on the one hand, and the caecal protein concentration and the ability of caecal flora to metabolise xylitol on the other. The diarrhoea associated with xylitol feeding and, in particular with the 10 and 20% (w/w) xylitol diets, would appear to be associated with a dilution of caecal flora per unit volume of caecal contents as measured by decreases in caecal protein concentration and the glucose-metabolising activity of caecal flora per unit volume of caecal contents.

Effect of Dietary Carbohydrates on Carbohydrate Metabolism by Caecal Flora

Since the ability of caecal flora to metabolise xylitol was found to be both time-dependent and related to the amount of xylitol in the diet, it was decided to investigate the effect of other dietary carbohydrates on the ability of caecal flora to metabolise various carbohydrates. Using a type B feeding regimen, rats were fed either 5% (w/w) carbohydrate in their diet for 4 days or 20% (w/w) carbohydrate for 7 days.

In general, caecal flora produced $^{14}CO_2$ from labelled carbohydrates in

the order: glucose>fructose>xylose>sorbitol>xylitol (Table 2.6), confirming the data in Fig. 2.4. The inclusion of either 5 or 20% (w/w) glucose or fructose in the diet had no effect on the ability of caecal flora to metabolise carbohydrates. Sucrose-feeding increased fructose metabolism and xylose-feeding increased its own metabolism. Like xylitol, sorbitol, and to a certain extent arabitol, caused visible changes in caecal size and fluidity of contents. Sorbitol-feeding increased the ability of caecal flora to metabolise fructose, xylitol and itself. Feeding 20% (w/w) arabitol in the diet increased both sorbitol and xylose metabolism. The effect of arabitol feeding on its own metabolism was not tested since |U-^{14}C| arabitol is not available commercially. These findings were for both the 5 and 20% (w/w) carbohydrate diets. The 5% (w/w) xylitol diet increased the ability of caecal flora to metabolise xylose, sorbitol and itself but the 20% (w/w) xylitol had no effect. The 20% (w/w) xylitol diet actually decreased the ability of caecal flora to metabolise glucose. This is consistent with the dilution of the caecal flora due to the greater degree of diarrhoea observed in rats on the 20% (w/w) xylitol diet (see above).

It can be seen from the standard errors of the means in Table 2.6 that there was considerable variation amongst individual rats in the ability of their caecal flora to metabolise various carbohydrates. Indeed, in preliminary experiments, it was the apparent inconsistency of observed increases in the xylitol-metabolising activity of the caecal flora of the rats fed 20 and 30% (w/w) xylitol diets that led to these experiments. The inter-individual variations reflect, in particular, the degree of diarrhoea in the sugar alcohol-fed rats (Fig. 2.5) (Wekell, Hartmann and Dong, 1980) and, in general, the small numbers of rats in each dietary study (Table 2.6).

DISCUSSION

Although a number of hexose sugars are absorbed by active or facilitated transport mechanisms in the intestine, there is no evidence for any specific transport mechanism for sugar polyols (Förster, 1978). Therefore, when sugar polyols are administered in relatively high doses, the ability

TABLE 2.6 A comparison of the effects of diets containing various carbohydrates on the ability of rat caecal flora to produce $^{14}CO_2$ from labelled carbohydrates.

Carbohydrate in the diet of rats	Weight of caecum plus contents (% body wt.)	Caecal protein concentration (mg/ml	Production of $^{14}CO_2$ by caecal flora samples from: (cpm X10^{-3}/mg protein)				
			|U-^{14}C| glucose	|U-^{14}C| fructose	|U-^{14}C| xylose	|U-^{14}C| sorbitol	|U-^{14}C| xylitol
5% (w/w) carbohydrate; 4 days (n=4)							
none	1.92 ± 0.12	61.0 ± 1.5	14.9 ± 0.9	14.7 ± 1.2	7.0 ± 1.5	2.7 ± 0.4	[illegible]3 ± 0.1
glucose	2.10 ± 0.10	58.5 ± 1.5	12.3 ± 2.0	11.6 ± 1.2	6.1 ± 1.2	2.1 ± 0.3	[illegible]2 ± 0.1
fructose	1.93 ± 0.05	58.5 ± 1.5	13.3 ± 1.5	12.0 ± 1.7	5.9 ± 1.0	2.5 ± 0.6	[illegible]3 ± 0.1
sucrose	2.42 ± 0.12	59.0 ± 6.0	15.4 ± 3.0	18.2 ± 4.4	7.0 ± 2.1	2.3 ± 2.1	[illegible]2 ± 0.1
xylose	2.12 ± 0.10	59.0 ± 5.5	15.6 ± 0.5	14.4 ± 0.8	10.7 ± 0.7	2.7 ± 0.3	1.6 ± 0.1
sorbitol	2.14 ± 0.21	54.5 ± 2.5	15.3 ± 2.0	18.9 ± 4.4	7.4 ± 2.3	19.2 ± 5.1*	[illegible]2 ± 2.1
xylitol	2.38 ± 0.17	65.5 ± 1.0	13.6 ± 0.6	12.9 ± 1.4	13.3 ± 0.7†	6.3 ± 1.7	[illegible]0 ± 1.6†
arabitol	2.32 ± 0.12	58.0 ± 7.5	15.3 ± 0.6	15.3 ± 1.7	7.9 ± 1.7	3.6 ± 0.5	1.8 ± 0.1
20% (w/w) carbohydrate; 7 days (n=3)							
none	2.03 ± 0.18	52.0 ± 6.5	11.1 ± 0.8	6.4 ± 1.1	2.6 ± 1.1	1.3 ± 0.2	[illegible]7 ± 0.1
glucose	1.77 ± 0.07	52.5 ± 3.5	12.2 ± 2.1	7.2 ± 1.7	2.5 ± 1.0	1.5 ± 0.3	1.1 ± 0.3
fructose	1.80 ± 0.06	54.0 ± 4.5	12.5 ± 2.5	6.2 ± 1.3	3.0 ± 0.4	1.4 ± 0.2	1.1 ± 0.3
sucrose	1.97 ± 0.03	47.5 ± 2.0	13.3 ± 1.7	9.0 ± 0.9	2.5 ± 0.8	1.8 ± 0.4	0.8 ± 0.1
xylose	2.46 ± 0.30	54.0 ± 2.0	16.5 ± 2.2	7.8 ± 1.8	9.5 ± 2.5*	1.2 ± 0.1	0.6 ± 0.1
sorbitol	3.30 ± 0.21†	39.0 ± 5.0	13.3 ± 4.1	10.3 ± 4.2	1.7 ± 0.9	15.1 ± 6.3	6.0 ± 2.8
xylitol	3.47 ± 0.35†	21.0 ± 3.5†	8.4 ± 2.3	4.3 ± 0.8	2.8 ± 0.5	0.8 ± 0.2	0.5 ± 0.2
arabitol	3.40 ± 0.21†	38.5 ± 5.0	12.2 ± 2.1	6.0 ± 1.9	11.6 ± 0.1†	4.9 ± 0.8†	0.8 ± 0.2

Values significantly different from control, $p < 0.05$(*); $p < 0.01$ (†).

of the small intestine to absorb passively the polyols is exceeded and, with the subsequent arrival of the administered polyols at the large intestine, an osmotic load is produced which in turn results in osmotic diarrhoea. The observations of diarrhoea reported in this chapter confirm the findings of others in that the incidence, severity and duration of the xylitol-induced diarrhoea are related to the amount of xylitol in the diet and the manner in which it was introduced into the diet (Hosoya and Iitoyo, 1969; Mäkinen and Scheinin, 1975a; Förster, 1978). Considerable individual variation in the degree of diarrhoea in rats (personal observations) as well as in humans (Mäkinen and Scheinin, 1975a) have been noted. Furthermore, the observation that animals can undergo some form of adaptation to the continued presence of xylitol in their diet resulting in a cessation of the diarrhoea was confirmed (Bässler, 1969; Förster, 1978).

The liver, being the major organ for xylitol metabolism (Lang, 1969), has been suggested to be the site of adaptation (Förster, 1978). There is, however, no evidence for any major form of liver adaptation from the enzyme or metabolic studies reported here (Tables 2.1 and 2.2). In fact none of the dietary carbohydrates had any major effect. Some carbohydrates, however, are known to induce a variety of liver enzymes, but in those studies the carbohydrate comprised more than 50% (w/w) of the diet (Rudack et al, 1971; Romsos and Leveille, 1974) whereas, in the studies reported here, the rats received only 10-20% (w/w) of their diet in the form of a carbohydrate. Nevertheless, Fujisawa et al, (1969) found that rats had significant changes in the activities of some of their liver enzymes within a few days of commencing a diet containing 10% (w/w) xylitol. A number of enzyme activities were increased in the glucuronate-xylulose and pentose phosphate pathways, decreased in gluconeogenesis and unchanged for the aminotransferases. On the other hand, Hosoya and Iitoyo (1969) showed that when the xylitol content of the diet was increased gradually from 5 to 20% over 4 weeks then the activity of the hepatic xylitol dehydrogenase only slowly doubled in the same period. Hepatic xylitol dehydrogenase activity was not altered in the feeding studies reported here (Table 2.1).

Förster (1978) argues that if the adaptation does occur in the liver then one might expect the more rapid conversion of xylitol by the liver to result in increased xylitol absorption from the gut lumen. The gut intraluminal concentration of xylitol exceeded 100 mmol/l (Fig. 2.2) and has

been reported to be as high as 200 mmol/l (Förster, 1978). However, the plasma xylitol concentration did not exceed 0.5 mmol/l even when the animals had full stomachs and post-prandial hyperglycaemia (Table 2.3). In all the liver studies described and quoted above, none of the observed changes were greater than two-fold, and this would not explain the more than 100-fold decrease in caecal intraluminal xylitol concentration that accompanies adaptation (Fig. 2.2).

There is the possibility of adaptation in the gut wall itself. The wide range of gut intraluminal xylitol concentrations and the fairly narrow range of plasma xylitol concentrations (Table 2.3), however, support the concept that there is no inducible transport mechanisms for polyols. The gut wall may contain enzymes capable of metabolising xylitol and these enzymes may be inducible, but this possibility does not appear to have been investigated. Certainly, when plasma values and histology are used as gross indicators of adaptation there is no evidence for cellular change in the liver, kidneys, bone or gut (Table 2.3).

On the other hand, there is some evidence for adaptation within the gut lumen from pH changes, presence of gas, formation of a water-insoluble polysaccharide like material and changes in xylitol concentration (Table 2.4; Fig. 2.2). In addition, other evidence of changes induced in the metabolic activity of caecal flora by dietary xylitol are seen in the thiamin sparing-action of xylitol in the rat. (See Appendix to this Chapter). Nevertheless, it is commonly assumed for at least two reasons that the microbial flora of the gut play no role in the adaptation (Bässler, 1969; Hosoya and Iitoyo, 1969). Bässler (1969) has shown that adaptation can be achieved by parenteral xylitol application. Animals treated in this way get no diarrhoea after changing to oral xylitol. Bässler (1969) suggests that increased liver polyol dehydrogenase activity will increase the elimination of xylitol from the blood and hence allow for increased absorption of xylitol from the gut. The only way that parenterally administered xylitol could be prevented from entering the gut lumen, however, is if some energy-dependent, one-way transport mechanism existed for xylitol, and there is no evidence for such a system for any of the sugar polyols. Secondly, since oral microflora appear unable to metabolise xylitol (Mäkinen and Scheinin, 1975a; Counsell, 1978), then, by inference, gut microflora cannot metabolise xylitol. However, in this chapter the xylitol-metabolising ability of the gut microflora was shown to be increased

by 10-fold or more following the exposure of the flora to only low concentrations of xylitol. That is, the increased tolerance to xylitol, in the short term at least is related to changes within the lumen of the gut.

Besides being able to metabolise xylitol, gut commensals can also undergo adaptation to increase their xylitol-metabolising activity (Fig. 2.5). In the carbohydrate-feeding experiments (Table 2.6), dietary glucose and fructose did not alter the $^{14}CO_2$ producing activities of caecal flora, whereas xylose feeding increased xylose metabolism, sorbitol feeding increased sorbitol and xylitol metabolism and xylitol feeding increased the metabolism of xylitol, sorbitol and xylose. The simplest explanation for these observations would be to propose that each carbohydrate induces the synthesis of an enzyme necessary for the initiation of its own metabolism. Induction of either a specific or non-specific polyol dehydrogenase would explain the increased metabolism of both xylitol and sorbitol following the feeding of either polyol. The induction of such enzyme(s), however, would not account for the increased xylose metabolism following xylitol feeding and the increased fructose metabolism following sorbitol feeding (Table 2.6). Xylitol could, instead, induce a somewhat more specific xylitol (or ribitol) dehydrogenase (Mortlock, 1976), but this mechanism may not necessarily increase sorbitol metabolism and would certainly not increase xylose metabolism. Besides that, xylitol feeding could induce xylulose kinase, an enzyme common to the metabolism of both xylitol and xylose, but this would imply that xylitol dehydrogenase and xylose isomerase were not rate-limiting and that would be inconsistent with the observation that xylose only induces its own metabolism (Table 2.6). From these observations, it seems unlikely that xylitol feeding results in bacterial adaptation by simply inducing a single bacterial enzyme but would involve a group of enzymes related to the metabolism of xylitol. These possibilities, however, are examined in more detail in Chapter Four. The adaptation to xylitol by caecal flora cannot be explained by a mutation affecting the activity of a single enzyme or transport protein. Indeed, such a mutation would be expected to be permanent and, therefore, to be expressed in the presence of xylitol. Furthermore, mutations are most unlikely since xylitol and its metabolites have been shown by both in vitro and in vivo tests to be non-mutagenic (Batzinger, Suh-Yun and Beuding, 1977).

The above arguments, however, show that the adaptation is much more complex than, say, the change in activity of one single enzyme. Several enzymes at least would appear to be involved in the effect of xylitol-feeding on the metabolism of xylitol, sorbitol and xylose (Table 2.6). The impressions of bizarre morphological changes in the gram-positive cocci of the caecal contents of rats fed 10% xylitol in their diets (Fig. 2.3), and the substantial morphological changes observed by Demetrakopoulos and Amos (1978), in their studies of the effect of xylitol in the media on fibroblasts from various animal species, indicate that the increased xylitol-metabolising activity is the result of changes affecting the whole bacterial cell.

In conclusion, the feeding of xylitol to rats increases the ability of rat caecal flora to utilise xylitol. A similar adaptation has been observed in the caecal flora of mice in our laboratory (Potezny et al, unpublished observations). Thus, the ability of man and animals to tolerate significantly greater amounts of dietary xylitol with prolonged intake of xylitol (Förster, 1978) can be accounted for, in part, by the increased xylitol-metabolising activity of the caecal flora. In order to gain an understanding of the mechanism for the increased xylitol-metabolising activity of the caecal flora, it was decided firstly to isolate the bacteria responsible for this phenomenon from the caeca of rats (Chapter Three) and then carry out enzyme induction and metabolic studies to characterise the mechanism involved (Chapter Four).

CHAPTER TWO: APPENDIX

A MECHANISM FOR THE THIAMIN-SPARING ACTION OF DIETARY XYLITOL IN THE RAT

In Chapter Two it was demonstrated that the inclusion of sorbitol or xylitol in the diet leads to a dramatic increase in the ability of the caecal microflora to metabolise these sugar polyols. Marked changes were also observed in the number, composition and morphology of the caecal microflora. Since xylitol-feeding has also been associated with the sparing of B group vitamins (Brin et al, 1978) it seemed probable that this effect was related to the changes observed in the caecal microflora.

When certain carbohydrates, and most notably the sugar polyols, are included in the diet of rats, the requirements for several, if not all, of the B group vitamins decrease (Morgan and Yudkin, 1962; Hotzel and Barnes, 1966). It has been suggested that this effect is due to the stimulation of enteral vitamin synthesis since, for example, in rats fed thiamin-deficient diets, the inclusion of sorbitol (10% w/w) restored growth rates to near those of thiamin-replete rats (Morgan and Yudkin, 1957). Other observations included enlarged caeca, indications of increased bacterial activity, increased thiamin content of the caeca and faeces, a requirement for coprophagy, and the inhibition of the thiamin-sparing effect by sulphaguanidine (review by Hotzel and Barnes, 1966). However, further attempts to define the involvement of bacteria in the vitamin-sparing effect were unsuccessful (Morgan and Yudkin, 1962; Hotzel and Barnes, 1966).

In this Appendix investigations of the contribution of the caecal microflora to the vitamin-sparing effect of xylitol are reported. These studies were carried out in conjunction with Dr. Allan Rofe (Division of Clinical Chemistry, Institute of Medical and Veterinary Science).

MATERIALS AND METHODS

The sources of reagents are described in Chapter Two.

Animals

Twenty male Porton rats weighing approximately 200 g were housed in groups of 3 or less in perspex cages fitted with wire mesh floors. Coprophagy was not prevented by this procedure. Rats had free access to food and water and were maintained on a 14 h light/10 h dark cycle at 22°C. Animal weights were recorded every 2 days and the amount of water and food consumed also noted.

Diet

The synthetic diet, from which thiamin, riboflavin and pyridoxine were excluded, was prepared by the method of Paquet et al, (1970). Sucrose is the major component in this diet. When included, xylitol (10% w/w) replaced an equal weight of sucrose in the diet.

In vivo experiments

All rats were fed the vitamin-deficient diet until they began to lose weight. Xylitol was then included in the diet of half the rats while the remainder were maintained on the vitamin-deficient diet alone. At various time intervals thereafter, pairs of animals from each group were killed and their livers and caeca removed. Caecal contents were suspended (20% w/v) in phosphate-buffered saline, pH 7.0, as described in Chapter Two. The livers were homogenised (20% w/v) in 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.2 mol/l KCl and 1.0 mmol/l $MgSO_4$, using a Sorvall Omnimix. The caecal suspensions and liver homogenates were then used in the analytical procedures described below. The caeca were rinsed in saline, blotted free of excess liquid and weighed. The caecal dry weights were recorded after drying for 48 h at 100°C.

In vitro experiments

Crude caecal contents from rats were suspended (20% w/v) in minimal media, used for growing Escherichia coli (Davis et al, 1973), and centrifuged at 500 x g for 2 min to remove debris. Conical flasks containing 50 mmol/l xylitol in minimal media (50 ml) were inoculated with

sufficient caecal suspension to give an absorbance at 600 nm (A_{600}) of 1.0-1.5. Appropriate controls without xylitol or inoculum were included. The flasks were loosely capped with aluminium foil and shaken at 37°C on a rotary shaker. At 24 h intervals, the A_{600}, pH and xylitol- and glucose-metabolising activities were measured. In addition, an 8.0 ml aliquot of the bacterial suspension was centrifuged at 3,000 x g for 5 min and the bacterial pellet resuspended to a volume of 0.5 ml in water. This was then used for thiamin determinations.

Measurement of xylitol and glucose metabolism by caecal microflora

Suspensions of caecal contents or cultured bacteria were assayed for xylitol- and glucose-metabolising activities as described previously in Chapter Two.

Measurement of thiamin and thiamin pyrophosphate

Aliquots of the suspensions of the caecal contents, liver homogenates or bacteria were mixed with 2 volumes of 5% trichloroacetic (TCA), frozen and stored. On thawing, 0.2 ml of the mixture was retained for protein determination. The remaining extract was then centrifuged at 3,000 x g for 5 min and 0.5 ml aliquots of the supernatant used for the assay of thiamin by the thiochrome method of Leveille (1972). An additional aliquot of the supernatant was incubated with 200 units of human, prostatic acid phosphatase for 5 h to hydrolyse thiamin pyrophosphate to thiamin (Burch, 1957). Thiamin pyrophosphate levels were then calculated by the difference between the phosphatase-treated and untreated samples. In each measurement, internal standards of thiamin (10 pmol) were included as well as an aliquot of each test which had been treated with benzene sulphonyl chloride to destroy thiamin. This latter step allows for a correction to be made for fluorescence produced by compounds other than the thiochrome.

Protein determination

Protein was determined by the standard method of Lowry et al, (1951) after first mixing the suspension to be measured in an equal volume of 1.0 M NaOH.

Statistics

Statistical significance has been calculated using the t-test for unpaired means.

RESULTS

Reversal of weight loss in vitamin-deficient rats by xylitol

After consuming a diet deficient in thiamin, riboflavin and pyridoxine for 18 days, the rats ceased growing and began losing weight (Fig. A.1). At this time (day 0 in Fig. A.1), 10% xylitol (w/w) was included in the diet of 10 of these rats. Within 10 days the xylitol-fed rats stopped losing weight and after 14 days they were gaining weight at a rate similar to that of the vitamin-replete animals. In contrast, those rats which did not receive xylitol continued to lose weight. The short time (16 days) required to cause cessation of growth in the rats on the vitamin-deficient diet is typical of thiamin deficiency (Bai, Bennion and Gubler, 1971). Therefore, whilst it was originally planned to study the combination of thiamin, pyridoxine and riboflavin deficiencies, the duration of the experiment shown in Fig. A.1 was such that marked deficiencies of pyridoxine and riboflavin would not have been manifested. It is assumed, therefore, that the effects reported here are the result of thiamin deficiency, though it is recognised that partial riboflavin and pyridoxine deficiencies may also have been corrected by xylitol-feeding. The weight losses in animals not receiving xylitol were accompanied by anorexia, whereas the group receiving xylitol continued to consume food at a rate equivalent to that observed during the first week of the experiment.

Effect of xylitol on the caecum of vitamin-deficient rats

Most of the caecal changes elicited by the inclusion of xylitol in the diet were near maximal within 3 days of commencing the diet, a finding which is in accord with previous data (Chapter Two). Therefore, the results in Tables A.1, A.2 and A.3 represent pooled data taken from the time xylitol was included in the diet until day 24. Table A.1 shows

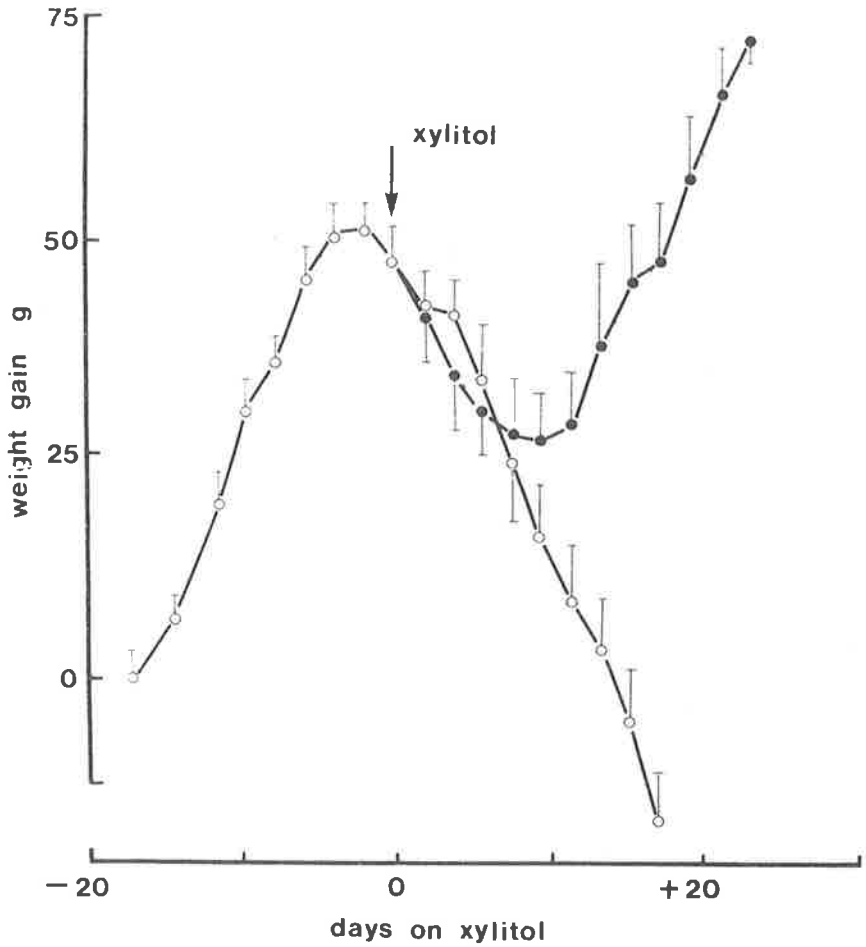


Fig. A.1 The effect of feeding xylitol on the growth of rats maintained on a diet deficient in thiamin, riboflavin and pyridoxine. Twenty rats were divided into two groups of 10 on day 0, one of which received xylitol (10% w/w) in the vitamin-deficient diet (closed circles). The control group (open circles) received the vitamin-deficient diet alone. The results are expressed as the mean ± S.E.M. for n = 10, 8, 6 and 4 for days 3, 6, 12 and 18, respectively. The results from day 18 are the average of 2 rat weights.

the marked increases which dietary xylitol caused in the wet and dry weights of the caecum and in the wet weight and protein concentration of the caecal contents. The change in these parameters after xylitol feeding indicates both changes in the number of microorganisms in the caeca as well as hypertrophy of the caecal wall.

Effect of xylitol on glucose and xylitol metabolism

The inclusion of xylitol in the diet of rats resulted in a dramatic increase in the ability of the caecal microflora to metabolise this sugar polyol (Table A.2), an effect reported in Chapter Two. This change occurs within 24 h of xylitol consumption and in this study was near maximal at 3 days. If calculated per ml of caecal contents, the metabolic activity with xylitol as substrate increased 17-fold, whereas that of glucose was not affected. If this activity is expressed on a total caecum basis, glucose metabolism was increased 2-fold by xylitol feeding. This coincides with the increase in the total caecal protein and the weight of the caecal contents (Table A.1) which indicate increased bacterial numbers in the caeca of these rats.

Thiamin pyrophosphate content of caecum and liver

Thiamin pyrophosphate was the predominant form of the vitamin in both the liver and caecal contents (Table A.3). Xylitol caused a marked increase in the thiamin and thiamin pyrophosphate concentration of the caecal contents and the thiamin pyrophosphate concentration in the liver. The increase in the thiamin pyrophosphate level of the caecal contents (Fig. A.2), like that of xylitol metabolism, occurred in the first 3 days following the inclusion of xylitol in the diet. A similar rate of increase, however, was not observed in the liver. As shown in Fig. A.2, the thiamin pyrophosphate content of the liver did not increase until 6-12 days after commencing xylitol feeding. This rise in the hepatic thiamin pyrophosphate level parellels the weight gain seen in these rats (Fig. A.1). Thus, the rapid increase in caecal thiamin pyrophosphate, seen at day 3, is not of obvious immediate benefit to the host.

TABLE A.1 The effect of dietary xylitol on the caecum of vitamin-deficient rats.

	Diet	
	Vitamin-deficient	Vitamin-deficient + 10% xylitol
Wet weight of caecum (g)	0.61 ± 0.06	0.97 ± 0.07*
Dry weight of caecum (g)	0.092 ± 0.012	0.207 ± 0.014**
Weight of caecal contents (g)	2.08 ± 0.26	4.57 ± 0.46**
Total protein in caecal contents (mg)	62 ± 7	185 ± 30*
Protein concentration of caecal contents (mg/ml)	30 ± 4	40 ± 7 NS
	n = 8	n = 10

The results are shown as mean ± S.E.M.

The significance of the xylitol effect is shown by * $p < 0.01$, ** $p < 0.001$, NS not significant.

TABLE A.2 Effect of dietary xylitol on xylitol and glucose metabolism by caecal contents of vitamin deficient rats

Substrate metabolised to CO_2 by the caecal contents	Diet	
	Vitamin-deficient	Vitamin-deficient + 10% xylitol
Xylitol (μmol/h/ml)	0.21 ± 0.04	3.54 ± 0.49*
Glucose (μmol/h/ml)	3.41 ± 0.30	3.23 ± 0.35 NS
Xylitol (μmol/h/total)	0.44 ± 0.08	16.2 ± 2.2*
Glucose (μmol/h/total)	7.09 ± 0.61	14.6 ± 1.6*
	n = 8	n = 10

The amount of xylitol or glucose metabolised to CO_2 was calculated from the percentage conversion of substrate to CO_2.
The initial substrate concentration was 10 mmol/l. The results are shown as the mean ± S.E.M., * $p < 0.001$, NS not significant with respect to the effect of xylitol feeding.

TABLE A.3 Effect of dietary xylitol on the thiamin and thiamin pyrophosphate content of the liver and caecal contents of vitamin deficient rats.

	Diet	
	Vitamin-deficient	Vitamin-deficient + 10% xylitol
Liver (pmol/g wet wt)		
Thiamin	32 ± 7	40 ± 9 NS
Thiamin pyrophosphate	112 ± 25	285 ± 38*
Caecal contents (pmol/ml)		
Thiamin	13 ± 7	82 ± 21*
Thiamin pyrophosphate	140 ± 39	443 ± 86*
	n = 8	n = 10

*$p < 0.01$, NS not significant.

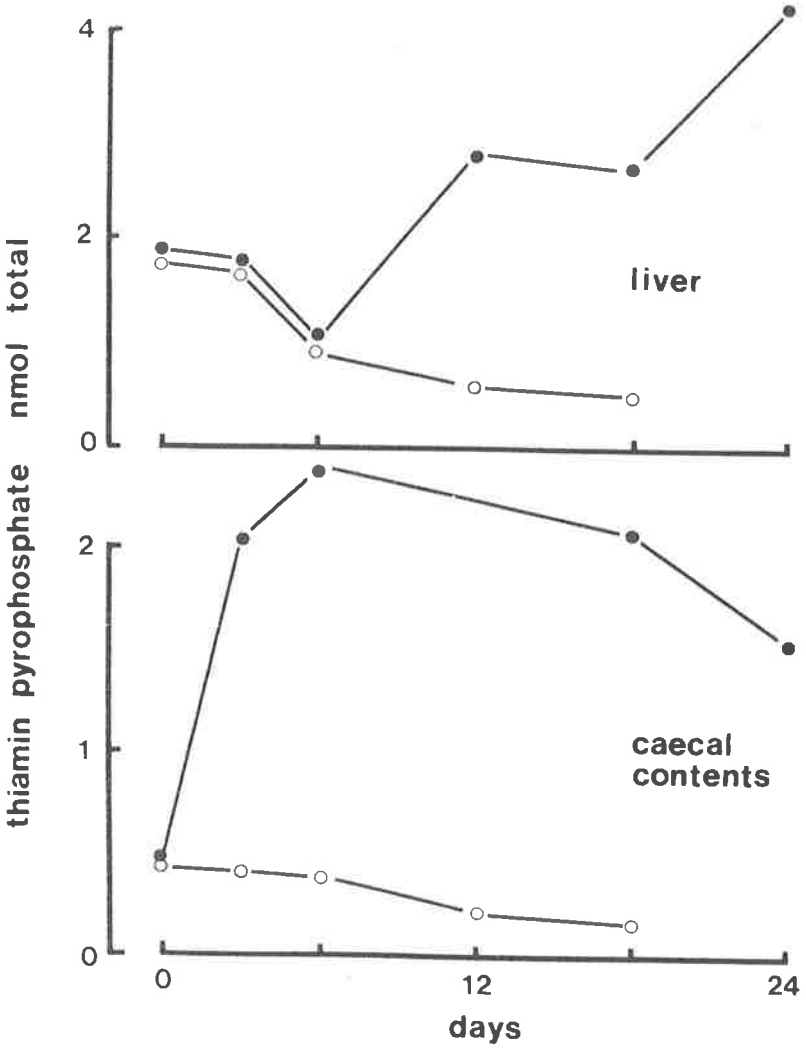


Fig. A.2 The thiamin pyrophosphate content of the caecal contents and livers from rats maintained on the vitamin-deficient diet plus xylitol (closed circles) and minus xylitol (open circles). Each point represents the average of duplicate determinations from two rats.

In vitro growth of caecal microflora on xylitol

Very few micro-organisms have the ability to utilise and grow on xylitol (Mortlock, 1976). In the experiments reported here, however, it was found that when caecal inocula were incubated in a minimal media containing citrate, ammonium sulphate, phosphate and magnesium sulphate, growth on xylitol occurred. This growth was accompanied by a marked increase in xylitol and glucose metabolism (Table A.4). The growth of these xylitol-metabolising organisms was associated with an increase in thiamin pyrophosphate levels, and this increase was essentially proportional to cell numbers (A_{600}) and the protein concentration.

DISCUSSION

The vitamin-sparing action of the sugar polyols is well documented and, with regard to xylitol, this study supports the findings of Brin et al, (1978). By including xylitol in the diet after the onset of signs of thiamin deficiency, it was clearly shown that xylitol stimulates thiamin synthesis rather than spares its catabolism. However, vitamin-sparing has become the accepted term to describe the phenomenon under discussion. The demonstration of thiamin synthesis within the caecum is not a new finding. Wostmann and Knight (1961) found that the caecum was the major, if not the only, site of thiamin synthesis in the gastro-intestinal tract of the rat. The experiments carried out in this study provide evidence which suggests that the bacterial thiamin synthesis is related to the increased growth and metabolism of caecal bacteria on xylitol (Table A.4).

There are a number of indications that the ingestion of xylitol and other sugar polyols causes alterations in the caecal microflora (Morgan and Yudkin, 1962; Chapter Two). These include caecal distension, gas production and changes in bacterial numbers. Despite these signs, and after considerable investigation, Morgan and Yudkin (1962) could not detect significant changes in the composition of the caecal microflora. They were therefore forced to conclude that while some patterns of caecal microflora were more conducive to vitamin synthesis than others, these changes in pattern could be subtle and therefore difficult to elucidate.

TABLE A.4 The *in vitro* growth of caecal microflora in minimal media containing xylitol.

	Time (h)		
	0	24	48
Xylitol metabolism (μmol/h/ml)	0.016 ± 0.001	0.370 ± 0.025	5.77 ± 0.47
Glucose metabolism (μmol/ml/h)	0.044 ± 0.009	3.05 ± 0.51	3.16 ± 0.26
pH	6.85 ± 0.01	7.05 ± 0.02	6.57 ± 0.05
A_{600}	1.30 ± 0.11	1.43 ± 0.15	2.72 ± 0.06
Protein (mg/ml)	0.55 ± 0.08	1.06 ± 0.02	3.01 ± 0.16
Thiamin pyrophosphate (pmol/ml)	17 ± 1	17 ± 1	54 ± 3

Xylitol and glucose metabolism were measured as the percentage conversion of substrate to CO_2 per ml of culture. The results are shown as the mean ± S.E.M. of independent caecal samples taken from 4 rats maintained on laboratory food.

Control incubations, without xylitol but containing all other components, were included. These showed little change in all parameters during the 48 h incubation.

In Chapter Two, however, xylitol feeding in rats has shown that changes do occur in the number, composition and morphology of the caecal microflora and this is a dramatic, reproducible and well defined effect. It is these changes which indicate the mechanism of the vitamin-sparing effect associated with xylitol-feeding.

From the evidence presented here and in Chapter Two, the series of events which lead to the phenomenon termed vitamin-sparing can now be postulated. When xylitol is consumed by rats it, like other sugar polyols, is poorly absorbed and consequently reaches the caecum where its concentration increases in proportion to the amount consumed (Bässler, 1969, Chapter Two). The consequence of the increased xylitol metabolising activity of the caecal is a fall in caecal pH (Chapter Two) which in turn will lead to further modifications in the bacterial population (Eyssen, DePauw and Paramentien, 1974). As shown in the in vitro studies presented here (Table A.4) bacteria in the caecum have the ability to synthesise thiamin and other essential vitamins when grown on xylitol as the sole carbon source. Therefore it can be suggested that the increased thiamin concentration of the caecal contents will extend to the faeces and, coupled with coprophagy, lead to an increased supply of the vitamin to the host. The manner in which the thiamin becomes available to the host animal has been covered in detail elsewhere (Hotzel and Barnes, 1966). As there is a lag period of at least 3 days between the time when the caecal thiamin concentration increases and the liver levels rise (Fig. A.2), then coprophagy is the most likely means by which this thiamin becomes available to the host. Prevention of coprophagy in rats has been shown to abolish the vitamin-sparing effect of sorbitol (Morgan and Yudkin, 1959; Hotzel and Barnes, 1966), but a single study in one man has shown the increased urinary excretion of B group vitamins following sorbitol ingestion (Watson and Yudkin, 1959). While the major site of thiamin absorption in the rat is the proximal small intestine (Sklan and Trostler, 1977), slow absorption from the caecum and large bowel cannot be excluded.

The ability of micro-organisms to synthesise certain vitamins is important in the nutrition of the host (Baker et al, 1950; Hotzel and Barnes, 1966) with the most often quoted example being that of enteral vitamin B_{12} synthesis. Examples of enteral thiamin synthesis are less well documented, though it is assumed that a number of bacteria must have the ability to manufacture all cellular requirements from elementary

nutrient sources. Thiamin synthesis has been discussed in terms of those organisms which can synthesise the cystine part of the thiamin molecule and those which can only manufacture the methionine moiety (Gibson and Pittard, 1968; Shimomura et al, 1957; Yamada, Sawaki and Kayami, 1957). Presumably, those organisms capable of metabolising and growing on xylitol as the major carbon source can do both. The *in vitro* studies indicate that thiamin synthesis does not proceed at a rate greater than that of protein synthesis (i.e. growth), thus suggesting that the thiamin produced is that required for metabolic integrity and is not the result of excessive secondary metabolism.

It could be suggested that the bacteria which metabolise pentoses and pentitols may have an additional need for thiamin in that the transketolase reaction in the pentose phosphate pathway requires thiamin pyrophosphate as a cofactor. The activity of the tricarboxylic acid cycle is also likely to be significant during xylitol metabolism, thus involving the thiamine pyrophosphate-dependent pyruvate and α-ketoglutarate dehydrogenase reactions. Therefore, these organisms must have salvage pathways or pathways for the *de novo* synthesis of thiamin if they are to remain viable and sustain their metabolic rates. As sugars other than xylitol show the phenomenon of vitamin-sparing (Hotzel and Barnes, 1966), the increased thiamin levels may be the result of a general increase in carbohydrate metabolism rather than the demands of specific pathways.

Therefore, in summary, it can be predicted that the set of events which will be common to all sugars and sugar polyols that show vitamin-sparing effects will include poor absorption (i.e. the polyol must reach the caecum) followed by the selection of caecal bacteria capable of xylitol metabolism and *de novo* vitamin synthesis.

CHAPTER THREE

THE ISOLATION AND CHARACTERISATION OF XYLITOL-UTILISING BACTERIA FROM THE RAT CAECUM

INTRODUCTION

Mortlock (1976), in his review of the catabolism of sugar polyols by microorganisms, described a number of yeasts, fungi and bacteria that have the ability to metabolise xylitol. Some of these studies, however, were performed with cell-free systems and thus the ability of xylitol to support microbial growth was not investigated. While several strains of yeasts have been reported to utilise xylitol as a sole source of carbon for growth (Barnett, 1968), reports of bacterial growth on xylitol are rare. Indeed, xylitol has only been described as a major carbon and energy source for the growth of Azotobacter agilis (Marcuss and Marr, 1961), mutant strains of Aerobacter aerogenes (Mortlock and Wood, 1964), Lactobacillus casei (London and Chace, 1979), Streptococcus avium, Lactobacillus salivarius subp. salivarius (Rogosa, 1974), Staphylococcus saprophyticus (Schleifer and Kloos, 1975) and propionibacteria similar to P.avidium, P.jensenii and P.thoenii (Gallagher and Fussell, 1979). Bacillus subtilis can also grow on xylitol but only when trace amounts of sorbitol are included in the growth medium (Horowitz and Kaplan, 1964).

Despite the small number of reports of xylitol-utilising bacteria in the scientific literature, evidence presented in Chapter Two indicates that bacteria in the caeca of rats can metabolise xylitol, albeit at low rates. In the continued presence of xylitol, these caecal bacteria are able to increase their ability to metabolise xylitol several-fold. This increased ability can be attributed to either the selection of bacteria which can preferentially grow on xylitol or to the induction of xylitol-metabolising enzymes.

In order to study the xylitol-metabolising activity of the rat caecal microflora in further detail, it was necessary to first isolate and identify the bacteria involved. The bacterial studies reported in this chapter were, however, restricted to the isolation of aerobic or facultative organisms. The radioisotopic assay, used to demonstrate the xylitol-metabolising activity of the rat caecal microflora (Chapter Two), and the batch cultures (see below) were performed only under aerobic conditions. In addition, to ensure that the bacterial inocula from the caeca of rats were exposed to xylitol as the major growth substrate, the addition of other nutrients to the media was kept to a minimum. This procedure, therefore, enables xylitol to select for, and also facilitate the growth of, xylitol-metabolising bacteria from the rat caecum. Once again radioactive substrates were employed to confirm that the isolated bacteria were able to metabolise xylitol. The subsequent identification of the bacteria which were capable of metabolising xylitol was carried out by the use of standard microbiological techniques.

MATERIALS AND METHODS

Male rats weighing approximately 250 to 300 g received either a control (mouse M and V cubes) or a 10% (w/w) xylitol diet for 7 days. Details of the diet and the sources of materials used in this experiment have already been described in Chapter Two.

Media and Culture Techniques

Two types of minimal media, namely ammonium sulphate and yeast extract, were used for the aerobic culture of rat caecal microflora. The media were prepared by modifying the minimal media described by Davies et al, (1973).

The ammonium sulphate medium consisted of 5.4 mmol/1 KH_2PO_4, 1.7 mmol/1 K_2HPO_4, 0.4 mmol/1 $MgSO_4$ and 7 mmol/1 $(NH_4)_2SO_4$. The yeast extract medium was prepared by the addition of 5 g/1 of yeast extract (GIBCO, USA) to the ammonium sulphate medium. The pH of both media was 7.2. The control-ammonium sulphate and the control-yeast extract media did not contain any added carbohydrates whereas the xylitol-ammonium sulphate and the xylitol-yeast extract media contained xylitol at a final concentration of 50 mmol/1. Control and xylitol agar plates were prepared by the addition of 15 g/1 agar (DIFCO, USA) and 0.002 g/1 bromthymol blue (pH indicator) to the respective ammonium sulphate and yeast extract media. All the inoculated agar plates and liquid growth media were incubated at 37°C.

Determination of Viable Numbers of Bacteria

The caecal contents obtained from control- and xylitol-fed rats were suspended in sterile phosphate-buffered saline (PBS; Chapter Two) to a concentration of 1 g/10 ml. Particulate matter in the caecal microfloral suspensions was thoroughly dispersed by vortex mixing for 5 min. The suspensions of caecal microflora were then serially diluted to 10^4-, 10^7- and 10^{10}- fold in PBS. Duplicate aliquots (0.1 ml) of the dilutions were spread onto control- and xylitol-ammonium sulphate and control- and xylitol-yeast extract agar plates. These plates were incubated for a period of 48 to 72 h at 37°C to allow for the optimal development of colony morphology and to facilitate the counting of colonies. Individual colonies were categorised with respect to both colony morphology and Gram stain pattern prior to the biochemical identification.

Identification of Bacteria

Colonies containing Gram-negative bacteria belonging to the Enterobacteriaceae family were identified by the Microbact-24E (MB-24E) miniaturised identification system (Mugg and Hill, 1981). The MB-24E consists of a test panel of 24 dehydrates substrates in a microtitre tray. The 24 biochemical tests that were examined in this bacterial identification system included the lysine decarboxylase, ornithine decarboxylase, H_2S, glucose, mannitol, xylose, O-nitro phenol glucose (ONPG), indole, urease, Voges Proskaven (VP), citrate, tryptophan deaminase (TDA), gelatin,

malonate, inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol(ribitol), raffinose, salicin and arginine dihydrolase. These tests are commonly used in microbiological laboratories and are described in detail by Mac Faddin,(1980). Saline suspensions of organisms to be identified were dispensed into the wells of the microtitre tray. The inoculated trays were then incubated at 35°C for 24 h before the test reactions were assessed qualitatively by the colour reactions produced. Computer based profile registers are available for the MB-24E system in the Institute of Medical and Veterinary Science and were used in the identification of the isolated Enterobacteria to the species level based on the test pattern obtained.

All Gram-positive bacteria were identified by standard biochemical reactions as outlined in Bergey's Manual of Determinative Bacteriology (1974). These tests included the catalase, glucose, Voges Proskaven (VP), nitrate reduction, arginine hydrolysis, sensitivity or resistance to Novobiocin, growth and degree of pigmentation in CLED (cysteine, lactose, electrolyte-deficient) and blood agar media tests.

Radioisotopic Assay of Bacterial Xylitol-metabolising Activity

Pure cultures of bacterial isolates were inoculated into 50 ml ammonium sulphate or yeast extract media containing either 50 mmol/l of glucose or xylitol. The inoculated media were shaken (150 oscillations/min) in 250 ml conical flasks for 36 h at 37°C. Each culture was then centrifuged at 10,000 x g for 20 min at 4°C, the supernatant discarded and the bacterial pellet resuspended in an equal volume of PBS and centrifuged again. The resultant pellet was finally resuspended in PBS and adjusted to an absorbancy (A_{600}nm) of 1.0. Protein determinations were performed on these samples by the method of Lowry et al, (1951). The metabolic activities of the bacterial samples were determined by incubating them with xylitol for 90 min at 37°C and measuring the production of $^{14}CO_2$ from |U-^{14}C| glucose or |U-^{14}C| xylitol as described in Chapter Two. The metabolic activities are expressed as cpm/mg of bacterial protein.

RESULTS

Aerobic Isolation of Bacteria from the Rat Caecum

The caecal contents obtained from rats fed either a control (n=3) or a 10% (w/w) xylitol (n=3) diet were serially diluted and inoculated onto control- and xylitol-ammonium sulphate and control- and xylitol-yeast extract agar plates. These agar plates were incubated aerobically and all organisms that were observed are shown in Table 3.1. This procedure would only detect those species of bacteria that were present at $\geq 10^4$ organisms/g of caecal contents (that is, the minimum dilution was 10^{-4}; see above).

Ammonium Sulphate Agar Plates

A comparison of the results obtained from the control- and xylitol-ammonium sulphate agar plates shows that Klebsiella pneumoniae, Escherichia coli, Corynebacterium sp. or Staphylococcus epidermidis were not preferentially selected for in the presence of xylitol; that is, these colonies appeared on both the control- and xylitol-ammonium sulphate agar plates (Table 3.1). On the other hand, the selective effect of xylitol was clearly shown for Serratia liquefaciens since distinct colonies grew only on xylitol-ammonium sulphate agar plates. This is the first time that S.liquefaciens has been reported to grow on xylitol as a major carbon source. When xylitol-ammonium sulphate agar plates were examined after longer periods (48 to 72 h) of incubation, it was found that the colonies of K.pneumoniae became larger in size. This observation implies that K. pneumoniae is capable of utilising xylitol as a growth substrate. Moreover, when all the isolated colonies were subcultured onto xylitol-ammonium sulphate agar plates, only S.liquefaciens and K.pneumoniae were able to grow. In the case of E.coli, Corynebacterium sp. and S.epidermidis the carry-over of substrates from the diluted caecal contents was the most likely explanation for their growth on ammonium sulphate agar plates, since subsequent subcultures of these bacteria failed to grow under the same conditions in these plates.

TABLE 3.1 Bacteria isolated under aerobic conditions on control- or xylitol-ammonium sulphate agar plates and control- or xylitol-yeast extract agar plates. The bacteria were initially obtained from the caecal contents of rats fed control diet or xylitol diet (see text).

Isolated bacteria	Ammonium sulphate agar plates		Yeast extract agar plates	
	Control n = 3	Xylitol n = 3	Control n = 3	Xylitol n = 3
Klebsiella pneumoniae	+	+	+	+
Escherichia coli	+	+	+	+
Proteus mirabilis	-	-	+(a)	+(a)
Serratia liquefaciens	-	+(a)	-	-
Corynebacterium sp.	+	+	-	-
Micrococcus sp.	-	-	+	+
Staphylococcus epidermidis	+	+	-	-
Staphylococcus saprophyticus	-	-	+(a)	+(a)
Streptococcus faecalis	-	-	+(b)	+(b)

(a) colonies detected only in caecal contents of rats fed 10% (w/w) xylitol in diet.

(b) colonies detected only in caecal contents of rats fed a control diet.

\+ indicates presence of bacteria at a detection level of 10^4 organisms/g of caecal contents.

\- indicates absence of bacteria at a detection level of 10^4 organisms/g of caecal contents.

Yeast Extract Agar Plates

The yeast extract medium has a greater nutrient content than the ammonium sulphate medium and so it was not unexpected to find bacteria with more specific nutrient requirements being detected on yeast extract agar plates. Indeed, bacteria which were not detected on the ammonium sulphate agar plates were found to grow on yeast extract agar plates. These included Staphylococcus saprophyticus, Micrococcus sp., Proteus mirabilis and Streptoccus faecalis (Table 3.1). These bacteria are known to have organic nutrient requirements for growth which can be provided by yeast extract. The inclusion of xylitol in the yeast extract agar plates, however, did not show any selective advantage for the growth of these bacteria. That is, all the colonies grew on both control- and xylitol-yeast extract agar plates (Table 3.1) and, furthermore, the subcultured isolates grew on both types of plates.

Glucose- and Xylitol-metabolising Activities of Bacterial Isolates from the Rat Caecum

The organisms, S.liquefaciens and K.pneumoniae, remained viable when subcultured on xylitol-ammonium sulphate agar plates and, hence, their glucose- and xylitol-metabolising activities were assessed after the growth of these bacteria in glucose- and xylitol-ammonium sulphate and glucose- and xylitol-yeast extract media. The other species of bacteria that were initially present on the xylitol-ammonium sulphate agar plates failed to grow on subsequent subcultures and were therefore not further investigated. Colonies of E. Coli, K.pneumoniae, P.mirabilis, Micrococcus sp., S.saprophyticus and S.faecalis which were isolated on xylitol-yeast extract agar plates were inoculated in glucose- and xylitol-yeast extract media and then, their glucose- and xylitol-metabolising activities were determined (Table 3.3).

In general, with the exception of E.coli and S.saprophyticus, the glucose-metabolising activity of the bacterial isolates was no different or lower with xylitol than with glucose as a growth substrate (Tables 3.2 and 3.3). This is probably a reflection of there being fewer bacteria in the xylitol-containing medium compared with those in the glucose-containing medium. If yeast extract is utilised in preference to xylitol, the bacterial growth yields in xylitol-yeast extract medium would be lower than

TABLE 3.2 The glucose and xylitol-metabolising activities of rat caecal bacteria previously isolated on xylitol-ammonium sulphate agar plates. The bacterial isolates which appeared on ammonium sulphate agar plates were subcultured and grown in glucose- and xylitol-ammonium sulphate and glucose- and xylitol-yeast extract liquid media and then assayed for their metabolic activities as described in Materials and Methods section. Results are shown as a mean of 3 independent observations.

Isolated Bacteria	Carbon Source in medium (50 mmol/l)	Carbohydrate-metabolising activity (cpm/mg of bacterial protein)		X/G Ratio
	ammonium sulphate	$\lvert U\text{-}^{14}C \rvert$ glucose	$\lvert U\text{-}^{14}C \rvert$ xylitol	
Serratia liquefaciens	glucose	24,500	1,900	0.08
	xylitol	20,700	12,900	0.63
Klebsiella pneumoniae	glucose	19,500	2,200	0.11
	xylitol	16,600	9,100	0.55
	yeast extract			
Serratia liquefaciens	glucose	78,500	7,100	0.09
	xylitol	61,000	32,200	0.52
Klebsiella pneumoniae	glucose	29,500	900	0.05
	xylitol	27,800	900	0.03

TABLE 3.3 The glucose- and xylitol-metabolising activities of rat caecal bacteria previously isolated on xylitol-yeast extract agar plates. The bacterial isolates which appeared on yeast extract agar plates were subcultured and grown in glucose- and xylitol-yeast extract liquid medium and then assayed for their metabolic activities as described in Materials and Methods section. Results are shown as a mean of 3 independent observations.

Isolated bacteria	Carbon source in medium (50 mmol/l)	Carbohydrate-metabolising activity (cpm/mg of bacterial protein)		X/G Ratio
	yeast extract	$\lvert U\text{-}^{14}C \rvert$ glucose	$\lvert U\text{-}^{14}C \rvert$ xylitol	
Escherichia coli	glucose	44,800	2,000	0.045
	xylitol	85,300	4,500	0.053
Proteus mirabilis	glucose	3,200	380	0.11
	xylitol	3,500	390	0.11
Micrococcus sp.	glucose	27,000	1,000	0.04
	xylitol	18,500	21,200	1.14
Staphylococcus saprophyticus	glucose	6,300	350	0.05
	xylitol	13,400	500	0.03
Streptococcus faecalis	glucose	4,000	150	0.04
	xylitol	750	40	0.05

that in glucose-yeast extract medium. That is, the bacterial growth in xylitol-yeast extract medium is probably diauxic (the term diauxic growth refers to the sequential and preferential use by bacteria for growth on individual substrates when a mixture of substrate is available). On the other hand, E.coli and S.saprophyticus grown in xylitol-yeast extract medium showed higher glucose-metabolising activities compared to the same activities for these bacteria grown in glucose yeast extract medium (Table 3.3). This may be due to the fact that glucose is utilised at a faster rate than xylitol and, hence, bacteria grown in glucose will loose their viability sooner than bacteria grown in xylitol. This loss of viability in bacteria grown in glucose was reflected by the lower glucose-metabolising activity.

The metabolic (or X/G) ratio is used to indicate the change in the xylitol-metabolising activity relative to the glucose-metabolising activity of the bacterial isolates and to compensate for any variation in growth that occurs between glucose- and xylitol-grown bacteria (Tables 3.2 and 3.3). This ratio is defined as:

$$\frac{^{14}CO_2 \text{ produced from } |U\text{-}^{14}C| \text{ xylitol (cpm/mg of bacterial protein)}}{^{14}CO_2 \text{ produced from } |U\text{-}^{14}C| \text{ glucose (cpm/mg of bacterial protein)}}$$

Hayes and Roberts (1978) have also defined a similar X/G ratio to express the degree of induction of xylitol metabolism in oral plaque bacteria. When S.liquefaciens and K.pneumoniae were grown on glucose-ammonium sulphate medium, the X/G ratios were 0.08 and 0.11, respectively, but, when xylitol was included in the growth medium, the X/G ratios increased to 0.63 and 0.55, respectively (Table 3.2). While S.liquefaciens maintained its enhanced xylitol-metabolising activity in xylitol-yeast extract medium, K.pneumoniae did not demonstrate induced xylitol-metabolising activity in yeast extract medium (Table 3.2). Amongst the bacteria isolated on xylitol-yeast extract agar plates, Micrococcus sp. showed enhanced xylitol-metabolising activity since it had an X/G ratio of 1.14 when grown on xylitol (Table 3.3). The other bacteria isolated on xylitol-yeast extract agar plates (Table 3.3), while not exhibiting enhanced xylitol metabolism did show low xylitol-metabolising activities. Although it has been previously

reported that S.saprophyticus is capable of metabolising xylitol (Schleifer and Kloos, 1975), the isolate obtained here (Table 3.3) did not exhibit any enhancement of xylitol-metabolising activity. Despite the constant X/G ratios for S.faecalis grown on either glucose or xylitol, the inhibitory effect of xylitol on the growth of S.faecalis is clearly shown by the markedly lowered, glucose-metabolising activity of xylitol-grown bacteria (Table 3.3). This inhibition of bacterial growth by xylitol has also been observed in Streptococcus mutans OMZ 176 (Assev, Vegarud and Rölla, 1980).

It is of interest to note here that K.pneumoniae grown in xylitol-ammonium sulphate medium produced an insoluble material which was observed in the supernatant of the centrifuged culture. On the basis of centrifugation properties, this material appears to be similar to the insoluble polysaccharide-like material observed in the caecal contents of rats fed xylitol (Chapter Two). Since the insoluble material produced by K. pneumoniae did not appear in glucose-ammonium sulphate medium or in glucose- and xylitol-yeast extract media, its synthesis may be related to the induction of xylitol-metabolising enzymes of K.pneumoniae. This aspect of the xylitol-metabolising activity of the caecal microflora of rats is examined in further detail in Chapter Five.

Effect of Dietary Xylitol on the Populations of Caecal Bacteria

Although the minimal media used in these experiments were suitable mainly for the isolation of certain types of aerobic or facultatively anaerobic bacteria, some changes in the pattern of the caecal microflora produced by dietary xylitol could, nevertheless, be discerned (Table 3.4). Therefore, the viable numbers of bacteria isolated from the caeca of control- and xylitol-fed rats were compared in order to determine if certain species of bacteria were selected for by dietary xylitol. Furthermore, the data available on the xylitol-metabolising activity of the bacterial isolates in association with the changes in the numbers of bacteria should enable one to speculate on the mechanism(s) involved in the increased xylitol-metabolising activity of the caecal microflora (see discussion).

It was found that, Corynebacterium sp., S.epidermidis, S.saprophyticus, P.mirabilis and S.liquefaciens had all increased in numbers in the caecal contents of xylitol-fed rats (Table 3.4). S.liquefaciens, P.mirabilis and

TABLE 3.4 The effect of dietary xylitol on rat caecal bacterial numbers as counts of viable, aerobically isolated bacteria. The procedure for the isolation and enumeration of bacteria is described in the Materials and Methods section. Viable numbers of bacteria are the numbers of colonies appearing on agar plates and are expressed as the numbers of bacteria/g of caecal contents. Results are reported as the range observed in 3 different rats on a particular dietary regimen.

Isolated bacteria	Viable Bacteria/gram Caecal Contents			
	Xylitol-ammonium sulphate agar plates		Xylitol-yeast extract agar plates	
	Control-fed rats (n=3)	Xylitol-fed rats (n=3)	Control-fed rats (n=3)	Xylitol-fed rats (n=3)
Klebstella pneumoniae	$3x10^5$-$6.6x10^6$	$2x10^5$-$5x10^5$	$1.5x10^5$-$2.5x10^5$	$1.1x10^4$-$1.5x10^5$
Excherichia coli	$1x10^7$-$4x10^7$	$2x10^6$-$4x10^7$	$7.3x10^6$-$1.4x10^7$	$5.2x10^5$-$6.1x10^6$
Corynebacterium sp.	$1x10^4$-$2x10^4$	$2.7x10^5$-$2x10^6$	ND	ND
Staphylococcus epidermidis	$1x10^6$-$2.5x10^6$	$0.6x10^7$-$1.5x10^7$	ND	ND
Serratia liquefaciens	ND	$1x10^6$-$2.5x10^6$	ND	ND
Staphylococcus saprophyticus	ND	ND	ND	$3.3x10^6$-$6x10^6$
Micrococcus sp.	ND	ND	$1.6x10^6$-$4.3x10^6$	$2.3x10^5$-$8.3x10^5$
Proteus mirabilis	ND	ND	ND	$2.3x10^5$-$5x10^5$
Streptococcus faecalis	ND	ND	$6.3x10^6$-$7.8x10^6$	ND

ND = not detected

TABLE 3.5 Biochemical reactions of the xylitol-metabolising bacterial isolates of the Enterobacteriacae Family, determined by the MB-24E identification system. The MB-24E identification system is described in the text.

Test of Substrate	S.liquefaciens	K.pneumoniae
Lysine	+	+
Ornithine	+	-
H_2S	-	-
Glucose	+	+
Mannitol	+	+
Xylose	+	+
ONPG	+	+
Indole	-	-
Urease	-	-
VP	+	+
Citrate	+	+
TDA	-	-
Gelatin	-	-
Malonate	-	+
Inositol	+	+
Sorbitol	+	+
Rhamnose	-	+
Sucrose	+	+
Lactose	-	+
Arabinose	-	+
Ribitol	+	+
Raffinose	-	+
Salicin	+	+
Arginine	-	-
Motility	+	-
DNase	+	-

+ = positive reaction
- = no reaction

TABLE 3.6 Biochemical reactions required for the identification of *Micrococcus sp.*

Test or substrate	Reaction
Catalase	+
Glucose	Oxidative
Voges proskaven	-
Nitrate reduction	-
Arginine hydrolysis	-
Novobiocin	Resistant
Pigment (a) CLED	Pigmented
Pigment (b) Blood agar	Weakly pigmented

S.saprophyticus were not detected in the caecal contents of rats on the control diet but were present in the caecum of xylitol-fed rats (Table 3.4). On the other hand, K.pneumoniae, E.coli and Micrococcus sp. were present at marginally lower numbers in the caecum of xylitol-fed rats compared with those in the caecal contents of rats on the control diet. S.faecalis was not detected in the caecal contents of rats fed xylitol but did occur in the caecal contents of rats on the control diet (6.3×10^6-7.8×10^6 bacteria/g of caecal contents).

DISCUSSION

Under the experimental conditions described in this chapter, it was possible to isolate certain species of aerobic and facultative bacteria from the rat caecum (Table 3.1). Consequently, because "fastidious" organisms (for example, aerobic, facultative and anaerobic organisms which require either special or a complete range of nutrients for growth) are unable to grow on the minimal nutrient content of the growth media used in these experiments, one cannot exclude the involvement of these organisms in the metabolism of xylitol in the caecum. Nevertheless, under these minimal growth conditions, K.pneumoniae, S.liquefaciens and Micrococcus sp. were isolated based on their uncommon ability to utilise xylitol as a major carbon source. These organisms were among the species of aerobic and facultative bacteria isolated from the caeca of rats fed xylitol (Table 3.1) and were able to display the typical biochemical reactions required for their identity at the species level (Tables 3.5 and 3.6). The finding that S.liquefaciens and Micrococcus sp. are xylitol-metabolising organisms has not previously been reported. While mutant strains of K.pneumoniae have been previously shown to grow on xylitol (Rigby, Burleigh and Hartley, 1974; Taylor, Rigby and Hartley, 1974; Rigby, Gething and Hartley, 1976), it is not known if the isolates of K.pneumoniae, obtained in these experiments from the caeca of rats fed xylitol, are mutants that were selected for by xylitol within the caecum.

The determination of the xylitol-metabolising activity of the bacterial isolates by the radioisotopic assay led to the detection of bacteria which could utilise xylitol as a sole carbohydrate source for growth (Tables 3.2 and 3.3). Moreover, it can be inferred from these results that bacterial growth in xylitol-yeast extract medium does not

necessarily confer on bacteria the ability to utilise xylitol. For example, the rat caecal isolates E.coli, P.mirabilis, S.saprophyticus and S.faecalis grown in glucose-yeast extract medium, showed a low xylitol-metabolising activity which was not induced even when these bacteria were grown in xylitol-yeast extract medium (Table 3.3). That is, the growth of these bacteria in the xylitol-yeast extract medium can be attributed mainly to the metabolism of nutrients in the yeast extract rather than xylitol itself. Knuuttila and Mäkinen (1975) have also demonstrated that Streptococcus mutans(Ingbritt) is able to grow on trypticase-phytone-based medium containing xylitol but that it is unable to metabolise xylitol. Indeed, they showed that S.mutans metabolised amino acids and peptides present in the growth medium in preference to xylitol for growth. Whilst yeast extract appears to be utilised in preference to xylitol in E.coli, P.mirabilis, S.saprophyticus and S.faecalis, it is not so in the case of S.liquefaciens and Micrococcus sp. (Table 3.3). Instead there is an enhancement of xylitol-metabolising activity when S.liquefaciens and Micrococcus sp. were grown in xylitol-yeast extract medium. In these bacteria, however, yeast extract is apparently supportive of bacterial growth and therefore does not interfere with the bacterial metabolism of xylitol. On the other hand, K.pneumoniae which showed enhanced xylitol-metabolising activity when grown in xylitol-ammonium sulphate medium did not maintain its increased xylitol-metabolising activity when grown in xylitol-yeast extract medium. This is presumably due to the utilisation of yeast extract ingredients in the presence of xylitol (i.e. diauxic growth).

Generally, when microorganisms are exposed to more than one substrate, the phenomenon of diauxic growth and sequential substrate utilisation are observed (Epps and Gale, 1942; Monod, 1947; Hsiao, Chiang, Ueng and Tsao, 1981). It has been suggested that catabolite repression is the mechanism that regulates the enzymatic response of microorganisms in a growth environment that contains more than one substrate (Magasanik, 1961). Therefore, it is likely that the repressed xylitol-metabolising activity observed in K.pneumoniae grown under multi-substrate conditions (i.e. in xylitol-yeast extract medium) may be a result of catabolite repression of the xylitol-metabolising enzymes by either the preferred substrate or its metabolites. In addition, while catabolite repression can occur at the transcriptional level to prevent the synthesis of xylitol-metabolising enzymes, catabolite inhibition, a commonly observed regulatory mechanism in bacteria (McGinis and Paigen, 1969), can regulate, by allosteric mechanisms, those constitutive enzymes involved in the metabolism of xylitol.

Although yeast extract medium was used in these experiments to promote the growth of bacteria which were unable to grow in ammonium sulphate medium, it was not realised that yeast extract might compete with xylitol as a growth substrate. It is therefore important to point out that the isolation of "fastidious" xylitol-metabolising bacteria by the use of complex media may be hindered because some of the components of the growth media may interfere with the xylitol-metabolising activity of bacteria.

The effect of dietary xylitol on the populations of bacteria in the rat caecum was investigated by comparing the changes in the population of bacteria in the caecum of xylitol-fed rats with those in the control-fed rats (Table 3.4). If the changes in the bacterial population and their xylitol-metabolising activity are considered together it is possible to suggest the mechanisms involved in the xylitol-adaptation phase (Chapter Two). Three possible mechanisms are postulated for the adaptation, namely:

(i) a selection process in the presence of xylitol leading to an increase in the numbers of xylitol-metabolising organisms.

(ii) an induction of xylitol-metabolising enzymes in bacteria due to the continued presence of xylitol.

(iii) a combination of both bacterial selection and enzyme induction.

Certainly these various mechanisms have been examined in the microflora of the oral cavity of animals fed sorbitol (Birkhed et al, 1978). The findings in this chapter indicate that the mechanisms which operate are different for individual bacterial species. For example, with S.liquefaciens there is a combination of bacterial selection and induction, since this organism was detected in increased numbers in the caecum of xylitol-fed rats (Table 3.4) and was also able to demonstrate inducible xylitol-metabolising activity (i.e. increased X/G ratios; Table 3.2). While there was no evidence of selection of K.pneumoniae and Micrococcus sp. in the caecum of xylitol-fed rats (Table 3.4), these organisms were shown to metabolise xylitol at increased rates (Table 3.2). On the other hand, S.saprophyticus and P.mirabilis were unable to grow on or metabolise xylitol in culture (Table 3.3) but were detected in increased numbers in the caecum of xylitol-fed rats (Table 3.4). What is more, the growth of S.faecalis was considered to be inhibited by dietary xylitol since it was

not detected in the caecum of xylitol-fed rats (Table 3.4) and showed poor growth in xylitol-yeast extract medium (Table 3.3).

The available scientific literature concerning the effects of dietary xylitol on the gastrointestinal microflora is limited. Apart from this study, there have been only two other studies that have examined the effects of dietary xylitol on the gastrointestinal microflora of rats (Wekell, Hartman and Dong, 1980) and man (Dubach et al, 1973). Both these studies support the finding, reported in this chapter, that dietary xylitol, at least in the short term (two weeks) feeding regimen, can produce obvious changes in the populations of some bacterial species of the gastrointestinal tract. However, in addition to the effects that can be attributed directly to dietary xylitol (see above), there are suggestions that the pattern of changes observed in the microflora could have been influenced by other gut ecological factors (Hill, 1981). That is, factors which determine the final composition of bacteria in the caecum as a result of the increased xylitol-metabolising activity of the caecal bacteria could include the following:

(i) pH: certain organisms prefer alkaline conditions (e.g. Klebsiella spp.) while others (e.g. lactobacilli and streptococcus) thrive under acidic conditions (Hill, 1981).

(ii) Oxygen tension: if oxygen is able to enter the colon in greater than normal amounts, for instance during diarrhoea, oxygen-resistant organisms might proliferate at the expense of the strict anaerobes (Hill, 1981).

(iii) Nutrient availability: the selection of xylitol-metabolising bacteria could be affected by the presence of other carbohydrate or non-carbohydrate nutrients within the caecum. Energy needs for bacterial growth and metabolism could be met by the degradation of macromolecules like proteins, cellulose and mucopolysaccharides (Miller and Wolin, 1979; Salyers, 1979).

(iv) Caecal physiology: the distribution of flora in the caecum may not be uniform, in that, the bacterial flora at the mucosa of the caecum could differ from those in the caecal lumen (Cheng et al, 1981).

(v) Bacterial interference: antibacterial substances produced by

> bacteria, such as bacteriocines, are active against strains of the same species and antibiotics are active against other species of bacteria in the gastrointestinal tract (Hill, 1981).

An understanding of the role of each of these factors in determining the bacterial composition of the caecal contents in animals fed xylitol is worthy of further study. However, the most appropriate and immediate study to be performed was considered to be the determination of the mechanism of the increased xylitol-metabolising activity in these bacteria in association with the induction of xylitol-metabolising enzymes. This area was pursued in Chapter Four.

CHAPTER FOUR

THE UTILISATION OF XYLITOL BY KLEBSIELLA PNEUMONIAE AND SERRATIA LIQUEFACIENS

INTRODUCTION

In relation to its metabolism by microorganisms, xylitol has been called an unnatural carbohydrate (Mortlock, 1976). While this description is misleading in the sense that it is now realised that xylitol is a ubiquitous natural substance (Mäkinen, 1978) it is, nevertheless, valid in that few bacteria can metabolise xylitol. Moreover, even when these bacteria do metabolise xylitol, it is often at a much lower rate than with hexoses, pentoses, hexitols and other pentitols such as D-arabitol and ribitol. The topic of microbial metabolism of xylitol has been extensively reviewed by Mortlock (1976) and Mäkinen (1978) and therefore, in this introduction, only the biochemical pathways associated with the bacterial metabolism of xylitol are considered. In particular, in order to understand the mechanism(s) for the increased xylitol-metabolising activity in K.pneumoniae and S.liquefaciens grown on xylitol (Chapter Three) it is appropriate to take into account the genetic changes in bacterial strains that have led to the induction of enzymes involved in the immediate metabolism of xylitol.

A variety of studies have shown that the mutant strains of Klebsiella aerogenes (also known as Aerobacter aerogenes in the earlier scientific literature), which are capable of growth on xylitol, contain the D-arabitol and ribitol operons. The adaptative mechanism used by these bacteria involves the merging of the D-arabitol and ribitol pathways in order for xylitol to be metabolised (Fig. 4.1). Clarke (1974) suggested that this mechanism demonstrates how enzymes with broad substrate specificities can function in previously unrecognised metabolic pathways. Firstly, ribitol dehydrogenase is induced by xylitol (Mortlock and Wood, 1964) either by the synthesis of excessively large amounts of enzyme (Rigby, Burleigh and Hartley, 1974) or by the alteration of enzyme structure (Burleigh, Rigby and Hartley, 1974). This results in improved specificity of the ribitol dehydrogenase for xylitol. Secondly, substantial oxidation of xylitol to D-xylulose by ribitol dehydrogenase can then lead to the increased phosphorylation of D-xylulose to D-xylulose-5-phosphate by D-xylulokinase, an inducible enzyme of the D-arabitol pathway. The further metabolism of xylulose-5-phosphate can then satisfy the energy demands for bacterial metabolism and growth (Fig. 4.1).

Aerobacter aerogenes 1033 (Lerner et al, 1964), A.aerogenes PRL-R3 (Mortlock and Wood, 1964) and K.aerogenes W 70 (Charnetzky and Mortlock, 1974) are some examples of mutant strains that grow on xylitol since they have acquired the genetic ability to constitutively synthesise ribitol dehydrogenase. These mutant strains, however, are only able to grow slowly on xylitol because the ribitol dehydrogenase can only oxidise xylitol at a low rate. In an attempt to select for mutants with an ability to grow more rapidly on xylitol, Hartley et al, (1976) subjected a mutant strain of A.aerogenes 1033 described as mutant strain X1 by Wu et al, (1968) to further possible mutations in the presence of N-methyl-N'nitro-N-nitrosoguanidine. By this procedure a mutant strain X2 was selected with an improved growth rate on xylitol due to an altered ribitol dehydrogenase which had a greater affinity for xylitol (i.e. lower Km for xylitol). Further mutations in mutant strain X2 produced strain X3 which had the fastest rate of growth on xylitol of all the mutants of A.aerogenes 1033. Not only did it produce an altered ribitol dehydrogenase with a lower Km for xylitol, it also showed an enhanced transport of xylitol due to an increase in arabitol permease activity. This observation is noteworthy in that the presence of a cytoplasmic polyol dehydrogenase in itself is insufficient to allow bacterial growth if xylitol cannot be transported into

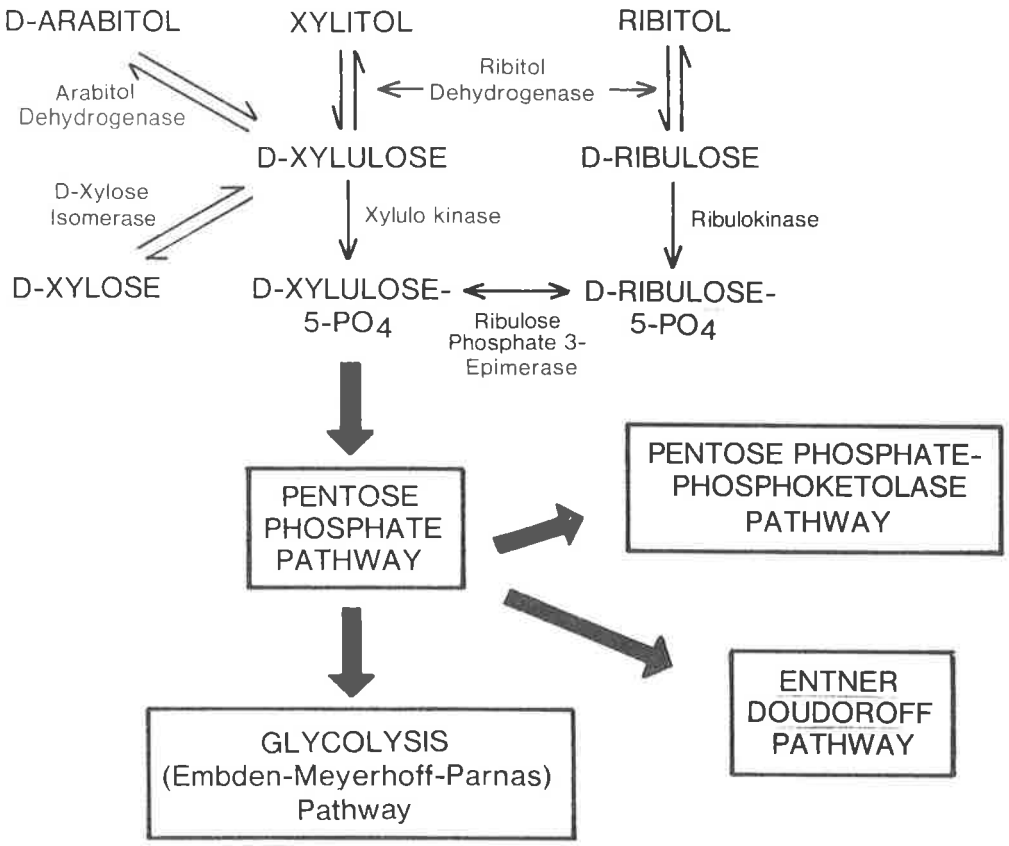


Fig. 4.1 Pathways for the Bacterial Metabolism of Xylitol.

the cell. In essence, bacterial growth on xylitol requires the presence of a transport system for xylitol, a polyol dehydrogenase with a suitable affinity for xylitol and a xylulokinase.

In this chapter, investigations of the mechanisms responsible for the observed increase in the xylitol-metabolising activity of K.pneumoniae and S.liquefaciens after growth on xylitol reported in Chapter Three are described. In one series of experiments, the xylitol-metabolising activities of these bacteria were determined after their growth on galactitol, mannitol, sorbitol, erythritol, arabitol, ribitol, glycerol and inositol as well as on xylitol. In another series of experiments, the activities for xylitol uptake, xylitol or polyol dehydrogenase and xylulokinase were determined in those cultures of K.pneumoniea and S.liquefaciens that grew on a particular sugar polyol and exhibited an increase in xylitol-metabolising activity. The observed increases in xylitol-metabolising activity are then discussed in terms of the induction of xylitol-metabolising enzymes and the enhancement of carbon flux from xylitol through the common and major carbohydrate pathways of K.pneumoniae and S.liquefaciens.

MATERIALS AND METHODS

Pyruvate, phosphoenol pyruvate (PEP), adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (reduced) (NADH) and lactatate dehydrogenase containing pyruvate kinase were all obtained from Boehringer, Mannheim, Australia. D-xylulose and D-ribulose were obtained from Sigma, U.S.A. The sources of all other chemicals, radiochemicals and reagents have already been described in Chapter Two. The bacterial isolates, K.pneumoniae and S.liquefaciens used in this study were initially obtained from the rat caecum (Chapter Three).

Media and Growth Conditions

In the growth experiments, the carbohydrates glucose, xylose, galactitol, mannitol, sorbitol, erythritol, arabitol, ribitol, xylitol, glycerol and inositol were added to give a final concentration of 50 mmol/l in ammonium sulphate medium (described in Chapter Three). Bromthymol-blue/lactose agar was prepared according to the instructions supplied by Oxoid Ltd., London, England.

Stored cultures of K.pneumoniae and S.liquefaciens, when required, were streaked onto bromthymol-blue/lactose agar plates and incubated for 48 h at 37°C. Single colonies were picked from these plates and used for the inoculation of glucose-ammonium sulphate medium. The exponentially growing bacteria in glucose-ammonium sulphate medium (50 ml) were centrifuged and washed once with sterile ammonium sulphate medium (without any added carbohydrate) prior to their use as inocula in the various carbohydrate-containing ammonium sulphate media. The growth media were regularly checked for contamination on bromthymol-blue/lactose agar plates.

Preparation of Bacterial Cell-Free Extracts

Bacterial cultures grown in 50 ml of carbohydrate-containing ammonium sulphate media were centrifuged, washed twice in 25 ml of 0.01 mol/l tris-HCl (pH 7.1) buffer containing 0.1 mmol/l Na_2EDTA, 0.4 mmol/l $MgCl_2$ and 1 mmol/l reduced glutathione and finally resuspended in 4 ml of the same buffer at 4°C. Cell suspensions were disrupted by sonication using a Branson Sonicator at maximum frequency for 5 min. To prevent samples from being denatured by the heat generated from the sonicator tip, the sample container was immersed in a water bath maintained at 4°C. Cell disruption by the sonicator was indicated by a decrease in turbidity of the bacterial cell suspension. The sonicated samples were centrifuged at 20,000 x 6 for 30 min at 4°C to remove the cell debris. The resultant, cell-free supernatants were used for enzyme assays.

Polyol Dehydrogenase Assay

Polyol dehydrogenase activities were determined on these cell-free extracts by measuring the rate of reduction of NAD at 340 nm using sugar polyols as substrates. The assay conditions are essentially those described by Bässler (1974) and were adapted for use in the Multistat Centrifugal Analyser (Multistat III, Micro Centrifugal Analyser, Instrumentation Laboratory, U.S.A.) The final concentration of reagents present in a total reaction volume of 250 µl were: 0.12 mol/l tris-HCl (pH 8.2), 0.1 mol/l sugar polyol, 10 mmol/l NAD and 5 µl diluted crude enzyme extract (1/2, 1/10 and 1/20 dilutions were used). The sugar polyol was not included in the blank reaction. The enzyme activities were measured from the linear portion of the plot. Enzyme activity is expressed

as U/mg of protein, where one unit (U) is equivalent to the amount of enzyme required to convert one µmole of substrate to product per min at $37^{o}C$.

Pentulokinase Assay

Pentulokinase (ribulokinase and xylulokinase) activities were determined using the method described by Simpson (1966). The amount of ADP formed is estimated by coupling the following enzyme reactions:-

D-pentulose + ATP $\xrightarrow{\text{D-pentulokinase}}$ pentulose-5-phosphate + ADP

ADP + PEP $\xrightarrow{\text{pyruvate kinase}}$ pyruvate + ATP

pyruvate + NADH $\xrightarrow{\text{lactate dehydrogenase}}$ L-lactate + NAD

The net effect of the coupled reactions is the production of NAD from NADH and the rate of this reaction is measured by the rate of decrease in absorbance at 340 nm. The final concentration of reagents present in a total reaction volume of 150 µl include: 0.1 mol/l tris-HCl (pH 7.8), 0.1 mol/l KCl, 0.2 mmol/l Na_2EDTA, 2 mmol/l phosphoenol-pyruvate, 1 mmol/l ATP, 1.8 mmol/l NADH, 1 mmol/l D-xylulose or D-ribulose 0.5 µl lactate dehydrogenase and pyruvate kinase and 5 µl crude enzyme (1/2, 1/10 and 1/20 dilution). This assay was also adapted for use in the Multistat. Blank reactions contributed by NADH oxidase and polyol dehydrogenase activities in the crude enzyme extract were determined by excluding pentulose or ATP from the reaction mixtures, respectively. Thus, the pentulokinase activity reported in the results section was calculated by deducting both the NADH oxidase and polyol dehydrogenase activities from the total test activity.

Transport Assay

Cultures of K.pneumoniae and S.liquefaciens that were grown on various carbohydrate sources were centrifuged at 10,000 x g for 10 min, washed and resuspended in PBS to yield an absorbance of 1.0 at 600 nm. The washed cells were pre-incubated at $37^{o}C$ for 10 min in a shaking water bath before the addition of $|U\text{-}^{14}C|$ sorbitol or $|U\text{-}^{14}C|$ xylitol. Specific activities of both polyols were 5,200 cpm/nmol and were added as required. The volume of the total assay mixture was 0.5 ml. After various time intervals of 1,

2, 4, 6, 8 and 10 min, 50 µl samples were withdrawn from the reaction incubated at 37°C, collected on membrane filters (Millipore Corp. HAWP, 25 mm diameter, 0.45 µm pore size) and washed twice with 5 ml of PBS containing either 10 mmol/l xylitol or sorbitol so as to minimise uptake of radioactive polyol during the washing procedure. The washed filters were placed in small glass vials and dried overnight at 100°C. An aliquot (3.5 ml) of scintillation fluid containing 0.4% (w/v) 2,5 Diphenyloxazole (PPO; Sigma, U.S.A.) in toluene was added to the glass vials which were then capped, inserted into large plastic vials and counted on a Model 3375 Tri-Carb Liquid Scintillation Spectrometer. The rate of uptake of |U-^{14}C| xylitol and |U-^{14}C| sorbitol were expressed as nmol/min/mg of protein.

Radioisotopic Metabolic Assay

The metabolism of |U-^{14}C| glucose and |U-^{14}C| xylitol to $^{14}CO_2$ by K.pneumoniae and S.liquefaciens after the growth of these organisms on the various carbohydrates was determined by the method described in Chapter Three.

RESULTS

Effect of Carbohydrate Growth Substrates on the Xylitol-metabolising activity of K.pneumoniae and S.liquefaciens

The growth of K.pneumoniae and S.liquefaciens, under the minimal growth conditions in ammonium sulphate medium, was not supported by the sugar polyols arabitol, erythritol and galactitol (Table 4.1). Cultures of K.pneumoniae and S.liquefaciens able to grow on sugar polyols other than xylitol, demonstrated X/G ratios (for definition see Chapter Three) that were only comparable to that obtained for glucose-grown cells. This observation may suggest the presence of constitutive sugar polyol-metabolising enzymes with a non-specific but low activity towards xylitol or may reflect the low specific activity of the repressed 'xylitol' dehydrogenase. The xylitol-metabolising activity of K.pneumoniae and S.liquefaciens after the growth on xylitol demonstrated a 6.6 and a 38-fold increase, respectively, compared to the X/G ratios of glucose-grown

TABLE 4.1 The effect of various carbohydrate growth substrates on the glucose- and xylitol-metabolising activity of K.pneumoniae and S.liquefaciens. The bacteria were previously grown on ammonium sulphate medium containing various carbohydrates at a concentration of 50 mmol/l before being assayed for their glucose- and xylitol-metabolising activity as described in the Materials and Methods sections of Chapters Two and Three. Some substrates were unable to support the growth of these bacteria (NG) when tested over a period of 72 h at 37°C. The results are expressed as a mean of 3 independent determinations.

Growth substrate in medium	Rate of $\lvert U\text{-}^{14}C\rvert$ glucose and $\lvert U\text{-}^{14}C\rvert$ xylitol metabolism (nmol $^{14}CO_2$/min/mg of bacterial protein)					
	K.pneumoniae			S.liquefaciens		
	Glucose	Xylitol	X/G ratio	Glucose	Xylitol	X/G ratio
Glucose	88.6	4.5	0.05	41.4	1.2	0.02
Xylose	115.4	10	0.09	NG	NG	-
Glycerol	112.2	8.4	0.07	36.4	2.4	0.06
Xylitol*	46.6	16.2	0.33*	43.4	33	0.76*
Arabitol	NG	NG	-	NG	NG	-
Ribitol	60	3.2	0.05	37.3	2.4	0.06
Sorbitol	65.2	2.3	0.04	45.6	2.6	0.05
Mannitol	92.1	3.6	0.04	26.6	1.3	0.05
Galactitol	NG	NG	-	NG	NG	-
Erythritol	NG	NG	-	NG	NG	-
Inositol	118.8	9.5	0.08	33.1	1.8	0.05

cells (Table 4.1). These results also indicate that the enhancement of the xylitol-metabolising activity (measured by the production of $^{14}CO_2$ from |U-^{14}C| xylitol) was solely due to the presence of xylitol in the growth medium and was not significantly affected by other sugar polyols or media constituents.

Effect of Carbohydrate Growth Substrates on the Uptake of Xylitol and Sorbitol

Since xylitol was the only sugar polyol to cause an increase in the xylitol-metabolising activity of both K.pneumoniae and S.liquefaciens, it was decided to restrict the comparison of transport activities to the effects of the substrates, glucose, ribitol, xylitol and sorbitol, on the uptake of |U-^{14}C| xylitol and |U-^{14}C| sorbitol. The uptake of |U-^{14}C| xylitol by K.pneumoniae and S.liquefaciens that were previously grown on xylitol was linear over a 15 min incubation period (Figs. 4.2a and 4.3a). Calculations of the rate (slope of the line) of xylitol uptake in these cells yielded values of 0.52 nmol/min/mg of protein for K.pneumoniae and 1.04 nmol/min/mg of protein for S.liquefaciens (Figs. 4.2a and 4.3a). K.pneumoniae grown on glucose and ribitol did not show a measurable xylitol uptake activity throughout the incubation period, and consequently no rates were determined (Figs. 4.2a and b). While sorbitol-grown K.pneumoniae demonstrated a xylitol uptake rate of 1.2 nmol/min/mg of protein, xylitol-grown K.pneumoniae did not show the uptake of |U-^{14}C| sorbitol (Fig. 4.2b). From these results it can be inferred that sorbitol induced a non-specific polyol transport system which allows xylitol to be transported but xylitol was only able to induce a specific transport system for itself.

S.liquefaciens grown on ribitol did not show any xylitol uptake but when grown on glucose or sorbitol, only low xylitol uptake activities were detected (Fig. 4.3a). On the other hand, when sorbitol uptake was measured in S.liquefaciens grown on xylitol and ribitol the activity was low but detectable (Fig. 4.3b). The |U-^{14}C| xylitol and |U-^{14}C| sorbitol uptake in S.liquefaciens was shown in these results to be specifically induced by xylitol and sorbitol as growth substrates respectively, with no evidence of cross-induction (that is, the induction of other substrates that were not present in the growth medium) of transport activity by structurally similar substrates.

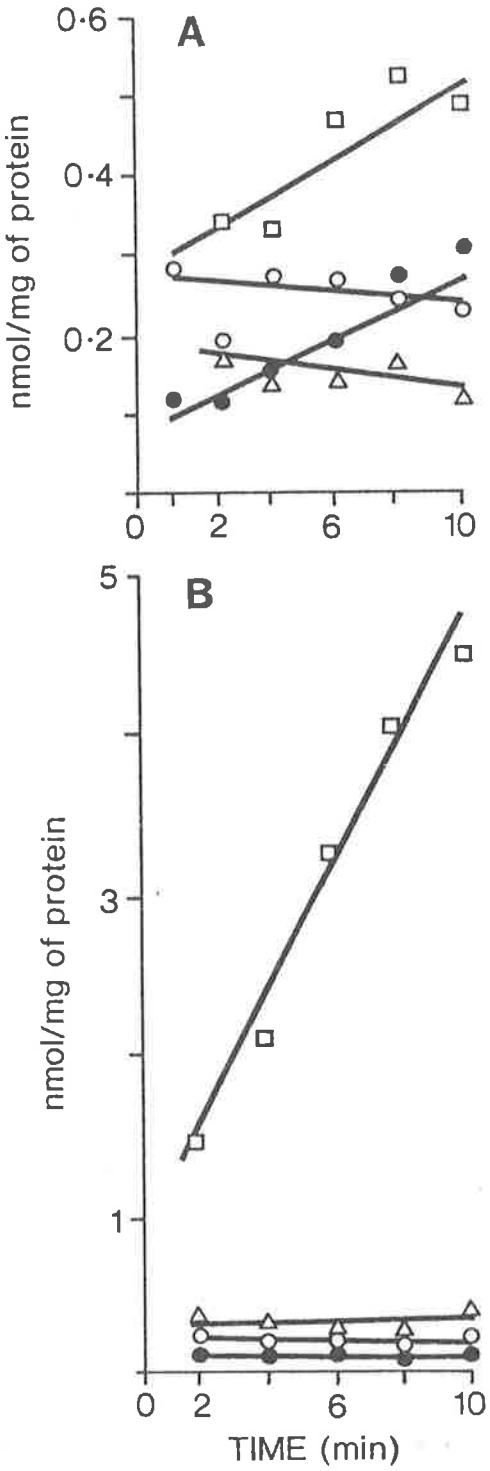


Fig. 4.2 The effect of various growth substrates on the uptake of (a) $|U^{14}\text{-}C|$ xylitol and (b) $|U\text{-}^{14}C|$ sorbitol in K.pneumoniae. Cells were grown on glucose (○), ribitol (△), xylitol (●) and sorbitol (□). The procedure for the radio-active uptake assay is described in the Materials and Methods section.

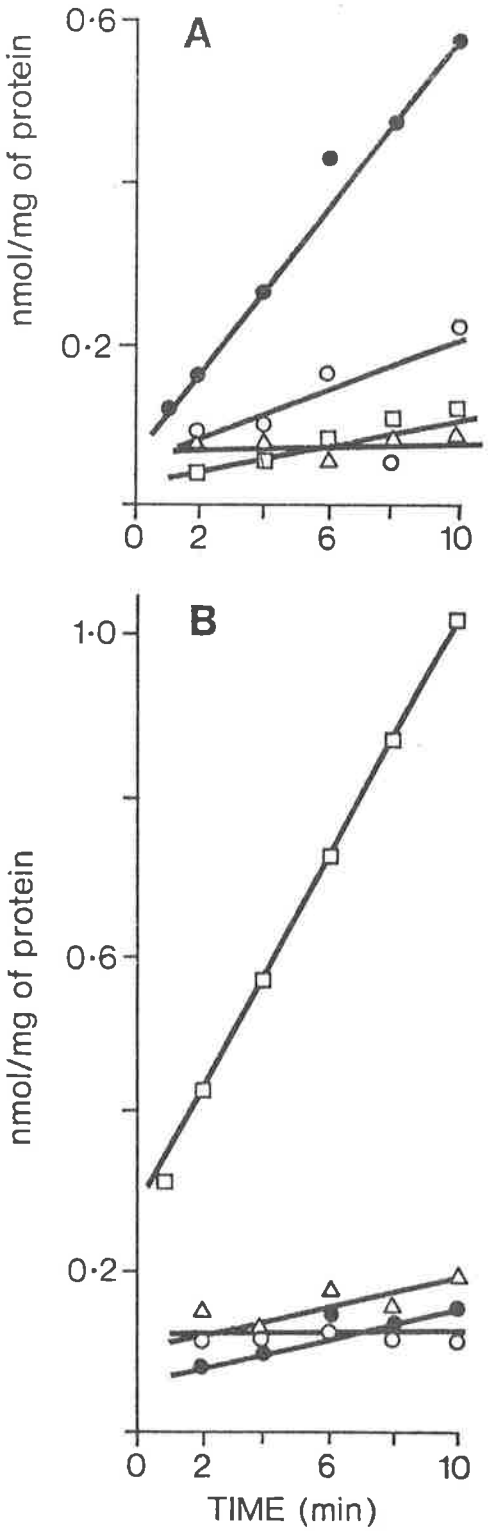


Fig. 4.3 The effect of various growth substrates on the uptake of (a) |U-^{14}C| xylitol and (b) |U^{14}-C| sorbitol in S.liquefaciens. Cells were grown on glucose (○), ribitol (△), xylitol (●) and sorbitol (□). The experimental procedures is described in the Materials and Methods section.

Competition of Xylitol and Sorbitol Uptake by other Sugar Polyols

The effect of sugar polyols on the uptake of xylitol or sorbitol in K.pneumoniae and S.liquefaciens is reported in Table 4.2. These uptake experiments were performed by adding other sugar polyols to a reaction mixture containing either $|U\text{-}^{14}C|$ xylitol or $|U\text{-}^{14}C|$ sorbitol at a concentration of 200 µmol/l. Under these conditions, the uptake of $|U\text{-}^{14}C|$ xylitol in K.pneumoniae was not affected by the addition of either 200 or 400 µmol/l of sorbitol. While, lower concentrations of ribitol (100 and 200 µmol/l) increased xylitol uptake in K.pneumoniae, higher concentrations (400 and 800 µmol/l) were inhibitory. Arabitol, however, inhibited xylitol uptake only at low concentrations (Table 4.2). In S.liquefaciens there was no evidence of competition between xylitol and the added arabitol, ribitol or sorbitol for the xylitol uptake system (Table 4.2). The uptake of sorbitol was not affected by the presence of arabitol, ribitol or xylitol in reaction mixtures of K.pneumoniae or S.liquefaciens.

Effect of Carbohydrate Growth Substrates on the Polyol Dehydrogenase Activities found in Cell-free Extracts of K.Pneumoniae and S.liquefaciens

Cell-free extracts of K.pneumoniae and S.liquefaciens that were grown on 50 mmol/l carbohydrate (xylose, glucose, sorbitol, ribitol and xylitol) were assayed for polyol dehydrogenase activities with the enzyme substrates arabitol, ribitol, xylitol and sorbitol (Tables 4.3 and 4.4). The enzyme activities reported for the substrates do not necessarily represent any one specific polyol dehydrogenase since the enzyme extracts used in these studies may contain more than one induced polyol dehydrogenase and, furthermore, any induced polyol dehydrogenase is likely to possess a broad substrate specificity (Primrose and Ronson, 1980). Cell extracts prepared from glucose-grown K.pneumoniae showed polyol dehydrogenase activities for arabitol and ribitol but no detectable activities were observed for xylitol or sorbitol (Table 4.3). Interesting patterns of enzyme induction, however, were noted when sugar polyols were present as growth substrates. For example, in K.pneumoniae, the growth substrate ribitol increased its own oxidation by approximately 12-fold (compared to the activity attained in glucose-grown cells) but had no effect on the low polyol dehydrogenase activities seen with sorbitol and xylitol (Table 4.3).

TABLE 4.2 The effect of added polyols on the uptake of xylitol and sorbitol in K.pneumoniae and S.liquefaciens The bacteria were grown on ammonium sulphate medium containing either 50 mmol/l of xylitol or sorbitol and then washed with phosphate-buffered saline and adjusted to a cell density (absorbance) of 1.0 at 600 nm. The final reaction mixture contained 200 µmol of labelled polyol.

Organism		K.pneumoniae		S.liquefaciens	
Growth Substrate		Xylitol	Sorbitol	Xylitol	Sorbitol
Uptake (nmol/min/mg of protein)		$\|U\text{-}^{14}C\|$ xylitol	$\|U\text{-}^{14}C\|$ sorbitol	$\|U^{-14}C\|$ xylitol	$\|U\text{-}^{14}C\|$ sorbitol
Without added polyols		0.52	7.5	1.04	2.1
Added polyols (µmol/l)					
Arabitol	50	0.17	-	-	-
	100	0.19	-	-	-
	200	0.38	9.6	1.28	2.14
	400	0.47	9.4	1.20	2.10
Ribitol	100	0.74	-	-	-
	200	0.65	7.2	1.26	2.06
	400	0.33	7.6	1.16	2.04
	800	0.05	-	-	-
Sorbitol	200	0.42	-	0.96	-
	400	0.52	-	1.16	-
Xylitol	200	-	8.8	-	2.0
	400	-	8.4	-	2.2

- Not tested

TABLE 4.3 The effect of various carbohydrate growth substrates on the polyol dehydrogenase activity in K.pneumoniae.
Cell-free extracts were prepared from K.pneumoniae, grown on ammonium sulphate medium containing 50 mmol/l of the carbohydrate indicated, and used for the assay of polyol dehydrogenase activity with various polyols as substrates. The results are expressed as a mean of 3 determinations.

Growth Substrate	Specific activity (units/mg of protein)			
	Arabitol	Ribitol	Sorbitol	Xylitol
Glucose	7	12.6	0	0
Ribitol	12.2	140	0	3
Xylitol	23	652	2	20
Sorbitol	10	230	0.8	3
Xylose	55	1,985	9	55

TABLE 4.4 The effect of various carbohydrate growth substrates on the polyol dehydrogenase activity in S.liquefaciens.
Cell-free extracts were prepared from S.liquefaciens, grown on ammonium sulphate medium containing 50 mmol/l of the carbohydrates indicated, and used for the assay of polyol dehydrogenase activity with various polyols as substrates. The results are expressed as a mean of 3 determinations.

Growth substrate (50 mmol/l)	Specific activity (units/mg of protein)			
	Arabitol	Ribitol	Sorbitol	Xylitol
Glucose	18	1	0.5	0
Ribitol	16	15	0	0
Xylitol	23	7	19	12
Sorbitol	21	0.3	0.2	0.2

Xylitol, sorbitol and xylose caused even greater induction that ribitol of ribitol dehydrogenase-like activity in K.pneumoniae. This induction was accompanied by a proportionate increase in xylitol oxidation (Table 4.3). The induction of ribitol dehydrogenase has been previously shown to be necessary for the oxidation of xylitol in A.aerogenes (Mortlock, Fossitt and Wood, 1965). Xylose appeared to be the best inducer of ribitol, xylitol and arabitol dehydrogenase-like activities indicating that polyol dehydrogenases can be induced by substances with a broad structural specificity.

The pattern of induction of polyol dehydrogenases in S.liquefaciens was different from that observed in K.pneumoniae. With respect to arabitol dehydrogenase activity it would appear that this activity is constitutive in S.liquefaciens since the sugar polyol growth substrates caused only minor increases in the activity when compared to glucose-grown cells (Table 4.4). The results in Table 4.5 demonstrate that in S.liquefaciens, ribitol as a growth substrate induced a ribitol dehydrogenase-like activity and xylitol had induced ribitol, sorbitol and xylitol dehydrogenase-like activities. Surprisingly, sorbitol as a growth substrate did not induce sorbitol dehydrogenase activity. Sorbitol may be metabolised via sorbitol-6-phosphate, which is oxidised by sorbitol-phosphate dehydrogenase (Kelker and Anderson, 1971). Such activity would not be detected by the polyol dehydrogenase assay used in these studies.

Pentulokinase Activities in K.pneumoniae and S.liquefaciens

The cell extracts prepared from K.pneumoniae and S.liquefaciens were assayed for the activities of D-xylulokinase and D-ribulokinase, since they are key enzymes involved in the metabolism of pentitols, in particular xylitol, ribitol and arabitol. Pentulokinase activity was not detected in the cell extracts of glucose-grown cells (Table 4.5). K.pneumoniae grown on ribitol showed the induction of D-ribulokinase whereas growth on sorbitol showed xylulokinase activity (Table 4.5). Both xylitol and xylose were able to induce both xylulokinase and ribulokinase activities but in different proportions. Xylitol had induced more ribulokinase activity than xylulokinase activity in K.pneumoniae. In S.liquefaciens, however, growth on xylitol and sorbitol showed induced xylulokinase activity. There were no detectable levels of ribulokinase in ribitol-grown S.liquefaciens (Table 4.5).

TABLE 4.5 The effect of various carbohydrate growth substrates on the pentulokinase activity in K.pneumoniae and S.liquefaciens. Cell-free extracts of bacteria were assayed for enzyme activity with D-xylulose and D-ribulose as substrates. The results are expressed as a mean of 3 determinations.

Growth substrate (50 mmol/l)	K.pneumoniae		S.liquefaciens	
	DXK[a]	DRK[b]	DXK	DRK
Glucose	0	0	0	0
Ribitol	0	200	0	0
Xylitol	350	65	20	0
Sorbitol	16	0	4	0
Xylose	320	250	-	-

(a) DXK: D-xylulokinase activity in units/mg of protein

(b) DRK: D-ribulokinase activity in units/mg of protein

(c) - not tested, since S.liquefaciens did not grow on xylose (see Table 4.1).

The phosphorylation of xylulose to xylulose-1-phosphate by fructokinase has been shown to occur in mammalian liver (Barngrover et al, 1981 and James et al, 1982). Therefore a preliminary experiment was carried out to determine fructokinase activity in cell extracts of K.pneumoniae and S.liquefaciens especially since fructokinase activity has been reported to be present in A.aerogenes (Sapico and Anderson, 1969). In these experiments, when fructose was used instead of a pentulose as the substrate no activity was detected. This implies that xylulose metabolism in these two bacterial species is via xylulose 5-phosphate and not xylulose 1-phosphate.

DISCUSSION

The experiments reported in this chapter confirmed that, as a growth substrate, xylitol was the only sugar polyol capable of increasing the rate of metabolism of $|U\text{-}^{14}C|$ xylitol to $^{14}CO_2$ in K.pneumoniae and S.liquefaciens (Table 4.1). In association with the increased xylitol-metabolising activity in these bacteria, there were increased activities in xylitol uptake, polyol dehydrogenase(s) (with a specific activity towards xylitol) and xylulokinase (Tables 4.2, 4.3, 4.4 and 4.5). Table 4.6 shows that the uptake of xylitol is the rate-limiting step in the overall metabolism of xylitol and therefore dictates the carbon flux. The xylitol uptake activity is about 6-fold lower than the rate of xylitol utilisation. This discrepancy may be accounted for by the differences in the methods used for the measurement of xylitol uptake (Figs. 4.2 and 4.3) and the rate of xylitol utilisation (Table 4.1) in K.pneumoniae and S.liquefaciens. The concentration of xylitol used in the uptake studies was low (200 µmol/l) and assays were performed for short periods of time in order to minimise the accumulation of metabolic intermediates. On the other hand, a higher concentration of xylitol (50 mmol/l) and longer incubation periods were used in the determination of the rate of bacterial utilisation of xylitol. These differences in the concentration of nutrient and incubation periods can affect the kinetics of nutrient uptake and therefore the rate of substrate utilisation (Button, 1983).

Among the polyols examined, galactitol, erythritol and arabitol were found not to support the growth of S.liquefaciens and K.pneumoniae (Table 4.1). Although samples of the inoculated bacteria in these polyol-

TABLE 4.6 Metabolic and enzymatic activities associated with the growth of K.pneumoniae and S.liquefaciens on xylitol. The interpretation of these activities is discussed in the text.

Activities (nmol/min/mg of protein)	K.pneumoniae	S.liquefaciens
Rate of xylitol utilisation	3.2	6.6
Xylitol uptake	0.5	1.04
Xylitol dehydrogenase activity	2×10^4	1.2×10^4
Xylulokinase activity	3.5×10^5	2×10^4

containing growth medium were not further investigated it is, nevertheless, appropriate to consider the mechanisms involved in this effect. One possibility is that phosphorylated intermediates accumulate intracellularly in a manner that is lethal to the bacteria. That is, inhibition of polyol metabolism could occur subsequent to a phosphorylation reaction. Indeed, in mutant strains of E.coli, which are capable of utilising xylitol by constitutively producing the ribitol-metabolising enzymes (ribitol dehydrogenase and ribulokinase), there is a concomitant acquisition of toxicity to galactitol, L-arabitol and to D-arabitol if they are unable to utilise it for growth (Scangos and Reiner, 1978). This implies that the constitutive production of ribulokinase is involved in the phosphorylation of these polyols to toxic metabolites. On the other hand, the lack of growth of S.liquefaciens on xylose (Table 4.1) can be attributed to inefficient transport of xylose (Mortlock and Old, 1979) or to repressed D-xylose isomerase (Mortlock and Wood, 1964) since mutant bacterial strains which lose these enzyme activities are also unable to grow on this substrate.

A comparison of the substrate specificities of the xylitol-metabolising enzymes in S.liquefaciens and K.pneumoniae after growth on xylitol, suggests that the types of enzymes induced by these bacteria are different. Differences in the specificity of xylitol transport in S.liquefaciens and K.pneumoniae were demonstrated by the competition of $|U\text{-}^{14}C|$ xylitol uptake with the addition of non-radioactive polyols (Table 4.2). In K.pneumoniae xylitol uptake was inhibited by D-arabitol and ribitol whereas, in S.liquefaciens these substrates did not affect the uptake of xylitol (Table 4.2). Sorbitol, on the other hand, had no effect on the uptake of xylitol in both S.liquefaciens and K.pneumoniae. It is interesting to note that arabitol had inhibited xylitol uptake in K.pneumoniae only in low concentrations (50 to 100 mmol/l) but had no effect at higher concentrations. This observation is consistent with the fact that a variety of carbohydrate permeases appear to show decreased activity when the concentration of intracellular metabolites such as sugar phosphates are elevated (Dills et al, 1980). In addition, Saier and Simoni (1976) demonstrated that the elevated levels of the intracellular sugar phosphates can inhibit the permease activity. Consequently, when higher concentrations of arabitol are added in the reaction mixture containing labelled xylitol, the inhibitory effect of arabitol on xylitol uptake is reduced (Table 4.2) because the accumulation of phosphorylated arabitol intermediates could inhibit the uptake of arabitol. The mechanism by which intracellular sugar phosphates

inhibit sugar uptake is not clearly understood (Dills et al, 1980). It would appear that arabitol permease may be involved in the transport of xylitol in K.pneumoniae since K.aerogenes which is a related species of bacteria has been shown to possess this permease (Wu et al, 1968).

In addition to the different transport systems involved in the uptake of xylitol in K.pneumoniae and S.liquefaciens, the types of polyol dehydrogenase activities that were induced were different (Tables 4.4 and 4.5). In K.pneumoniae, for example, the induction of xylitol dehydrogenase-like activity was associated with the induction of ribitol dehydrogenase-like activity and this is consistent with the results obtained for A.aerogenes (Mortlock, Fossitt and Wood, 1965). Whereas, when S.liquefaciens was grown on xylitol, induced polyol dehydrogenase activities were observed for the enzyme substrates ribitol, sorbitol and xylitol. The xylitol dehydrogenase-like activities were greater than that obtained for ribitol as an enzyme substrate (Table 4.5). Further experiments are warranted here to ascertain if a structurally modified ribitol dehydrogenase with improved specificity for xylitol was involved or a specific xylitol dehydrogenase was induced. Although K.pneumoniae and S.liquefaciens were able to grow on sorbitol as a major carbon source, the sorbitol dehydrogenase activities in these cells were very low (Tables 4.4 and 4.5). Nevertheless, other pathways for sorbitol metabolism have been implicated, for example, in A.aerogenes PRL-R3. In this organism the metabolism was shown to proceed via the pathway: sorbitol $\longrightarrow$ sorbitol-6-phosphate $\longrightarrow$ fructose-6-phosphate (Kelker and Anderson, 1971). It was also demonstrated that the transport of sorbitol and its phosphorylation occur simultaneously by the phosphoenol pyruvate: sorbitol-6-phosphotransferase system and the sorbitol-6-phosphate formed is oxidised by sorbitol-6-phosphate dehydrogenase. If this pathway was functional in K.pneumoniae and S.liquefaciens, any sorbitol-6-phosphate dehydrogenase activity could not be detected in cell-free extracts by the sorbitol dehydrogenase assay.

In general, it can be inferred that the pathways for the metabolism of xylitol in S.liquefaciens and K.pneumoniae have evolved independently, without there necessarily being any transfer of genetic information between these species of bacteria. In support of this observation, genetic studies with E.coli show that only 10 to 20% of natural strains possess the operons for ribitol and arabitol metabolism and these genes tend to be located in the bacterial chromosome (Link and Reiner, 1982) rather than in plasmids. That is, the transfer of genetic material responsible for

inducible xylitol-metabolising activity between species of such bacteria could be very limited. Furthermore, it can be concluded that the induction of xylitol-metabolising enzymes as shown in these bacterial isolates causes enhanced xylitol-metabolising activity and the rapid removal of unabsorbed xylitol in the rat caecum. As a consequence of this bacterial mechanism, the osmotic load in the caecum is reduced and the host 'adapts' with the disappearance of diarrhoea.

CHAPTER FIVE

AN EXAMINATION OF THE INSOLUBLE MUCUS-LIKE MATERIAL THAT ACCUMULATES IN THE CAECUM OF XYLITOL-FED RATS

INTRODUCTION

Whilst it was clearly shown that dietary xylitol caused changes in the populations and metabolism of the bacterial microflora in the rat caecum (Chapters Two, Three and Four), it was not possible to determine whether the occurrence of the insoluble mucus-like material in the caecal contents of xylitol-fed rats (Chapter Two) was a metabolic product of the caecal microflora or a result of xylitol-induced changes in the cellular metabolism of the host gastrointestinal wall. This viscous, mucus-like material was referred to as the fluffy layer because of its appearance. That is, the fluffy layer appeared as a layer in the supernatant when the caecal contents obtained from xylitol-fed rats were centrifuged at 15,000 x g for 30 min at $4^{o}C$ (Fig. 5.1).

Under normal physiological conditions the intestinal goblet cells secrete mucus to protect and lubricate the gastrointestinal tract (Guyton, 1976). An increase in the production of mucus can occur if there is irritation or inflammation of the intestinal wall. For example, in man, pathological inflammatory changes with increased secretions of mucus is a

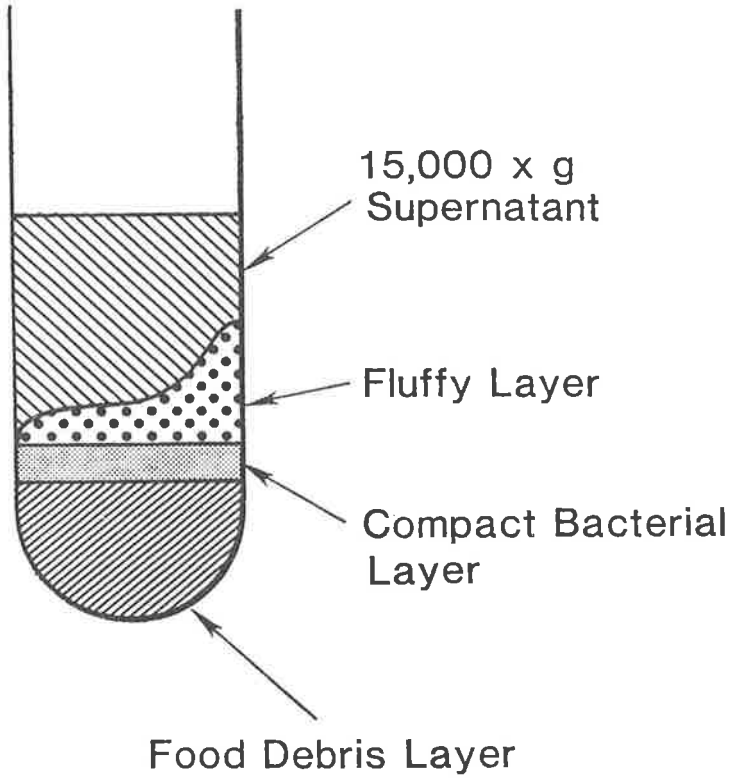


Fig. 5.1 Fluffy layer definition. The fluffy layer is a viscous, low density, insoluble material that is observed by centrifuging caecal contents obtained from 10% (w/w) xylitol-fed rats. The fluffy layer is easily resuspendable compared to the compact layers of bacteria and food debris in the pellet obtained when the caecal contents have been centrifuged at 15,000 x g for 15 min at 4°C.

common feature of ulcerative colitis and Crohn's disease (MacDermott et al, 1974; Clamp et al, 1981). It is thus possible that any host involvement in the increased production of the fluffy layer could be a result of xylitol or its metabolites causing an irritation or inflammation of the intestinal wall. Another possibility is that changes in the rat caecal microflora, induced by dietary xylitol (Chapters Two and Three), could contribute to the accumulation of any host-synthesised fluffy layer in the caecum. Evidence that is suggestive of this latter mechanism comes, firstly, from studies of germ-free rats which accumulate mucus in their caeca in the absence of bacterial enzymes that can degrade it (Spiro, 1969; Gordon and Wostmann, 1973). Indeed, the normal faecal microflora of conventional laboratory rats have the necessary bacterial enzymes to degrade the mucus present in the stools from germ-free rats (Hoskin and Zamchek, 1968). Secondly, there are suggestions that dietary constituents or drugs, which can alter the gastrointestinal microflora, can upset the normal bacterial degradation of host mucus with the consequent accumulation of mucus in the caecum (Wostmann et al, 1973).

On the other hand, the fluffy layer could be a slime or bacterial extracellular polysaccharide that is extruded by the metabolically active caecal microflora. It is possible that certain physiological and environmental conditions are favourable for the biosynthesis of bacterial extracellular polysaccharides and that such conditions may exist in the caecum of xylitol-fed rats. For instance, when large amounts of utilisable carbohydrates are present under situations where other substrates such as nitrogen, phosphorus or sulphur occur in relatively lower proportions to the carbon source, there is an increased production of bacterial extracellular polysaccharides (Sutherland, 1979). Since orally ingested xylitol is poorly absorbed through the intestinal wall (Asano et al, 1973), the relatively high concentrations of xylitol in the caecum of the xylitol-fed rat (Fig. 2.2) could favour the *in situ* production of bacterial extracellular polysaccharide or slime.

A role for bacteria in the synthesis of the fluffy layer was strongly suggested by the observation of a material, with similar centrifugation properties, in *in vitro* cultures of *K.pneumoniae* grown on xylitol (Chapter Three). Furthermore, the involvement of the host in the production of the fluffy layer was considered to be unlikely because histological sections of the intestinal wall of xylitol-fed rats failed to show any signs of tissue damage or inflammation (personal communication; Dr. J. McClure, Division

of Tissue Pathology, I.M.V.S.). On the basis of these findings, it was considered that the fluffy layer was most likely of bacterial origin rather than produced by the host cells.

It was recognised that samples of the fluffy layer from the caecum would be contaminated with dietary constituents as well as endogenously produced substances and that the methodology involved in the purification of macromolecules such as polysaccharides and glycoproteins would need to be employed. In this chapter the physico-chemical properties of the fluffy layer obtained from the caeca of rats fed xylitol are compared to the properties of the material obtained from the in vitro cultures of K.pneumoniae grown on xylitol. An animal feeding study was also conducted to determine whether the fluffy layer was related to the consumption of large quantities of carbohydrates in general, restricted to the consumption of sugar polyols in particular, or due to a more specific effect of xylitol. To complement this study, experiments with K.pneumoniae grown on various carbohydrate substrates in culture were also assessed for the bacterial production of fluffy layer.

MATERIALS AND METHODS

Preparation of Fluffy Layer

(i) From Caecal Contents of Rats

The fluffy layer was obtained from the pooled caecal contents of rats fed 10% (w/w) xylitol for a period of 7 days. The caecal contents were made up to 20% (w/v) with phosphate-buffered saline (see Chapter Two) and centrifuged at 500 x g for 5 min to remove food debris. The turbid supernatant was removed and centrifuged for a further 30 min at 15,000 x g. The clear amber-coloured supernatant that was obtained was carefully removed to avoid the resuspension of the fluffy layer that occurred above the firmly pelleted caecal contents (see Fig. 5.1). By this procedure 120 ml of fluffy layer was obtained from the caecal contents of 50 xylitol-fed rats. The fluffy layer was dialysed against water (3 x 20 l) for 48 h at 4^{o}C and then used for further analysis (see below).

(ii) From Cultures of K.pneumoniae

A pure culture of K.pneumoniae which had previously been isolated from the rat caecum was grown in ammonium sulphate medium (containing 50 mmol/l xylitol) at 37° for 48 h. The cultures were then centrifuged at 15,000 x g for 30 min at 4°C. The fluffy layer was separated from the firm bacterial pellet with a Pasteur pipette. By this procedure 200 ml of fluffy layer was obtained from one litre of growth medium. The samples were dialysed extensively as described above and stored at 4°C before analysis.

Comparative Carbohydrate Study

The effect of other carbohydrates such as glucose, fructose, sucrose, sorbitol and mannitol on the production of the fluffy layer in the caecum was assessed by feeding these carbohydrates (10% (w/w) of diet) to rats. Experiments using these carbohydrates as growth substrates in cultures of K.pneumoniae were also performed.

^{14}C-Labelling of the Fluffy Layer Produced by K.pneumoniae in Culture

Fluffy layer production by K.pneumoniae was maximal after 24 h of growth on xylitol. The incorporation of $|U\text{-}^{14}C|$ xylitol into the fluffy layer was achieved by the addition of the $|^{14}C|$-labelled substrate (50 mmol/l; 80,000 cpm/mmol) after cultures were grown for 24 h. The resulting $|^{14}C|$-labelled fluffy layer was washed and dialysed in the same manner as the unlabelled fluffy layers.

Alkaline Extraction of the Fluffy Layers

Samples of the fluffy layer obtained from the cacea of xylitol-fed rats and the $|^{14}C|$-labelled fluffy layer produced by cultures of K. pneumoniae were extracted with 1 mol/l sodium hydroxide for 5 min at room temperature (22-24°C). These samples were centrifuged and the resulting clear supernatant, containing the dissolved fluffy layer, was removed and dialysed against 1 mol/l sodium hydroxide for 24 h.

Aliquots containing 4 ml of the dialysed sodium hydroxide extracts of the fluffy layers were loaded onto a Sepharose CL-6B column (1,150 x 35) mm. Fractions (4 ml) were eluted with 1 mol/l sodium hydroxide at a rate of 1 ml/min. The fractions were monitored for total carbohydrates, proteins and, in the case of the $|^{14}C|$-labelled fluffy layer, for radioactivity (see below).

Isolation of the Polysaccharide Component of the Combined Fluffy Layer and Bacterial Pellet

An extensive purification of the polysaccharide components of both the fluffy layer and the bacterial pellet was performed using a combination of the methods of Thurow et al (1975) and Jann et al (1965). A schematic summary is given in Fig. 5.2. This procedure entailed the use of a 500 x g supernatant of 20% (w/v) caecal contents from xylitol-fed rats. Cultures of K.pneumoniae which were grown on xylitol and contained the fluffy layer were treated in a similar manner. Three 100 ml samples of these suspensions containing the fluffy layer and bacteria were incubated in 2% (w/v) phenol for 24 h at 37°C and were then precipitated with 900 ml of absolute ethanol. This precipitate was resuspended once in a solution of 86% (v/v) ethanol containing 14% (v/v) saturated sodium acetate solution. Each suspension was then centrifuged at 10,000 x g for 10 min and the precipitate suspended in 100 ml of water and extracted with an equal volume of 90% (w/v) phenol at 68°C for 10 min. The extracted mixtures were cooled to 10°C and centrifuged at 3,000 x g for 45 min. Three layers resulted on centrifugation. The upper, water phase contained the extracted polysaccharide, the middle phase consisted of cellular debris and the lower phase contained phenol. The water phase was removed and saved and the phenol phase was extracted twice more by the same procedure.

The pooled aqueous phases (150 ml) were dialysed against water (3 x 20 l) for 24 h. The dialysed samples were lyophilised and dissolved in water. This sample was ultracentrifuged twice at 110,000 x g for 4 h to remove cell-wall lipopolysaccharide. To this supernatant, Cetavolon (cetyltrimethylammonium bromide) and NaCl were added to a final concentrations of 2% (w/v) and 0.5 mol/l, respectively. This mixture was fractionally precipitated by the addition of water to give a final NaCl concentration of 60 mmol/l. This procedure removed nucleic acids by cetavolon precipitation. To obtain the sodium salt of the polysaccharide, the 60 mmol/l NaCl fraction was centrifuged and the pellet was twice dissolved in 1 mol/l NaCl and

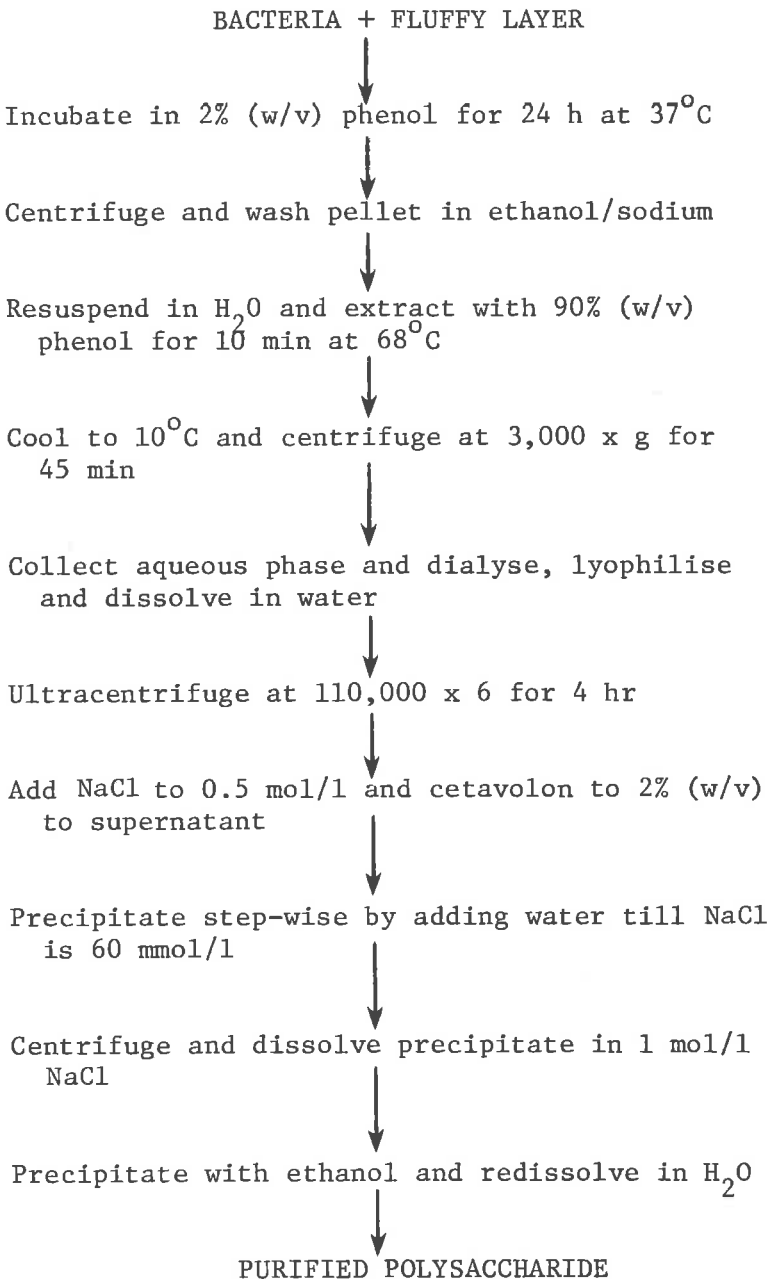


Fig. 5.2 A schematic summary of the method used in the isolation of the polysaccharide component from the fluffy layer.

The method is based on those of Jann et al, (1965) and Thurow et al, (1975).

precipitated with 80% (v/v) ethanol. This precipitate was dissolved in water (20 ml) and dialysed against water (3 x 2 l) before being lyophilised. The purified polysaccharide was subjected to fractionation on a Sepharose Cl-6B column (1,150 x 35 mm). The samples were eluted with 1 mol/l NaCl, at a flow rate of 1 ml/min. The 4 ml fractions were tested for total carbohydrate and counted for radioactivity. Lyophilised samples of the purified polysaccharide were also hydrolysed in 2 mol/l H_2SO_4 at 100°C for 6 h. The acid hydrolysates were neutralised with NaOH and chromatographed on both the Sepharose Cl-6B column and a Sephadex G-10 column (830 x 18 mm). One ml fractions were collected from the Sephadex G-10 column which was eluted with water at a flow rate of 0.56 ml/min. The column characteristics were determined by using the molecular weight markers, potassium ferricyanide and $|U\text{-}^{14}C|$ glucose in separate runs.

Chemical Analysis

Total carbohydrates were determined by the method of Dubois et al (1956). This method involves the reaction between the reducing groups of carbohydrates (e.g. simple sugars, oligosaccharides, polysaccharides and their derivatives, including the methyl ethers with free or potentially free reducing groups) with phenol and concentrated H_2SO_4 to give an orange-yellow colour. By use of this phenol-sulphuric acid reaction, microgram quantities of sugars and related substances were determined colourimetrically. The procedure used was as follows: one ml of the test solution was added to 0.025 ml of 80% (w/v) phenol and mixed rapidly with 2.5 ml of concentrated H_2SO_4. The reaction mixture was allowed to stand for 10 min at room temperature and then the absorbance was read at 490 nm. β-D-Glucose was used as a standard.

Protein estimations were performed by the method of Lowry et al (1951) using albumin as the standard.

Identification of Sugars

The monosaccharides in the acid hydrolysate of the purified polysaccharide were analysed as trimethylsilyl ether derivatives on a gas chromatograph linked to a mass spectrometer. The acid hydrolysates were neutralised with NaOH, dried under a stream of nitrogen and extracted with

pyridine. An aliquot of the pyridine extract containing approximately 1 to 2 mg of the hydrolysate was dried and derivatised using 0.5 ml of trimethylsilyl imidazole (TRISIL-Z, Pierce Chemical Co.). After the reaction had proceeded for 30 min at room temperature the excess reagent and pyridine solvent were evaporated in a stream of nitrogen with gentle warming. The sugar derivatives were dissolved in 0.5 ml of n-hexane for gas chromatography.

The column used for gas chromatography was 1.4 m long and packed with 3% OV-17 (Applied Science) stationary phase coated onto high performance Chromosorb -W (80-100 mesh) support medium (Johns Manville). A sample volume of 2 µl was injected into the entry port of the column. The carrier gas was 35 ml/mm helium and the gas chromatograph oven was temperature programmed from 120°C to 250°C at 8°C/min. The injection port temperature was 250°C. The effluent from the column was fed directly into a mass spectrometer interfaced via a membrane separator. The instrument was a Hewlett Packard 5980A quadrupole mass spectrometer with an interactive data system. Compounds sequentially eluted from the gas chromatograph were ionised and their spectra recorded and stored by the interactive data system for subsequent reduction and evaluation.

RESULTS

Characterisation of the Fluffy Layer

Attempts to remove the bacteria from the fluffy layer were made by differential centrifugation. In this procedure the fluffy layer was separated from the firm bacterial pellet at each centrifugation step and resuspended in phosphate-buffered saline and centrifuged again to remove more bacteria. The resuspended fluffy layer was assessed for total carbohydrate, protein and absorbance (A_{600}). It can be seen in Table 5.1 that as the absorbance (A_{600}) decreases (i.e. removal of bacteria), the carbohydrate/protein ratio increases. The fluffy layer obtained in this manner still contained bacteria and, when samples were analysed by Indian ink staining and light microscopy, encapsulated bacteria were observed (not shown). Further analysis with the use of ruthenium red staining (a stain for polysaccharides) and electronmicroscopy, indicated that extracellular

TABLE 5.1 The effect of removing bacteria from fluffy layer by differential centrifugation of the caecal contents of rats fed xylitol. Samples of fluffy layer obtained from the caecal contents of rats fed xylitol were centrifuged at 15,000 x g for 15 min at 4°C. At each centrifugation step the fluffy layer was separated from the bacterial pellet and resuspended in an equal volume of phosphate-buffered saline and centrifuged again under the same conditions. Bacterial density was measured as absorbance at 600 nm (A_{600}) and the carbohydrate and protein content are expressed as a ratio.

	A_{600}	Carbohydrate/protein ratio
1st centrifugation step		
Unwashed fluffy layer	2.1	0.25
2nd centrifugation step		
Fluffy layer (1st resuspension)	1.2	0.30
3rd centrifugation step		
Fluffy layer (2nd resuspension)	0.70	0.40
4th centrifugation step		
Fluffy layer (3rd resuspension)	0.50	0.70

polysaccharide-like material was associated with bacteria found in the fluffy layers obtained from both caecal contents of xylitol-fed rats and cultures of K.pneumoniae grown on xylitol (Plates 5.1 and 5.2).

The Effect of Carbohydrate Substrates on the Production of the Fluffy Layer

The production of fluffy layer in the caecal contents of rats fed various sugars and sugar polyols and in cultures of K.pneumoniae grown in the presence of the same carbohydrates was investigated. In both caecal contents and cultures, the fluffy layer was produced only in the presence of the sugar polyols (Table 5.2). Of the polyols, mannitol was associated with the highest amount of fluffy layer. On the basis of the growth culture experiments (Table 5.2), it was concluded that K.pneumoniae, among the isolates tested, was the organism producing the fluffy layer within the caecal contents.

Alkaline Extract of the Fluffy Layer

The insolubility of the fluffy layer, which is presumably due to its links with the bacterial cell wall (Plates 5.1 and 5.2), makes characterisation of the fluffy layer by chromatography difficult. In a preliminary experiment where NaOH was added to the crude fluffy layer and the solution centrifuged, it was found that the fluffy layer was no longer visible and only a firm bacterial pellet remained. The solubility of the fluffy layer in sodium hydroxide allowed it to be chromatographed on Sepharose CL-6B which is resistant to NaOH. Since the 1 mol/l NaOH fraction had the highest carbohydrate/protein ratio (Table 5.3) and also contained the dissolved fluffy layer, it was used for the Sepharose CL-6B chromatography. The elution profile of the $|^{14}C|$-labelled fluffy layer of K.pneumoniae shows three major peaks of radioactivity (Fig. 5.3). Peak I has the highest incorporation of $|^{14}C|$-label into carbohydrate and would therefore appear to represent the polysaccharide component of the fluffy layer. Peak II, which contains highly labelled protein, indicates that non-specific labelling of macromolecules cannot be avoided. Peak III, which has the highest protein content, may reflect the non-specific extraction of the bacterial protein by NaOH. Fractions of the NaOH-extracted fluffy layer obtained from the caecal contents were estimated for total carbohydrate. The similarity of the carbohydrate profile of the polysaccharide from caecal

Plate 5.1 Electronmicrograph of a bacterium in the fluffy layer obtained from the caecal contents of rats previously fed 20% (w/v) xylitol for 7 days. The bacterium shows extrusion of polysaccharide (magnification x 90,000).

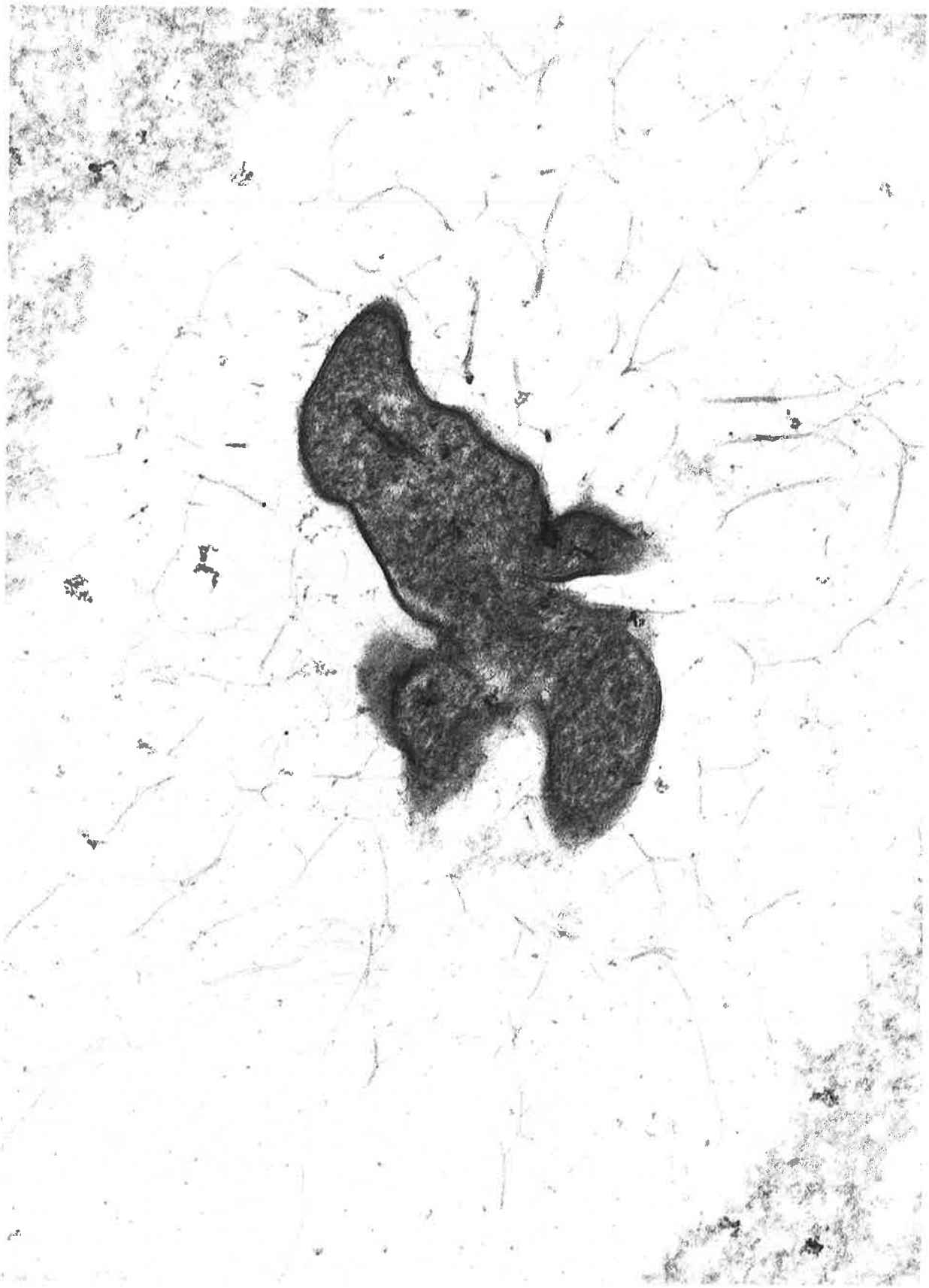


Plate 5.2 Electronmicrograph of a K.pneumoniae bacterium grown on xylitol in culture showing extrusion of polysaccharide. (Magnification x 90,000).

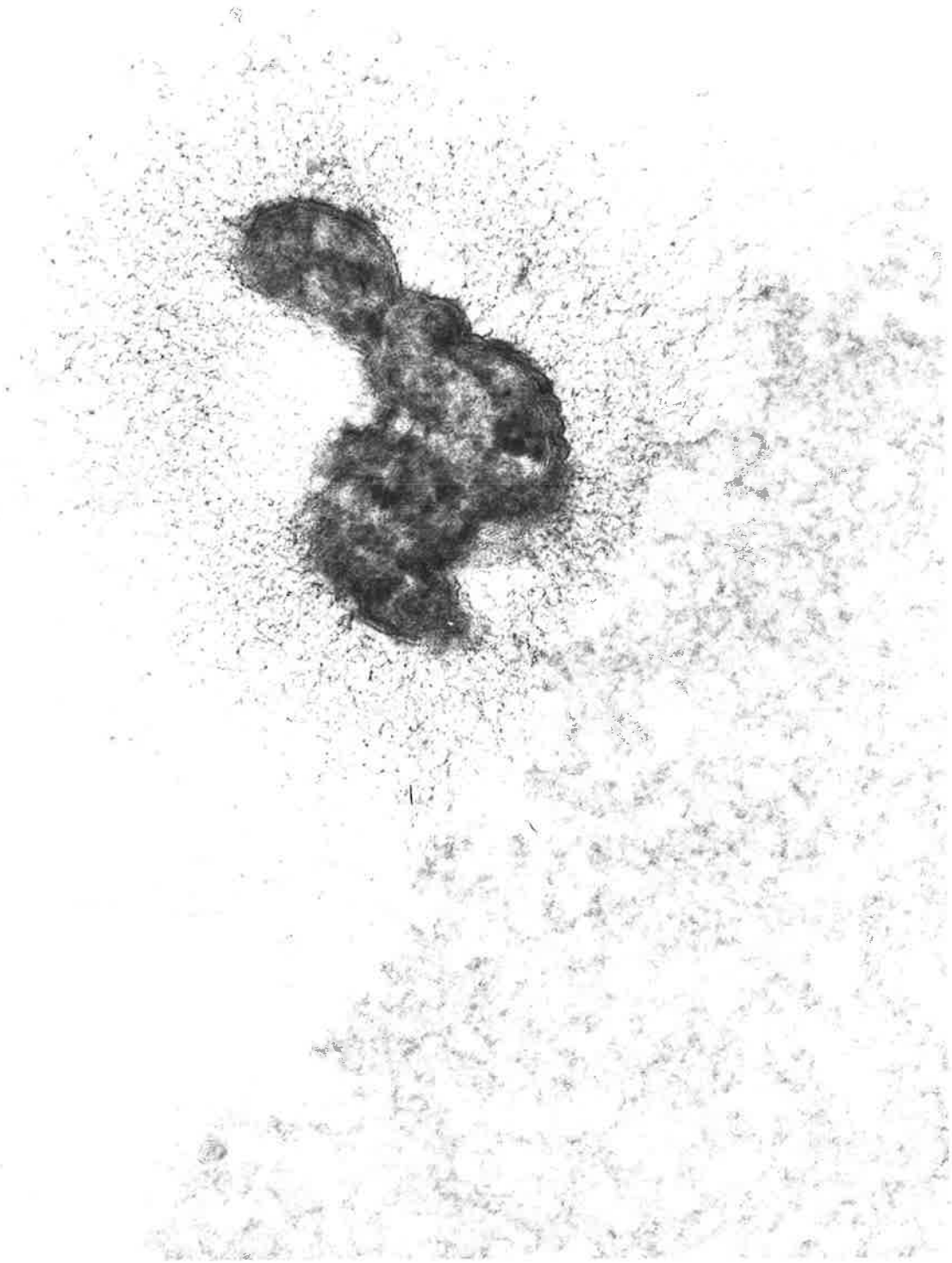


TABLE 5.2 The effect of sugars and sugar polyols on the production of fluffy layer in the caecal contents of rats and in cultures of K.pneumoniae

Carbohydrate (Diet/Growth medium)	Caecal Contents* (ml of fluffy layer/ 20% (w/v) caecal contents)	K.pneumoniae** (ml of fluffy layer/ ml of growth medium
No addition	0	0
Sucrose	0	0
Glucose	0	0
Fructose	0	0
Xylitol	0.08	0.20
Sorbitol	0.08	0.20
Mannitol	0.16	0.38

* Caecal contents were made up to 20% (w/v) in phosphate buffered saline and centrifuged at 15,000 x g for 15 min at 4°C. The fluffy layer that appeared on centrifugation was removed and its volume measured in a graduated cylinder. The volume of fluffy layer produced is expressed as a proportion of the total volume of a 20% (w/v) suspension of caecal contents.

** The fluffy layer produced in culture after 48 h of growth on xylitol was removed after centrifugation and volume measured. The volume of fluffy layer is expressed as a proportion of the total volume of growth medium.

TABLE 5.3 The alkaline extraction of the fluffy layer obtained from the caecal contents of xylitol-fed rats. The fluffy layer was prepared as described in Material and Methods section. The extraction of the fluffy layer was performed with 1mol/l NaOH. This was followed by a 4mol/l NaOH extraction of the insoluble pellet. The carbohydrate and protein estimations were performed on the fractions obtained. Results are expressed as a mean of 3 determinations.

Fraction	Protein (μg/ml)	Carbohydrate (μg/ml)	Carbohydrate protein ratio
(a) Fluffy layer	1,123	225	0.20
(b) 1 mol/l NaOH-soluble supernatant	109	53	0.48
(c) 4 mol/l NaOH-soluble supernatant	1,142	84	0.07
(d) 4 mol/l NaOH-insoluble pellet	171	38	0.22

contents and the $|^{14}C|$-profile of the NaOH-extracted fluffy layer from K.pneumoniae (Fig. 5.4), suggests that both NaOH extracts represent the same macromolecules (i.e. the polysaccharide component of the fluffy layer).

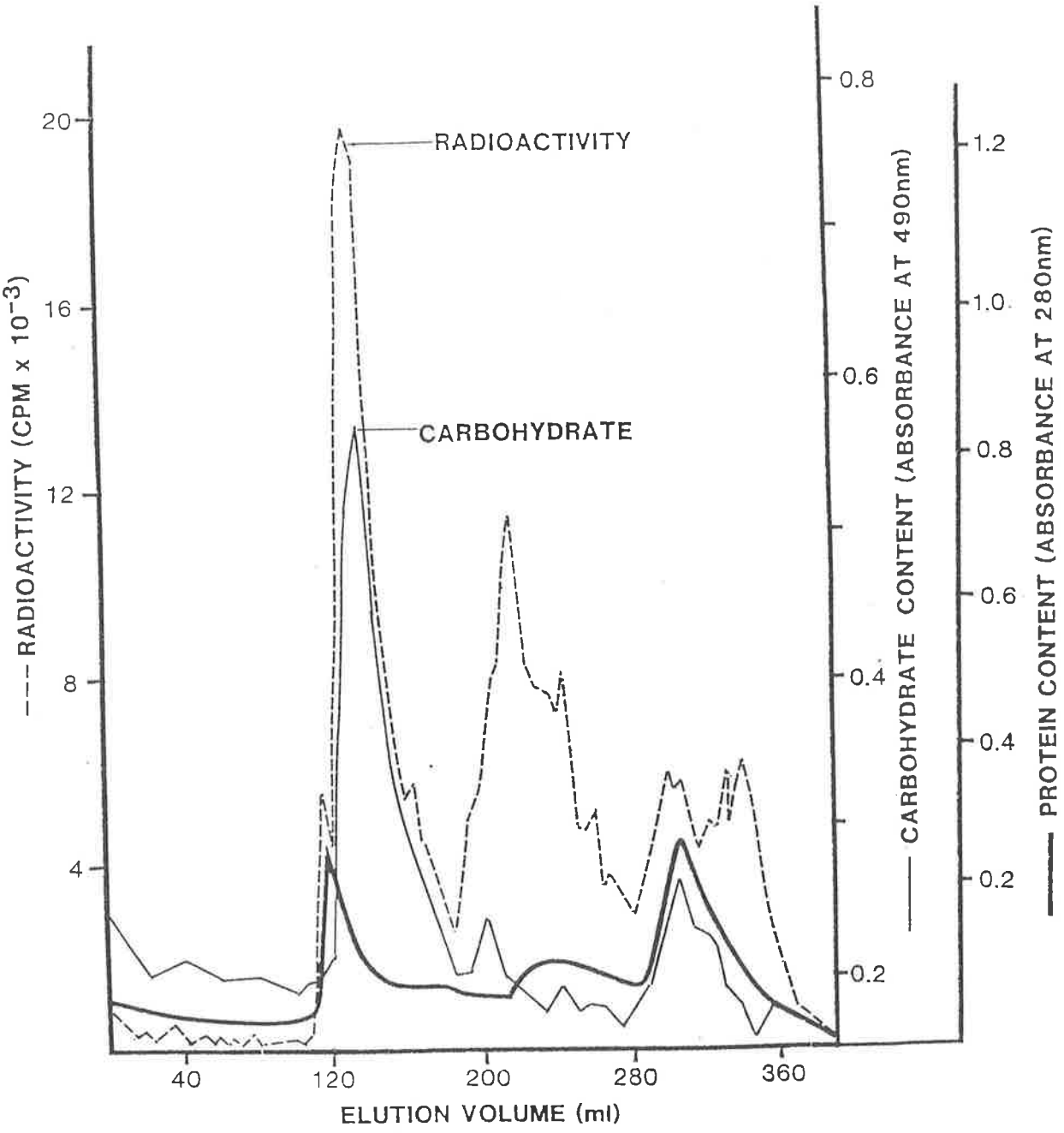
Purification of the Polysaccharide Component of the Fluffy Layer

The extracellular polysaccharide of the bacteria was isolated by a combination of the methods of Jann et al (1965) and Thurow et al (1975). The yield of polysaccharide based on radioactivity was 3.25% of the labelled bacterial cells and fluffy layer (Table 5.4). The purified polysaccharides from the fluffy layers obtained from both the caecal contents of rats fed xylitol appear to elute in the same volumes on the Sepharose CL-6B column (Fig. 5.5). Compared to the elution volume of blue dextran, the molecular weight of the purified polysaccharides was greater than 10^6 daltons. The acid hydrolysates of the purified polysaccharides had a molecular weight of about 180 daltons based on the elution volumes of potassium ferricyanide and $|U\text{-}^{14}C|$ glucose which were used as standards (Fig. 5.6).

Identification of Neutral Sugars in Acid Hydrolysates of Polysaccharides

The neutral sugars in the polysaccharide hydrolysates were identified by comparing their gas chromatogram retention times and their mass spectra with standards run under the same gas chromatogram and mass spectral condition. Glucose was the predominant hexose identified in both the hydrolysates of the polysaccharide components of the fluffy layers (Fig. 5.7). Monosaccharide identifications were based on known retention times. The two glucose anomers, which were present in different relative proportions in each sample, had retention times of 2.7 and 3.2 min.

No sugar alcohols were identified in the polysaccharide hydrolysates of the caecal contents and cultures of K.pneumoniae grown on xylitol. The pentitols and hexitols (TMS derivatives) are characterised by a significantly strong fragment ion at m/2 319. No gas chromatogram/mass spectrogram peaks showed this ion to be present. Furthermore, hexitols produce a fragmentation ion at m/2 421 but no evidence of this was seen in any of the gas chromatogram/mass spectrogram peaks. Peaks eluting earlier in the chromatograms (retention times between 1.3 and 1.5 min) are characteristic of pentoses.



Fig, 5.3 Chromatogram of alkaline extracted $|^{14}C|$-fluffy layer from cultures of K.pneumoniae grown on $|U\text{-}^{14}C|$ xylitol.

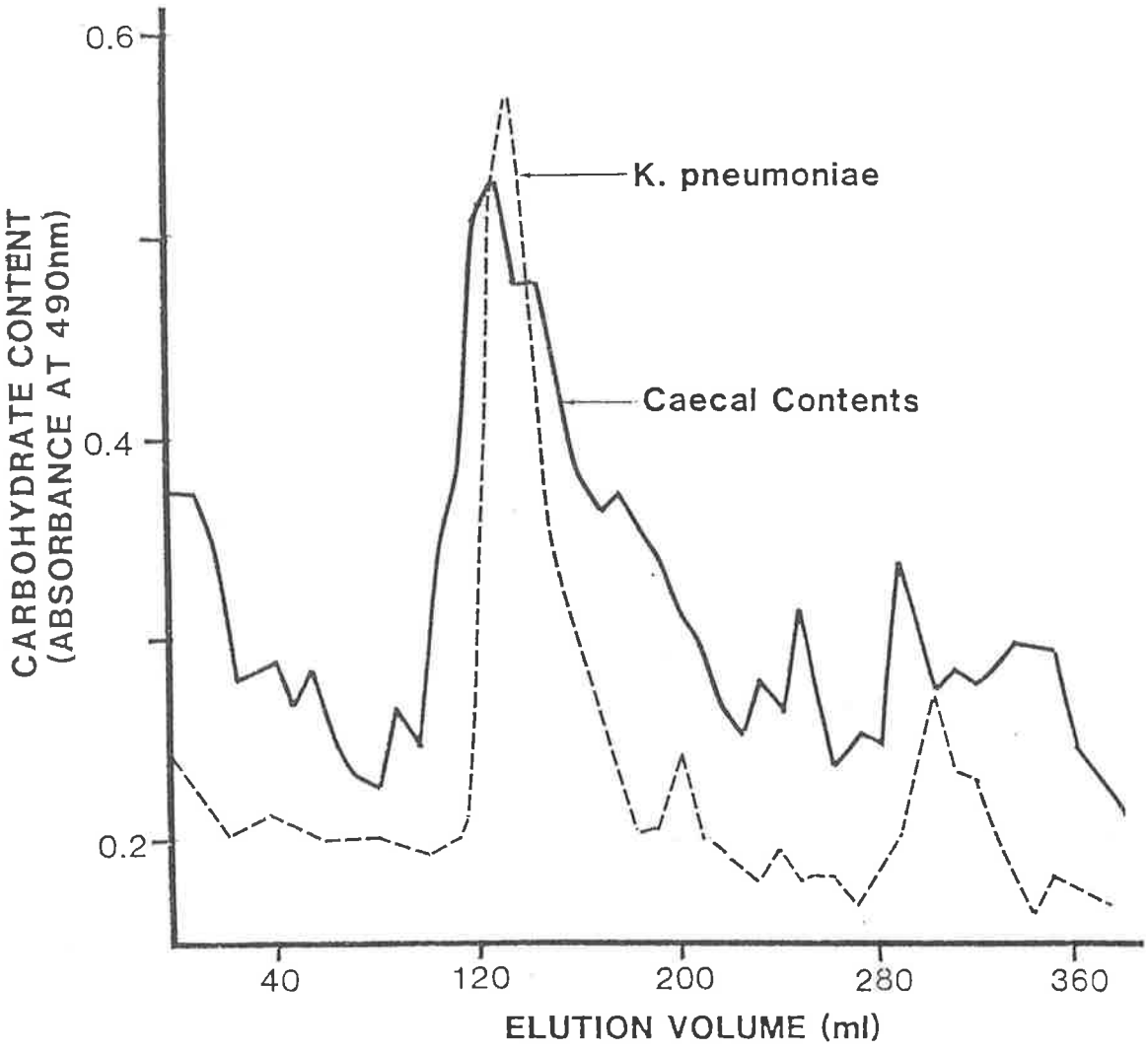


Fig. 5.4 A comparison of the elution profiles of alkaline extracted fluffy layers from the caecal contents of xylitol-fed rats and cultures of K.pneumoniae grown on xylitol.

TABLE 5.4 Purification of the polysaccharide component of $|^{14}C|$-labelled fluffy layer and bacteria from cultures of K.pneumoniae grown on xylitol. Further details are given in Fig. 5.2.

	Total cpm
(a) $|^{14}C|$-labelled bacteria and fluffy layer	4.0×10^6
(b) Phenol extraction (aqueous phase)	0.9×10^6
(c) Cetavolon and NaCl precipitation (supernatant)	0.19×10^6
(d) Soluble ethanol precipitate (purified polysaccharide)	0.13×10^6

$$\% \text{ yield of polysaccharide} = \frac{d}{a} \times \frac{100}{1} = \frac{0.03 \times 10^6}{4.0 \times 10^6} \times \frac{100}{1}$$

$$= 3.25\%$$

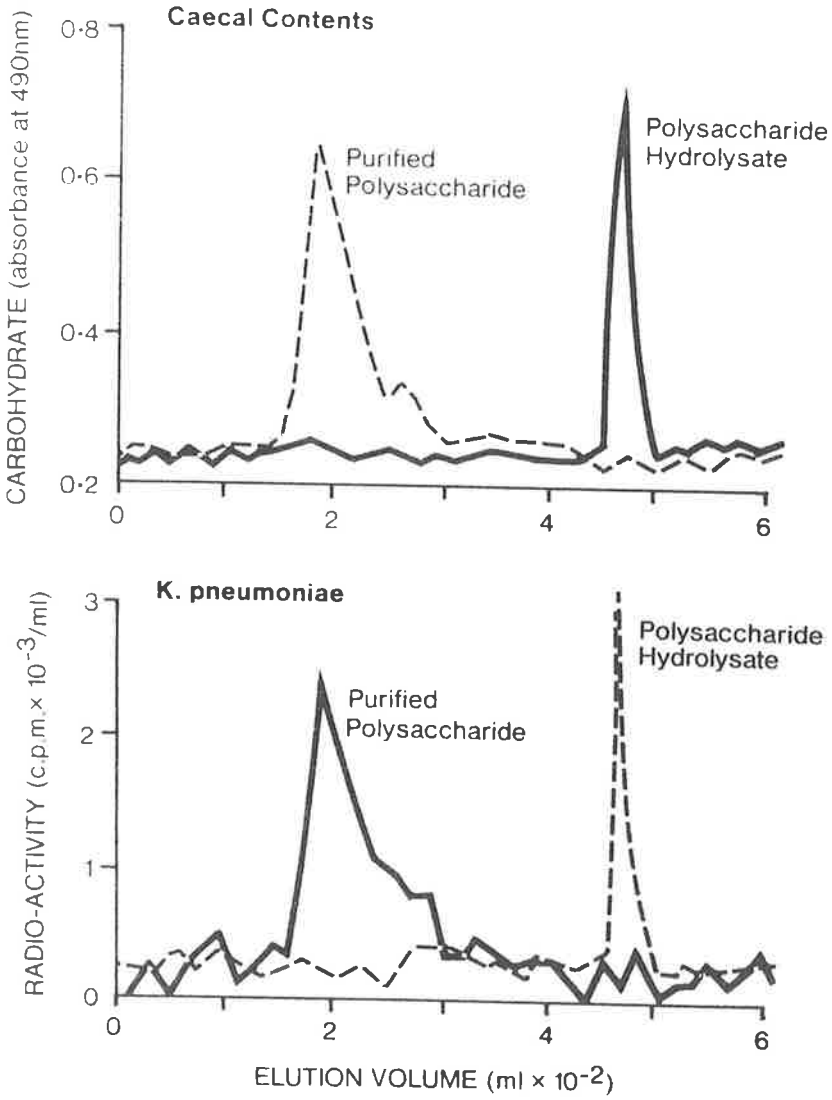


Fig. 5.5 Sepharose CL-6B chromatograms of the purified polysaccharide component and its hydrolysate from fluffy layers obtained from caecal contents and K.pneumoniae cultures.

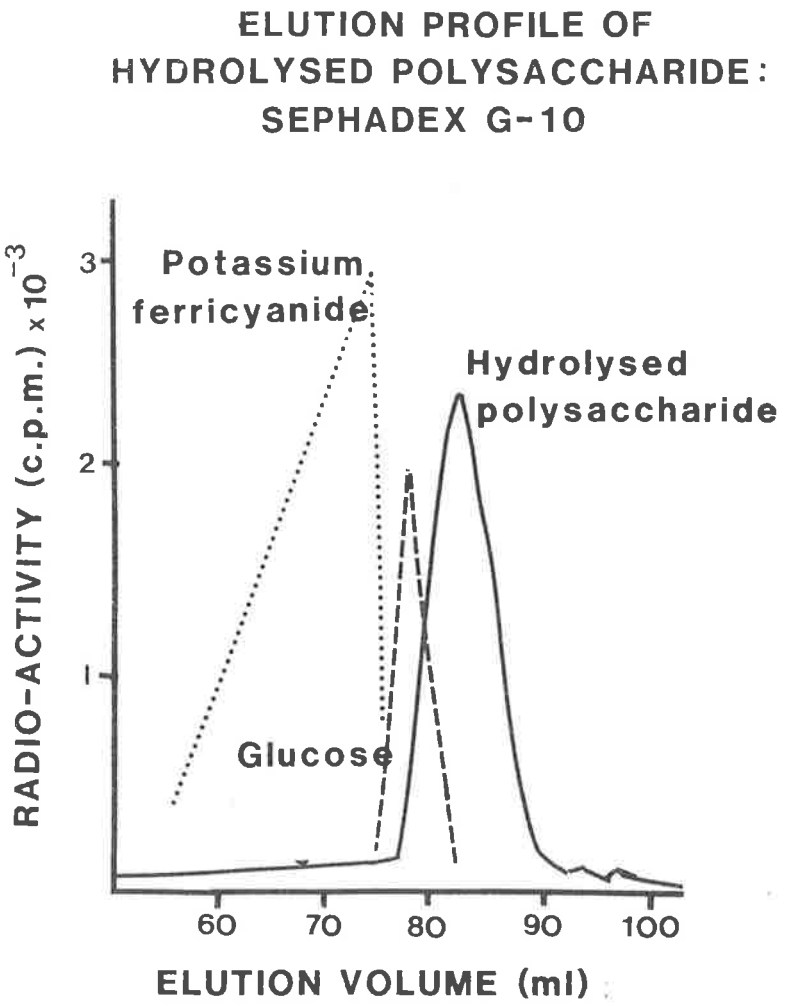


Fig. 5.6 Sephadex G-10 chromatograms of polysaccharide hydrolysate from caecal contents, glucose and potassium ferricyanide.

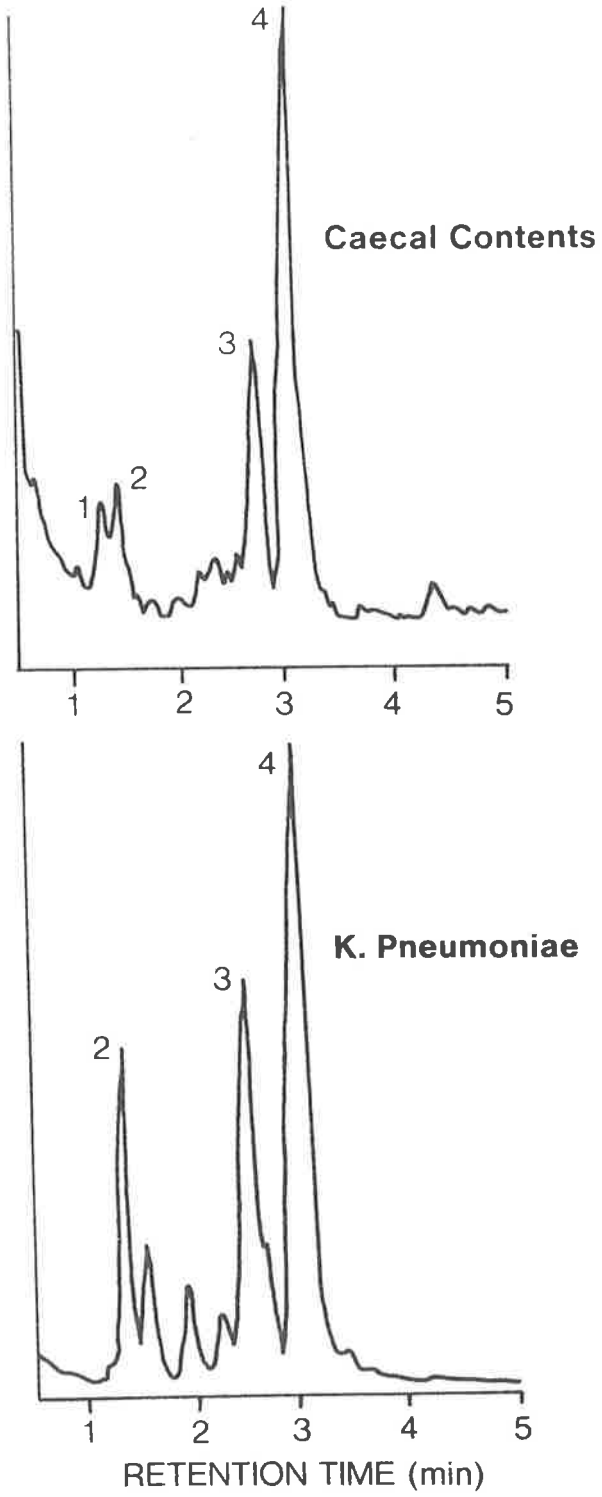


Fig. 5.7 Gas chromatogram elution profile of trimethylsilyl-derivatives of neutral sugars obtained from the hydrolysates of polysaccharides from caecal contents and K.pneumoniae. Peaks have been tentatively identified as (1) D-xylose; (2) D-ribose; (3) α-D-glucose; and (4) β-D-glucose.

The pentoses, xylose and ribose have been tentatively identified in the polysaccharide hydrolysates of both the caecal contents and the cultures of K.pneumoniae by comparisons with authentic sugars.

DISCUSSION

In Chapter Two dietary xylitol was shown to cause alterations in both the bacterial populations and the metabolic activity of the caecal microflora of rats. Despite these changes, however, it was not clear whether the fluffy layer, observed in the caecal contents, was synthesised by the microflora or by the gastrointestinal tract of the host. The experiments reported in this Chapter strongly support the conclusion that the fluffy layer results from dietary xylitol causing the caecal bacterial flora to produce excess extracellular polysaccharide rather than the gastrointestinal tract producing excess mucus.

Additional evidence that the fluffy layer is bacterial in origin came from the observation of its production in cultures of K.pneumoniae; a xylitol-metabolising organism isolated from the rat caecum (Chapter Three). This organism produced the fluffy layer when grown in batch cultures with xylitol as a growth substrate. In a comparative study of various carbohydrate substrates only the sugar polyols, when added either to the diets of rats or to growth medium in cultures containing K.pneumoniae, caused the production of fluffy layer in the caeca and in the culture (Table 5.2). Furthermore, the electronmicroscopy of the fluffy layers taken from the caeca and bacteria cultures which were prepared with ruthenium red, a polysaccharide stain, showed extrusions of polysaccharide on the bacteria (Plates 5.1 and 5.2). Since it was not possible to separate the fluffy layer from bacteria by millipore filtration using 0.22 and 0.45 μm filters (personal observation), the fluffy layer may be defined as an extracellular polysaccharide strongly adherent to the bacterial capsule (Roth, 1977; Politis and Goodman, 1980). That is, the insolubility of the fluffy layer is due to the adherence of the polysaccharide to the bacterial cell wall.

Further characterisation of the fluffy layers was achieved because it was possible to label the fluffy layer produced by K.pneumoniae by growing it in the presence of $|U\text{-}^{14}C|$ xylitol. This enabled further comparisons

to be made between the fluffy layers obtained from the caecum and culture. Extractions of the polysaccharide component of the fluffy layers with sodium hydroxide (Table 5.3 and Figs. 5.2 and 5.3) and with phenol (Table 5.4 and Fig. 5.5) followed by column chromatography on Sepharose CL-6B showed that the polysaccharide component of the fluffy layers from both the caeca and the bacterial culture had similar elution volumes and a molecular weight of the order of 10^6 daltons. This observation is consistent with the molecular weights of insoluble polysaccharides produced by Streptococcus mutans (Inoue and Koga, 1979).

The role of the fluffy layer in the adaptation involved in xylitol-induced diarrhoea has not been directly investigated. However, it may be implied that the xylitol-induced synthesis of macromolecules, such as extracellular polysaccharides would of necessity lead to a reduction in the osmotic load of xylitol in the caecum. Furthermore, Roth et al (1977) have demonstrated that bacterial extracellular polysaccharides are able to 'mop up' (i.e. bind) substrates in the medium. The increased production of the fluffy layer in the caecum could thus further reduce the osmotic load.

In conclusion, the presence of the fluffy layer in the caecum of xylitol-fed rats and in cultures of K.pneumoniae grown on xylitol further confirms that caecal bacteria can metabolise xylitol not only for energy (Chapters Two and Four), but also to produce complex macromolecules.

CHAPTER SIX

CONCLUDING REMARKS

While a variety of side-effects and toxic complications have been associated with the enteral and parenteral use of xylitol, the only universal side-effect of moderate, oral doses of xylitol is osmotic diarrhoea (Chapter One). The intestine lacks an active or facilitated transport system for xylitol (Förster, 1978) and, consequently, if the ability of the small intestine to absorb xylitol is exceeded then substantial quantities of xylitol reach the caecum and large intestine to produce an osmotic load and diarrhoea ensues (Fig. 2.2). The subsequent adaptation (i.e. cessation of diarrhoea), which has been reported in animals and humans fed xylitol continuously (see Chapter One), was confirmed in the dietary experiments described in Chapter Two. The nature of the adaptation to dietary xylitol was investigated by studying the biochemical changes in the gut lumen, gut wall and liver using the rat as an experimental model (Chapter Two).

The liver is considered to be the major organ for xylitol metabolism (Lang, 1969) but both enzymic and metabolic studies failed to show any evidence of adaptative changes with dietary xylitol or, indeed, with a range of sugars and sugar polyols at concentrations of 10 and 20% (w/w) in the diet (Tables 2.1, 2.2 and 2.3). In general, where other workers have demonstrated induction of a variety of liver enzymes by carbohydrates, the dietary concentrations of those carbohydrates have been greater than 50% (w/w) (Rudack et al, 1971; Romsos and Leveille, 1974). The observations of Barngrover et al, (1983) support the results in this thesis in that dietary xylitol did not affect the activities of liver xylitol dehydrogenase. These studies, however, do not agree with the findings of Fujisawa (1969) or Hosoya and Iiotoyo (1969). Fujisawa (1969) demonstrated changes in the liver activities of the enzymes in the pentose phosphate, glucuronate-xylulose and the gluconeogenic pathways within a few days of the rats commencing a diet containing 10% (w/w) xylitol.

Hosoya and Iiotoyo (1969) showed that, in rats, when the concentration of dietary xylitol was gradually increased from 5% to 20% (w/w) over 4 weeks there was a doubling of the activity of the liver, NAD-linked, cytoplasmic, xylitol dehydrogenase. The discrepancies between these studies and the one reported in this thesis could, however, be accounted for by differences in the dietary regimens. That is, if animals are exposed to increasing amounts of dietary xylitol gradually, rather than to large amounts from the beginning, then it is possible that intestinal absorption and hence hepatic concentrations of xylitol in such animals are greater. On the other hand, animals fed xylitol in high concentrations from the beginning of the diet appear to have a greater incidence of diarrhoea (Table 2.1). It might therefore be expected that these animals would have lower plasma concentrations and hence lower liver concentrations of xylitol compared to animals given gradually increasing amounts of dietary xylitol. Under these conditions, it might be expected that induction of xylitol-metabolising enzymes in the liver would not be obvious.

The possibility that the gut wall is a site for host adaptation to dietary xylitol was also investigated. The experiments reported in Chapter Two confirmed that there was no active transport system for xylitol (Bässler, 1969; Förster, 1978), since only a narrow range of low plasma xylitol concentrations were observed in rats fed xylitol (Table 2.3) despite there being a wide range of gut intraluminal xylitol concentrations (Fig. 2.2). The absence of any histological changes in the caecal wall of xylitol-fed rats, compared to normal-fed rats (Chapters Two and Five) further supports the lack of any adaptation by the gut wall in response to dietary xylitol. These studies provide only indirect evidence for a lack of gut wall adaptation, however, because the gut wall may contain xylitol-metabolising enzymes (Müller, 1967) which could be inducible.

The most convincing evidence for an adaptative response to dietary xylitol was observed within the caecal lumen of rats. These observations included the presence of increased amounts of gas, a decrease in the pH of caecal contents, formation of a water-insoluble, polysaccharide like-material (fluffy layer) and reductions in xylitol concentration (Table 2.4 and Fig. 2.2). In addition, it was observed that the caecal contents of xylitol-fed rats showed an increase in the bacterial synthesis of thiamin and thiamin pyrophosphate (see Chapter Two: Appendix). It was also demonstrated that the xylitol-metabolising activity of the caecal microflora

had increased by 10-fold or more within a few days of rats receiving low concentrations of dietary xylitol and up to 40-fold with high doses (Fig. 2.5). Moreover, changes in the population of the caecal microflora of xylitol-fed rats were also noted with, in particular, increases in gram positive organisms (Fig. 2.3). There are few published studies of the effect of dietary xylitol on the normal gut microflora, namely, the work of Wekell, Hartman and Dong (1980) on the gastrointestinal contents of rats and the work of Dubach et al, (1973) with human faeces. These authors provided evidence which supports the findings in this thesis in that dietary xylitol in short-term feeding trials can cause changes in the population of certain species of bacteria present in the normal gut microflora. In summary, the increased tolerance of the host to dietary xylitol in the short-term feeding studies, as reported in Chapter Two, can, in general, be attributed to those changes that have occurred within the gut lumen rather than any adaptative change in the liver or gut wall. In particular, the increased xylitol-metabolising activity of the caecal microflora (Fig. 2.5) is responsible for the rapid decrease in xylitol concentration in the caecum and the subsequent cessation of xylitol-induced diarrhoea (Fig. 2.2).

The change seen in the caecal microflora could be due to a selection process in which the presence of xylitol in the caecum leads to an increase in xylitol-metabolising bacteria. Alternatively, there could be an induction of xylitol-metabolising enzymes in the caecal bacteria exposed to xylitol. Another possibility is that there is a combination of both bacterial selection and enzyme induction (Chapter Three; Birkhed et al, 1978). The role of these mechanisms in the adaptation process was investigated by first isolating caecal bacteria capable of growth on xylitol-containing media (Table 3.1) and then by screening for increased xylitol-metabolising activity in those bacteria (Tables 3.2 and 3.3). While quantitation of bacteria from the caecum was restricted to those that grew aerobically on minimal medium containing xylitol (Table 3.4), the adaptative changes observed could be attributed, at least in part, to the increased xylitol-metabolising activity of K.pneumoniea, S.liquefaciens and Micrococcus sp. (Tables 3.2 and 3.3). K.pneumoniae and Micrococcus sp. showed only an enhancement of their xylitol-metabolising activity but S.liquefaciens showed an increase in its population as well (Chapter Three). It has been shown by other workers that the metabolic activity of some bacteria can change in relation to the amount and type of substrate(s) present in the caecum without there necessarily being any concomitant change

in viable bacterial numbers (Watanabe, Kawai and Mutai, 1980; Brockett and Tannock, 1982). That is, bacteria possess enzymes which can be induced or repressed by changes in the organisms environment and, especially, the nutrient energy source. In this study such an example is the enhancement of the xylitol-metabolising activity in K.pneumoniae observed in xylitol-ammonium sulphate medium (Table 3.2) but not seen in xylitol-yeast extract medium (Table 3.3). Such alterations in enzyme activity cannot often be detected by methods of enumeration of bacteria even with selective media (Watanabe, Kawai and Mutai, 1980).

In Chapter Four, the activities of key enzymes involved in xylitol metabolism in the caecal isolates, K.pneumoniae and S.liquefaciens, are reported. With both K.pneumoniae and S.liquefaciens, xylitol was the only sugar polyol tested as a growth substrate that could enhance the metabolism of |U-^{14}C| xylitol to $^{14}CO_2$ (Table 4.1). Furthermore, xylitol transport (Figs. 4.2 and 4.3), xylitol dehydrogenase (Tables 4.3 and 4.4) and xylulokinase (Table 4.5) activities were all induced in both of these bacteria when grown on xylitol. The induction of all three enzyme activities was essential for the enhanced production of $^{14}CO_2$ from |U-^{14}C| xylitol since bacterial growth on other substrates such as ribitol, D-arabitol or sorbitol did not demonstrate the induction of these three enzymes or the enhancement of xylitol metabolism (Tables 4.1, 4.2, 4.3, 4.4 and 4.5). Therefore the increased carbon-flux of xylitol to CO_2, through the common and major carbohydrate pathways, can be attributed to the induction of the key enzymes involved in xylitol metabolism.

In Chapter Five the presence of the fluffy layer, an insoluble polysaccharide-like material in the caecal contents of xylitol-fed rats was investigated. Despite the alterations in both the bacterial populations and the metabolic activity of the caecal microflora caused by dietary xylitol, it was not clear whether the fluffy layer was synthesised by the microflora or by the gastrointestinal tract of the host. Conclusive evidence that the fluffy layer is a bacterial product was shown by the production of the fluffy layer in cultures of K.pneumoniae isolated from the caeca of rats and grown on xylitol (Chapter Five). When the fluffy layers from the caecal content and K.pneumoniae culture were examined by electronmicroscopy with a polysaccharide stain (ruthenium red) it was shown that the polysaccharide was attached to bacteria (Plates 5.1 and 5.2). Further examination and comparison of the fluffy layers was made possible by radio-labelling the fluffy layer produced in culture. The polysaccharide

component of the fluffy layers from both sources were shown to be similar when chromatographed on Sepharose CL-6B after extractions with sodium hydroxide (Figs. 5.3 and 5.4) and phenol (Fig. 5.5). The gas chromatographic/mass spectrographic analysis showed that the polysaccharide components contained glucose, xylose and ribose residues (Fig. 5.7) and, therefore, illustrates how xylitol can be metabolised to other sugar residues and stored in the form of polysaccharides.

FUTURE EXPERIMENTS

Long-term Dietary Studies

In this thesis, only the effect of feeding xylitol to rats for periods up to two weeks was studied. While it was shown that the caecal microflora play an important role in the adaptation to xylitol-induced diarrhoea in rats in the short-term, it is not known how consistent or persistent these changes would be in rats fed xylitol over longer periods of time. It is still important to determine if host adaptative (i.e. liver and gut wall) mechanisms become important in rats (and, for that matter, humans) that have been exposed to long-term dietary ingestion of xylitol (e.g. 1 year). In order to examine these possible, longer term changes in the host and the gut microflora the following experiments are warranted:-

(i) A systematic study should be carried out to measure the activities of the pentose phosphate, glucuronate-xylulose and gluconeogenic pathway enzymes in liver and gut wall homogenates of both starch-fed and xylitol-fed rats. In addition, any increased ability of the liver and gut wall to metabolise $|U\text{-}^{14}C|$ xylitol to $^{14}CO_2$ and other metabolites, after exposure to dietary xylitol, may be pursued with the use of liver homogenates (Chapter Two), isolated rat hepatocytes (Rofe et al, 1977), gut mucosal homogenates and isolated enterocytes (Watford, Lund and Krebs, 1979).

(ii) While examination of the caecal wall by light microscopy showed no obvious changes after dietary xylitol it would still be important to determine if any ultrastructural changes occur in the gut wall

after prolonged exposure to xylitol (e.g. electronmicroscopy).

(iii) While invasive techniques such as caecectomy have been used to obtain bacteria for examination of their ability to metabolise xylitol, the use of non-invasive techniques, such as determination of faecal bacteria and metabolites and the measurement of hydrogen respired by intact animals (Schell-Dompert and Siebert, 1980) after dietary xylitol, would be an important aspect of examining gut bacterial changes in humans and animals following long-term xylitol intake.

Anaerobic Bacterial Metabolism of Xylitol

In the studies reported in this thesis, the use of aerobic culture conditions may have led to an underestimation of the xylitol-metabolising activity of the caecal microflora (Chapter Two) and of the numbers of species of xylitol-metabolising bacteria (Chapter Three). Furthermore, the products of the anaerobic metabolism of xylitol are more likely to be volatile-fatty acids (Poutiainen, Touri and Sirvio, 1976), which can be easily absorbed by the host, and not CO_2 as measured in the in vitro assays in Chapter Two. Consequently, it would be important to estimate whether the caecal microflora can metabolise xylitol under anaerobic conditions and also to attempt to isolate from the caecum such bacteria capable of metabolising xylitol anaerobically.

Continuous Culture Experiments with Caecal Contents

The batch culture growth experiments reported in this thesis represent a closed system, where substrates are consumed and metabolites accumulate with the result that the cellular environment is changing continuously and the cells, themselves, pass through a series of transient physiological states. The cellular environment is difficult to control and generally is far removed from the natural environment. On the other hand, in an open system (or continuous culture system) there is a balanced, continuous input of nutrients and output of metabolites and cells. Under these conditions steady-state growth can be established in which neither the cell population density nor the cell environment changes with time (Bull, 1974). The large intestines and the caecum among several other microbial

ecosystems can be characterised as open and dynamic, and analogous to continuous culture systems (Wolin, 1974). The use of a chemostat could thus provide more natural environments than batch culture for the study of bacterial metabolism. This would enable *in vitro* studies on the caecal microflora obtained from either starch-fed or xylitol-fed rats to be conducted under more controlled conditions.

Evidence for steady-state growth of xylitol-metabolising bacteria in the caecum of xylitol-fed rats can be seen in the data presented in Figs. 2.2 and 2.5 and represented schematically in Fig. 6.1. With the use of a chemostat, samples of caecal contents could be used as inocula and grown in continuous cultures. The effect of xylitol on the steady-state bacterial population of the caecum could then be mimicked under both aerobic and anaerobic conditions in the chemostat. Such an approach would lead to a clearer understanding of the mechanisms (i.e. population and enzymatic changes) that are responsible for the increased xylitol-metabolising activity of the caecal microflora.

Effect of Antibiotics on Fluffy Layer Production

Whilst the evidence in Chapter Five strongly suggests that the fluffy layer is of bacterial origin rather than host secretion, a more conclusive experiment is warranted. Since it is known that *K.pneumoniae* produces fluffy layer in culture, it would be of interest to see if rats, which are fed xylitol and also treated with oral antibiotics against *K.pneumoniae*, are still capable of producing the fluffy layer in their caeca.

CONCLUSION

It has been clearly established in this thesis that caecal microfloral metabolism plays a major role in the recovery of the host animal to xylitol-induced diarrhoea, at least in short-term feeding studies. Studies of this nature will eventually provide more information on the use of xylitol as a nutritive sweetener in both health and disease in humans. For example, a therapeutic use which has been suggested for polyols in gastrointestinal diseases such as diverticulitis and constipation (Burkitt, 1971) can also be suggested for xylitol, since gas and acid produced by

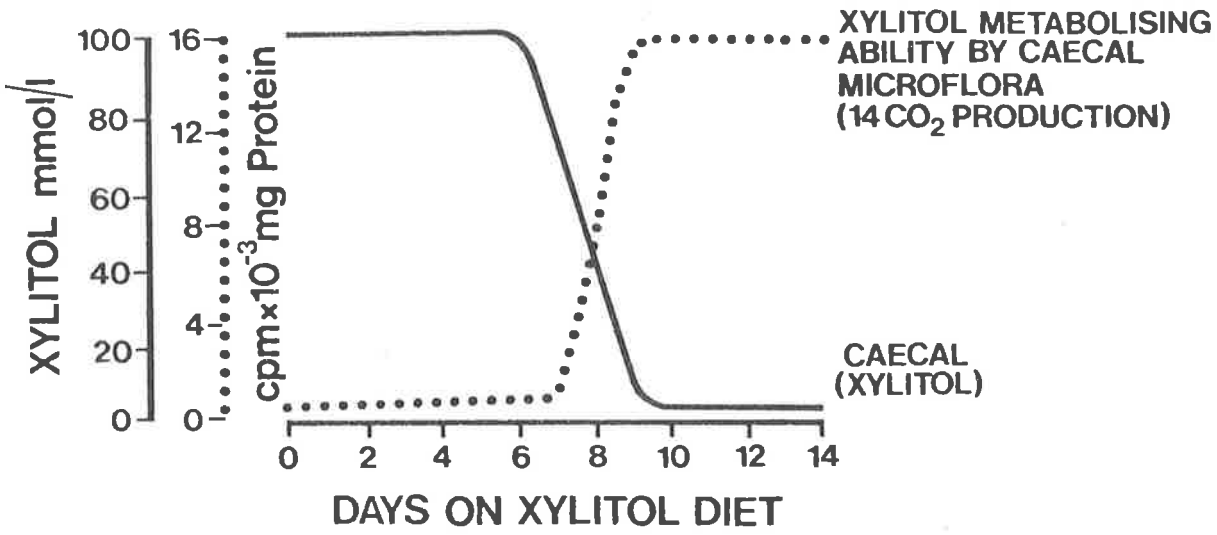


Fig. 6.1 Schematic representation of the steady state changes induced by dietary xylitol in the microflora of the caecum of rats. (Based on data obtained from Figs. 2.2 and 2.5).

xylitol metabolism in the gut (Chapter Two) can cause an increase in stool bulk and frequency (Hill, 1978). In addition, the acidification of the lower gut contents by dietary sugar polyols and in particular, xylitol, could reduce the incidence of large bowel carcinogenesis since the bacterial transformation of bile acids to carcinogenic unsaturated metabolites does not occur under conditions of low pH (Hill, 1975; 1978). Such speculation, however, must await further experimental clarification.

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PUBLICATIONS

During the course of the work reported in this thesis some findings were presented at Scientific Meetings or published in Scientific Journals. These communications are listed below. Specific contributions of other research colleagues to this work has been detailed in the acknowledgements and, as appropriate, within the text.

Publications

KRISHNAN, R., WILKINSON, I., JOYCE, L., ROFE, A.M., BAIS, R., CONYERS, R.A.J., and EDWARDS, J.B. (1980): 'The effect of dietary xylitol on the ability of rat caecal flora to metabolise xylitol'. Aust. J. Exp. Biol. Med. Sci., 58, 639-652.

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