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The molecular basis of lactose intolerance

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ABSTRACT

A staggering 4000 million people cannot digest lactose, the sugar in milk, properly. All mammals, apart from white Northern Europeans and few tribes in Africa and Asia, lose most of their lactase, the enzyme that cleaves lactose into galactose and glucose, after weaning. Lactose intolerance causes gut and a range of systemic symptoms, though the threshold to lactose varies considerably between ethnic groups and individuals within a group. The molecular basis of inherited hypolactasia has yet to be identified, though two polymorphisms in the introns of a helicase upstream from the lactase gene correlate closely with hypolactasia, and thus lactose intolerance. The symptoms of lactose intolerance are caused by gases and toxins produced by anaerobic bacteria in the large intestine. Bacterial toxins may play a key role in several other diseases, such as diabetes, rheumatoid arthritis, multiple sclerosis and some cancers. The problem of lactose intolerance has been exacerbated because of the addition of products containing lactose to various foods and drinks without being on the label. Lactose intolerance fits exactly the illness that Charles Darwin suffered from for over 40 years, and yet was never diagnosed. Darwin missed something else – the key to our own evolution – the Rubicon some 300 million years ago that produced lactose and lactase in sufficient amounts to be susceptible to natural selection.

Keywords: *lactose, lactase, lactose intolerance, milk, hypolactasia, evolution, Darwin, bacterial toxins*

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Introduction

Many individuals can ingest milk without any problem. However, many thousands of others suffer debilitating symptoms from just one glass of milk or less because they are intolerant to lactose, the sugar in milk. If we are to help these people, whose lives are misery because of their sensitivity to lactose, we need to answer five key questions:

- What is the precise cause of lactose intolerance?
- How does lactose produce gases and toxins in the gut?
- How do these toxins cause the wide range of gut and non-gut symptoms that make the lives of people with lactose intolerance a misery?
- What was the evolutionary origin of this biochemical system, unique to mammals?
- How has it evolved over the following 300 million years to influence our diet and health in the 21st century?

An unusual case

A few years ago we discovered a 53 year old woman whose life was a misery because of severe irritable bowel syndrome (IBS), diarrhoea, nausea and sickness, as well as skin rashes, breathing problems, muscle and joint pain, and lack of concentration. She had suffered these since childhood. But they were now so severe that she thought she had Alzheimer's disease. Her doctor told her she had eczema, asthma and osteo-arthritis. She was awaiting a knee replacement operation, and was on a range of drugs – skin creams, antihistamines, asthma inhalers, antibiotics, anti-diarrhoeals and strong pain relief. She was surprised when we decided to investigate her for lactose intolerance – 50 g of oral lactose, followed by an analysis of breath for hydrogen gas. But this was negative. According to the text books, this lady did not have lactose intolerance, as she had many non-gut symptoms and did not produce hydrogen when she ingested lactose. But she did become ill during the lactose test, recording gut and non-gut (systemic) symptoms several hours after taking the lactose – gut pain, nausea and vomiting, headache, light headedness, feeling drunk, heart palpitations, and joint and muscle pain. These remained severe for three days. We advised her to remove all lactose from her diet for one month, involving avoidance of 'dairy' products, and foods and drinks where lactose can be present

without being clear on the label. Within one month she described her skin as 'wonderful'. Her asthma and sinusitis had gone, and her joints were much improved. She no longer needed any medication, and was taken off the list for a knee replacement. Similar dramatic stories have been repeated among the 700 patients we have now diagnosed as having 'systemic' lactose intolerance[1–3].

What is lactose intolerance?

The fact that many southern Europeans become ill after drinking milk was first described by Hippocrates. But it took 2000 years to discover that this was caused solely by a biochemical intolerance to the sugar in milk. Lactose intolerance was first identified in the early years of the 20th century[4,5]. However, it was not until the 1960s that the biochemical basis of lactose intolerance, and its ethnic distribution, were properly defined[5–7].

The disaccharide lactose, 4-O-β-D-galactopyranosyl-D-glucopyranose (Figure 1), is found widely in Nature attached to polysaccharides, glycoproteins and glycolipids. The latter involve gluco- and galacto-ceramides, and lipids involved in the vesicles of endocytosis, *e.g.* lactose attached to the sialic acid N-acetyl glucosamine as neuramin lactose (Figure 2) found in small quantities in milk. Large amounts of free lactose are only found naturally in mammalian milk, where it can exist in the interconvertable α or β forms (Figure 1). α lactose is the principle form in milk, and that supplied in a Pharmacy, and used in the lactose test. Lactose dissolves in water up to about 1.5 M in boiling water. But it is not as soluble as glucose, fructose or sucrose. Molar solutions of lactose come out of solution when frozen, unlike glucose and sucrose. Milk does not taste sweet because α lactose has only a faintly sweet taste, the β form being slightly sweeter. Both forms rotate the plane of polarised light ($\alpha + 92.6^\circ$, and $\beta + 34^\circ$ at 20°C). Warming either form leads to an equilibrium value of the $\alpha + \beta$ forms with an optical rotation coefficient $[\alpha]^{20}_{D}$ of 52.3°.

All mammalian milk, apart from that of the Pinnepedia (sea lions and walruses), contains 40–75 g lactose per litre, depending on species, providing 40% of the energy needs of a suckling infant. Yet some two-thirds of the world's population cannot digest lactose properly (Table 1). Each of us has a different threshold to lactose. Many white Northern Europeans can drink 1–2 glasses (250–500 ml) of milk with no adverse effects, while others are so sensitive to lactose that just 10–20 ml in a cup of tea can make them ill. This is because:

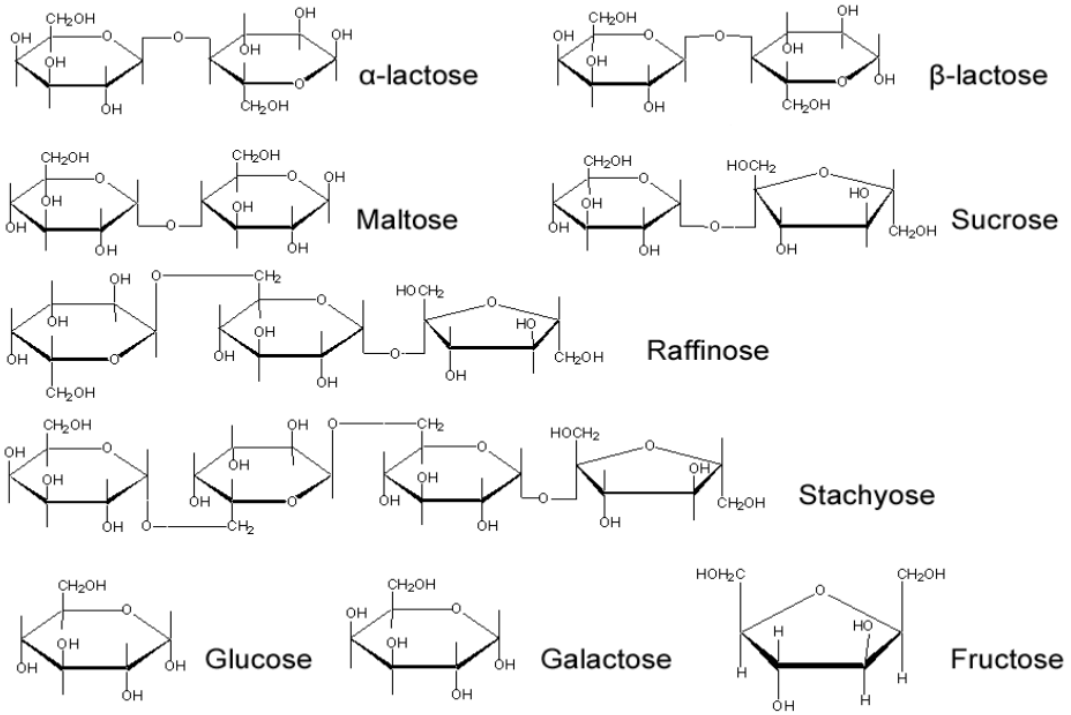

Fig. 1. *The chemical structure of some sugars. The α form of lactose has the hydroxyl at position 1 on the glucose up, rather than down as in the β form.*

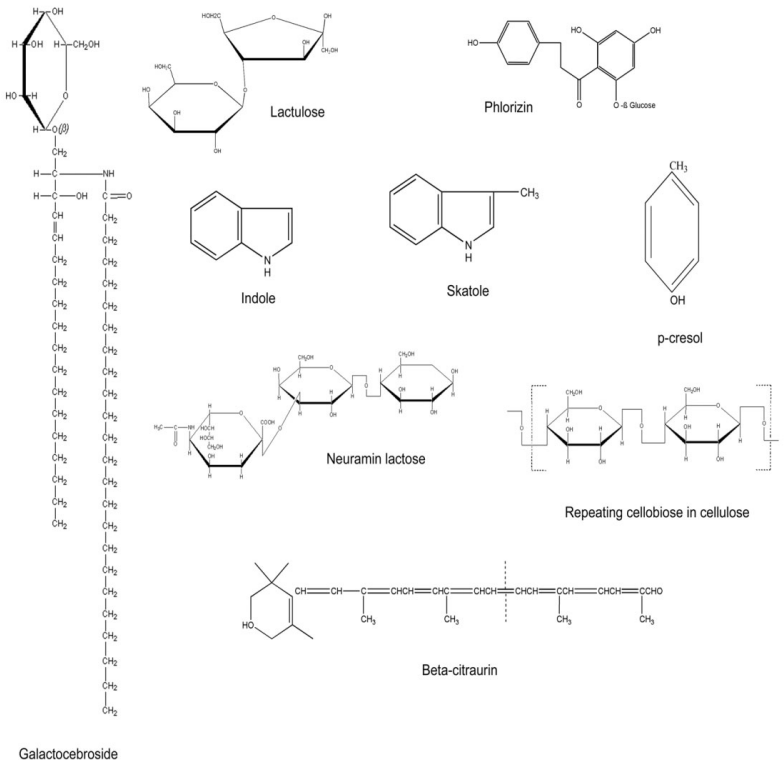

Fig. 2. *The chemical structure of some other substances relevant to lactose intolerance.*

1. A person labelled as lactose intolerant has a low level of lactase, the enzyme in the small intestine that cleaves lactose into glucose and galactose, which can then be absorbed.
2. Bacteria in the large intestine convert any lactose undigested in the small intestine into gases and toxins.
3. The tissues are sensitive to the bacteria toxins, after they have been absorbed into the rest of the body.

We are dealing with a biochemical intolerance, and not an allergy, though lactose intolerance can exacerbate allergic symptoms[8,9]. An allergy involves an immune response to a foreign protein, resulting in a reaction with antibodies IgE or IgG. These antibodies bind the allergen, and then the antigen-antibody complexes activate cells in the immune system – lymphocytes to generate more antibodies, phagocytes to release oxygen metabolites and proteases, and importantly, mast cells to release histamine. These substances then cause contraction of smooth muscle and inflammation, with

Table 1 *Different ethnic groups with low lactase and likely lactose intolerance*

Ethnic group (adult, unless stated)	% with low lactase and potential lactose intolerance
Chinese	>90%
Japanese	>90%
Indian and other Asian groups	>80%
Aboriginal Australian	>80%
Black African	>75%
American Red Indian	>70%
Eskimo	>70%
South American (total adults)	>50%
Mexican	>50%
West Indian	>50%
Spanish	>40%
Italian	>40%
Greek	>40%
Mid European (*e.g.* Hungarian and gypsy)	>40%
American (total adults)	30%
Finnish	20%
White Northern European	10%
White Australian	10%
Children under 2 years old (any ethnic group)	0–20%
Children between 2 and 10 years old	0–40%
Patients with IBS	>50%

These numbers are very approximate.

resulting breathing difficulties, skin itching and rashes. In its severest form a patient may suffer anaphylactic shock that can be lethal. In contrast, an intolerance is a biochemical defect that prevents the normal metabolism of a specific substance. Most commonly, such biochemical intolerances are to a carbohydrate, amino acid or other small organic molecule[8,9]. Lactose intolerance is caused by an impaired capacity to digest lactose properly, and thus a reduced capacity to absorb into the body its two constitutive sugars, galactose and glucose. To understand fully lactose intolerance five questions need to be answered:

1. How is lactose normally digested, and what mechanisms can prevents this occurring?
2. When lactose is not digested normally, what happens to it?
3. What are the symptoms that result from lactose not being digested normally?
4. What is the molecular basis of these symptoms, and what causes someone to cross the Rubicon and feel ill?
5. What is the evolutionary significance of lactose?

The discovery of lactose and lactase

Lactose was discovered in milk in the 17th century[10]. But it took a further 300 years before lactose was synthesised in the laboratory[5], and thus its precise chemical structure determined. The fact that lactose can induce diarrhoea was reported over 100 years ago[4]. It was then shown that animal and human intestine contained an enzyme, lactase, that could cleave lactose into its two constituent sugars[11]. Consistent with the fact that sea lion milk has no lactose, the intestine of these animals seemed devoid of lactase[5].

Free lactose is synthesised from UDP-galactose and glucose in the mammary gland. Lactose synthase has two sub-units, galactosyl transferase and a protein modifier, α-lactalbumin. Galactosyl transferase normally catalyses the formation of N-acetyl lactosamine, on glycoproteins:

$$\begin{aligned} \text{UDP} - \text{galactose} + \text{N} - \text{acetyl glucosamine} \rightarrow \\ \rightarrow \text{UDP} + \text{N} - \text{acetyl lactosamine} \end{aligned} \quad (1)$$

However, when the modifier sub-unit, α-lactalbumin, binds to galactosyl transferase the resulting complex changes its specificity to become a lactose synthase, transferring galactose to glucose rather than N-acetyl glucosamine:

$$\text{UDP} - \text{galactose} + \text{glucose} \rightarrow \text{UDP} + \text{lactose} \quad (2)$$

Galactosyl transferase is found in most tissues, but lactose synthase is found only in the mammary gland, where, in pregnancy, its gene switches on. At birth, the hormone prolactin then induces the modifier sub-unit α-lactalbumin, so that the breast can produce lactose in the milk for the new born baby.

The biochemistry of lactase

Lactase is a special type of β-galactosidase. There are three β-galactosidases found in human tissues:

1. Specific lactase on the apical surface of the enterocytes in the brush border villi, facing outwards, having a pH optimum of about 6. It breaks lactose ($C_{12}H_{22}O_{11}$) into D(+)-galactose and D(+)-glucose, and is found only in the small intestine in mammals.
2. β-galactosidase in the cytosol of cells, with a similar pH optimum but which may not hydrolyse lactose.
3. β-galactosidase in the lysosomes, with a pH optimum of 4.5.

In bacteria, the classic lac operon codes not only for a β-galactosidase but also for a permease, necessary if the bacteria are to take up lactose and access cytosolic β-galactosidase. It is not clear whether eukaryotic cells have such an active lactose permease in the plasma membrane. Yeast metabolises external lactose poorly[3]. Thus, lactose can be added to drinks, and even beers and lagers, without generating unnecessary CO_2. In most eukaryotic cells, the β-galactosidases see only β-galactosides generated from internal metabolism, such as those attached to lipids and proteins. Krabb's disease (globoid cell leukodystrophy) is caused by a deficiency in intracellular galactosylcerebrosidase. But it is loss of intestinal lactase that is responsible for lactose intolerance. It is found first in the duodenum (5–6 cm long), reaches a peak in the jejunum (2.5 m long), and decreases gradually down the ileum (4–5 m long). There are no significant amounts normally found in the large intestine, first shown over 100 years ago[5].

Intestinal lactase is a unique enzyme, since it has two active sites within one polypeptide chain. One hydrolyses lactose, while the other was identified originally by its ability to hydrolyse an aryl glycoside called phlorizin (Figure 2), discovered in apple bark, being hydrolysed to glucose and phloretin (Figure 2), a diabetogenic substance. Phlorizin is an aryl α-glucoside linked to phloretin (Figure 2), and was originally discovered as an inhibitor of the glucose uptake mechanism in the small intestine SGLT1. Phlorizin is a competitive inhibitor of the lactose site. But lactose does not appear to inhibit the phlorizin site. Small intestinal lactase is competitively inhibited by a number of other substances, including the common buffer Tris, and colchicine (Figure 2) that binds to tubulin in microtubules. Unlike the acid pH β-galactosidase in lysosomes, intestinal lactase is not blocked by SH reactive reagents such as *p*-chloro-mercurobenzoate (PCMB).

The full name is therefore lactase-phlorizin hydrolase with two enzyme commission (EC) numbers – EC 3.2.1.62 for its phlorizin hydrolase (LPH) activity and EC 3.2.1.108 for its β-galactosidase activity. Care should be taken to use the correct EC numbers, as there are some publications that have used the incorrect numbers with the bacterial β-galactosidase number, EC 3.2.1.23. Small intestinal lactase has no amino acid sequence similarities to the β galactosidase in bacteria. It is also different from enzyme supplements sold as 'lactase' in health food shops, and the other types of β galactosidases found in eukaryotic cells.

The natural substrates for the phlorizin site are cerebrosides (Figure 2) – glycolipids made up of a hexose sugar, usually

galactose, linked by a β link to sphingosine with a fatty acid attached. The non-sugar moeity is known as a ceramide. This enzyme activity is thus really a glycosyl ceramidase. This explains why we need to keep some lactose after weaning. Hydrolysis of cerebrosides provides sphingosine, particularly important in the membranes of the brain. Lactase, thus, has a number of enzymatic activities in addition to hydrolysing lactose, including a range of β-glycosides (phlorizin, glycolsyl and aryl β-ceramides, cellulose, cellobiose, cellotriose, cellotetraose), β-glucans found in the cell walls of plants and fungi we eat, *e.g.* laminaribose and to a lesser extent gentiobiose[12], and β-galactosides. It also hydrolyses o- and m- nitro-phenyl β-glycosides, useful as artificial substrates in assays. The hydolysis of flavonoid glycosides and pyridoxine-5′-beta-D-glucoside[13] may be important sources of flavonoids and vitamin B6, forming pyridoxal phosphate used in several energy system enzymes such as phosphorylase, and amino-transferases. Lactose is restricted to the milk of terrestrial mammals, but glycosyl ceramides are present in the diet of all vertebrates.

Lactase is not a particularly powerful enzyme, there being variations in the maximum enzyme activity (V_{max}) when saturated with substrate, and the affinity for the substrate (K_m) for lactase and phlorizin hydrolase activity between species[14,15]. The ratio of activities of the lactase/phlorizin-glycosyl ceramidase activities also varies between species, from 40 or 35 : 1 in rats or monkeys to 5 : 1 in humans. This means that rats and monkeys are better at digesting cerebrosides than humans. Human lactase has a K_m for lactose of about 20 mM, compared with pig at 5 mM, consistent with human milk having the higher lactose concentration. The Km for phlorizin is 0.4 mM in the human and pig enzymes, but $< 30\,\mu M$ in pig. Human lactase has a moderate V_{max} of 20 U/mg pure protein ($1\,U = 1\,\mu mol\ min^{-1}$). If one assumes a molecular weight of about 150,000, then this gives a turnover number of about $50\,s^{-1}$. This compares with $600{,}000\,s^{-1}$ for carbonic anhydrase, $1000\,s^{-1}$ for lactate dehydrogenase and $1\,s^{-1}$ for firefly luciferase. This has important implications for the evolution of such enzymes. At this stage in their evolution they can simply be considered as 'solvent cages'. Natural selection has yet to force improvement of biochemical properties through covalent and other interactions with their substrates. But the V_{max} and K_m are sufficient for lactase to be maximally active with a lactose concentration in cow's milk of 130 mM, and in human milk of 190 mM. From a turnover number of $50\,s^{-1}$, it is possible to estimate a total lactase activity in the entire human small intestine of 2500 U[14]. It

would thus take less than 15 min to digest all the lactose (33 mmole) in a 250 ml glass of milk. But, in someone who is severely hypolactasic, with a total lactase level of just 250 U (*i.e.* 10%), then it would take over 2 h to digest all this lactose. By this time it has reached the bacteria in the large intestine. The pH optimum for lactase is about 6, with little activity below pH 3. So it would be inactive in the stomach, where the pH is 1–3. However the pH rises in the duodenum to 6–6.5, and then in the jejunum and ileum to pH 7–8 as the food gets further away from the stomach. Since the pH activity curve of lactase is skewed towards alkaline pH, at pH 8–9 lactase still retains 50% of it maximal activity at pH 6, suitable for full activity throughout the small intestine, becoming more alkaline as food moves from the stomach to duodenum and then the ileum. The pH of the large intestine is 5.5–7.

Cellulose is the major polysaccharide in all plant cell walls, made of long chains of 1–4 β linked glucoses, unlike starch where the glucoses are linked by 1–4 and 1–6 α bonds. α – amylase cannot hydrolyse cellulose. Ruminants have bacteria in their multiple stomachs to achieve this efficiently. However lactase can hydrolyse cellulose, and the di-, tri- and tetra- saccharides[14], its initial degradation products – cellobiose, cellotriose and cellotetraose (Figure 2). In monkeys[15], the specific activity ratio for lactose : cellulase is 6 : 1, compared with 40 : 1 for phlorizin. So lactase could be more active in hydrolysing products from cellulose than ceramides. Lactase does not hydrolyse the laxative lactulose (4-O-β-D galactopyranosyl-D-fructose; Figure 2), slightly sweeter than lactose. Lactulose can be used to measure gut transit time, when it generates gas in the large intestine and induces diarrhoea.

Although intestinal lactase has no sequence homology to the β-galactosidase in *E.coli*, a comparison of amino acid sequences, using the software programme BLAST, of human lactase against the Genbank database, including genomes, identifies over 1800 proteins with some sequence similarity. However, the only major sequence similarities are with other intestinal lactases – rabbit, rat, mouse, cow, dog and pig, either from cloning or predicted from the genome sequence. Human lactase is 83% identical to that in rabbit, and 77% identical to rat. But rat is only 75% identical to rabbit. These produce a score of >3000 bits. Other proteins only have 'bit' scores of 600 or less. These include the Klotho precursor, a range of cytosolic eukaryotic and prokaryotic β-glucosidases, gentobiase, and myrosinase. BLAST and CLUSTAL data suggest that the active site for β-glucosidases and β-galactosidases has arisen several times independently in evolution.

There are four domains in the initial full lactase prosequence translated from mRNA, designate I, II, III and IV. Domains I and II are lost in ER cleavage of the N-terminus. Site directed mutagenesis and affinity labelling identified domain III as the phlorizin/cerebroside hydrolase, and domain IV is the lactase site[16]. The key amino acids are two glutamates, at position 1273 for human phlorizin hydrolase and 1749 for human lactase. The same glutamates have been identified in rabbit and rat, but at slightly different numbered sites, because of the different lengths of the full sequence. In contrast, the negative amino acid at the active centre of sucrase appears to be an aspartate. Sucrase-isomaltase can be isolated as one enzyme complex. However, unlike lactase, the two enzymatic activities are on different polypeptide chains. Lactase has no sucrase, isomaltase or amylase activity.

The molecular biology of lactase

Human lactase is located on the long arm of chromosome 2 (2p21q). The 55 kb DNA sequence contains 17 exons, and lies within a 70 kb sequence containing regulatory response elements[3,17] (Figure 3). It is on the reverse strand. In humans regulation involves both transcriptional and post-transcriptional mechanisms, transcriptional regulation controlling appearance of lactase in the foetus just before birth, and its loss on weaning. The developmental element responsible for the large increase in lactase just prior to birth is cis acting, CE-LPH[17]. In spite of extensive searching, no mechanism causing hypolactasia after weaning has been identified. The main reason for this is that potential mechanisms have focussed on regulation of the lactase response element itself, rather than the development or survival of cells expressing lactase. Using luciferase reporters, yeast hybrids, gel shift assays with binding of putative transcription factors, specific antibodies, mutants, and co-transfection of particular transcription factors, the main lactase response element in humans, pig and rat has been located within a 1 kb stretch immediately upstream from the gene, with four key regions at −894 to −798, −227 to −142, −299 to −227 and −142 to −17 in the pig, and potential regulation by the caudal homeodomain transcription factor cdx2[18], HNF1α, HIF (hypoxia-inducible factor), HOXC11, FREAC, and GATA transcription factors 4, 5 and 6[19], known to be gut and stomach homeodomain factors. Mutations within the 1 kb that prevented transcription factor binding identified the minimal promoter being −200 to −17

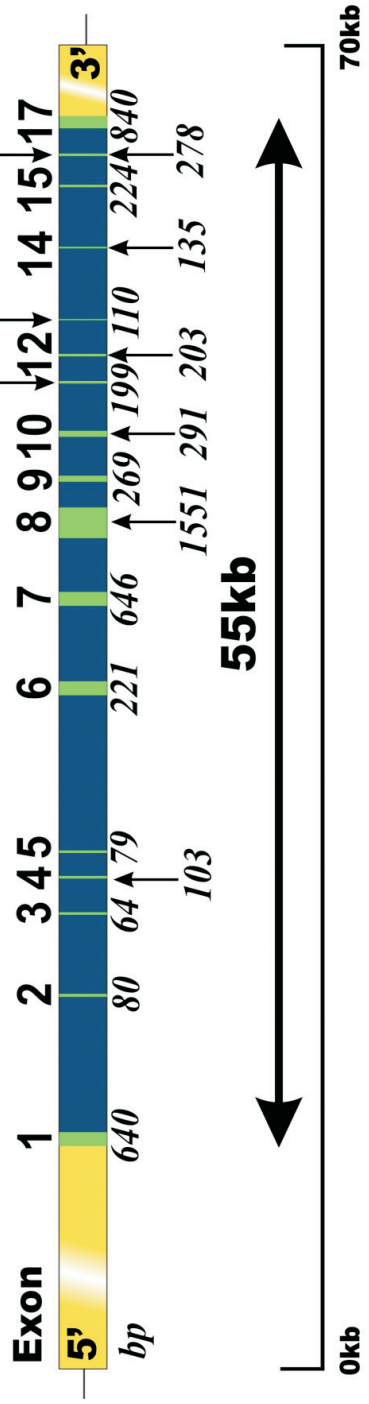

Fig. 3. The lactase gene.

from the translation start site, with two cdx2 sites, one HNF1α site, and the GATA site being at −100 to −73. There are homologies between the human, pig and rat lactase promoters[18], though the human promoter has two tandem alu elements within it. But none can be linked causally to lactase non-persistence that starts on weaning. This loss is highly specific for lactase, and does not occur with other disaccharidases such as sucrase. Lactose does not regulate the lactase promoter, unlike the induction of β-galactosidase (EC 3.2.1.23) in bacteria.

In order to reach the plasma membrane, lactase undergoes considerable post-translational modification, involving glycosylation and proteolytic cleavage[20] (Figure 4). Human lactase is synthesised as a pre-proprotein of 1927 amino acids[21] (1926 in rabbits) (Figure 4). But, the mature protein at the apical plasma membrane of the human enterocyte is only 1059 amino acids (1060 in rabbits). This consists of 1014 amino acids at the N-terminus facing the gut lumen with a terminal A869, a single membrane spanning domain of 19 amino acids, and a short C-terminus of just 26 amino acids facing the cytosol (25 in rabbits). As the lactase is synthesised from mRNA on the ribosome, the N-terminal signal peptide translocates it into the endoplasmic reticulum (ER), where the signal peptide of 19 amino acids is cleaved. Once inside the ER, the 1908 amino acid polypeptide is cleaved, almost in two. But, although the final protein in the membrane has an N-terminal A869, this is not the cleavage site in the ER. Mutation of R868A does not prevent proteolytic cleavage and processing of lactase in caco-2 cells[22]. We have shown, using a genetically engineered 'Rainbow' protein[23] that no cleavage occurred around R868 at this site in caco-2 cells.

Two proteolysis steps are required to produce the final 1059 amino acid protein in the plasma membrane. The first cleavage occurs between R734 and L735 via a furin-like protease, though the role of furin is not fully established. This occurs through the trans Golgi network, mutant R734/L735 retaining prolactase in the ER[22], and processing being inhibited by monensin and brefeldin A. Transportation on microtubules is also needed, since colchicine inhibits formation of the mature enzyme, causing precursor accumulation. After cleavage, lactase is glycosylated (15 N-linked predicted) and transported to the plasma membrane, where gut lumen trypsin trims the protein to the final 1059 peptide with an N-terminal alanine. The cleaved N-terminal 866 (847 + 19) amino acids contain domains I and II (87-172 and 363-848), with sequence similarity to the active site domains III and IV, 883-1365 and 1370-

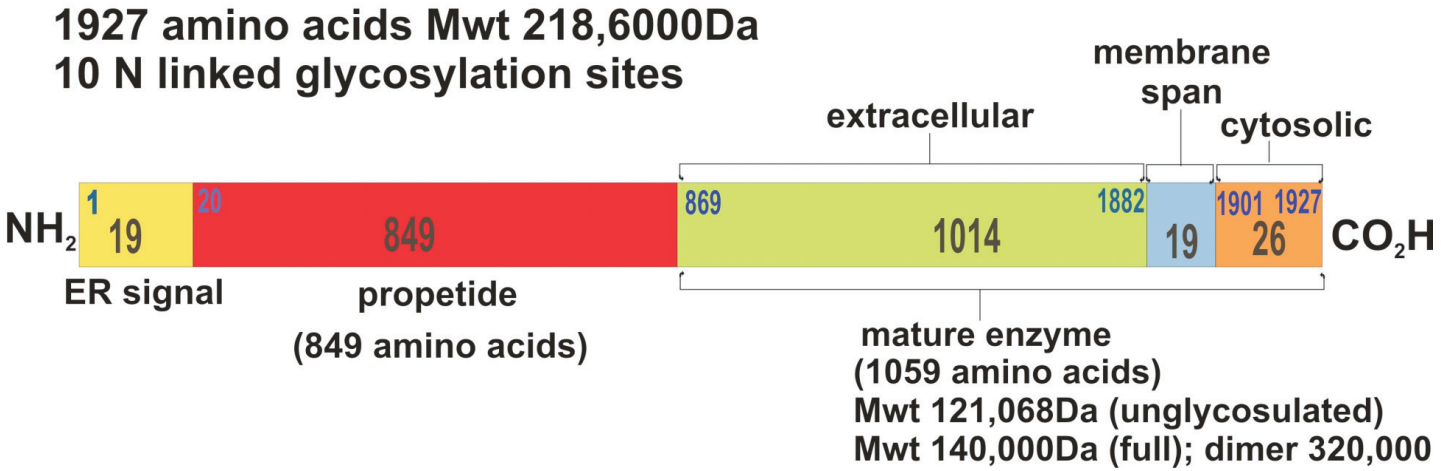

Fig. 4. *Lactase-phlorizin hydrolase (EC 3.1. 2.62 and 108), with cleavage and other sites marked.*

1841. But the N-terminus does not appear to have any β-galactosidase activity. Rather it is a chaperone, helping folding of the final protein, and its successful trafficking to the plasma membrane[24]. The final lactase enzyme monomer has a molecular weight of about 145 kDa, some 24 kDa greater than that estimated from the amino-acid sequence alone (121 kDa), consistent with its heavy N-linked sites via asparagines and O-linked glycosylation via serines and threonines. These are necessary for efficient folding and activity of the enzyme on the cell surface. The final dimer has a measured molecular weight of about 320 kDa.

There are thus are five ways to reduce intestinal lactase activity:

1. Reduced transcription of the lactase gene and splicing of the mRNA product.
2. Reduced translation of the mRNA.
3. Impairment of enzyme processing in the ER-Golgi, through protein mal-folding, improper proteolysis or glycosylation, or reduced protein transport to the plasma membrane.
4. Inhibition of enzymatic activity by substances in the gut lumen.
5. Loss of 'lactase' expressing cells through microbial or viral damage, mechanical loss, or apoptosis.

Loss of lactase expressing cells is the major mechanism responsible for inherited hypolactasia, and with loss in coeliac disease. There has been some debate about the role of transcriptional versus post transcriptional regulation of lactase. Too much emphasis has been placed on these as the explanation for lactase persistence/non-persistence. Enterocytes expressing lactase in the villi of the small intestine exhibit a patchy appearance[6], showing that they are generated by a non-clonal mechanism[25].

Eating lactose gradually over several weeks or months may increase the threshold to lactose, before becoming ill. This is not caused by specific induction of intestinal lactase. Plimmer[11] in 1906 showed that lactase was not induced by its substrate lactose. Any apparent dietary induction of lactase is caused by general intestinal hypertrophy. The inability of lactose to affect the level of intestinal lactase in mammals is in striking contrast to the famous induction by lactose of the lac operon in *E.coli*, and intestinal sucrase and isomaltase, which do not decrease after weaning and are induced by ingestion of their substrates. Changes in lactose sensitivity reported by patients are most likely caused by changes in microflora in the large intestine.

Hypolactasia versus lactose intolerance

Most of the world's adult population are 'hypolactasic' (Table 1), compared with a suckling infant. They have a low lactase, and are thus lactase non-persistent. There are three mechanisms that can cause this:

1. Congenital loss. This is very rare, though the genetic defect is found particularly in the Finnish population[26], and appears to be complete loss of lactase. Until recently, the mechanism of such alactasia was unknown. Initial studies suggested it maps 5′ to the lactase gene, *i.e.* towards the MCM gene, so may be a regulatory defect rather than a mutation within the lactase gene itself. However, mutations have now been identified in the lactase gene itself, causative of congenital lactase deficiency[27]. Characterisation of five mutations in the coding region of the lactase gene have shown 84% were homozygous for a nonsense mutation, T4170A (Y1390X), designated 'Fin (Major)'. Four other rare mutations included two that result in a frameshift and early truncation at S1666fsX1722 and S218fsX224, and two point mutations that result in substitutions Q268H and G1363S of the 1927aa polypeptide. All four lead to a protein structure with inactive enzyme.
2. Inherited loss on weaning. This is the norm in all mammals, apart from white Northern Europeans and some other ethnic groups (Table 1).
3. Secondary loss. This can occur as a result of intestinal bacterial, viral or protozoan infections. These include rotavirus, the protozoan *Giardia* and gut trypanosomes. Endocrine control through sex and thyroid hormones[28,29], and ageing, also may affect levels of lactase in the small intestine.

It is important to distinguish these when treating someone clinically, or when investigating the genetics. Only secondary loss of lactase is potentially reversible, and thus treatable. Lactase can also be reduced in a number of other conditions of food intolerance such as coeliac disease[30].

In all eukaryotic cells the endoplasmic reticulum (ER) has a signalling system that communicates to the cytosol, plasma membrane, and the nucleus[31]. This system determines whether a cell fires a Ca^{2+} signal to switch on an end response, traverses the cell through its division cycle, whether the cell defends itself against stresses such as the generation of large amounts of unfolded protein in the ER, or dies by apoptosis. Stress to the

ER will lead to a reduction in the lactase reaching the plasma membrane[31]. This is likely to be particularly relevant to loss of lactase in gut infections.

The genetics of lactase

The genetics of lactose intolerance have been studied extensively in white European populations and non-white populations throughout the world[7,17,32]. The presentation of the genetics is not very clear. Most geneticists argue that lactase persistence is an autosomal dominant trait, whereas non-persistence is recessive. People who are homozygous for lactase persistence retain high levels of lactase into adulthood. Those who are homozygous for lactase non-persistence have low levels of lactase in adulthood. Adults who are heterozygous have intermediate lactase levels. But clinically, these distinctions are not so clear. There is a huge variation between individual phenotype, both in hypolactasia and the threshold for lactose intolerance. Some patients with most severe symptoms are heterozygote for certain genetic markers linked closely to lactase non-persistence. The phenotype is not 'all or none'.

A problem is how to assess hypolactasia. This is usually done from a small biopsy. The small intestine is an incredible absorbing machine, folded over and over again to compact it into the peritoneal cavity. Fully opened out, as a single cell layer, estimates of the surface area of the small intestine vary from the size of a tennis court to half the size of the football pitch at Old Trafford! This poses problems when interpreting measurements from just a single biopsy. It is like taking a blade of grass from Cardiff's Millennium Stadium to discover whether the whole pitch is fit to play the Cup Final. It is the overall level of lactase in the entire small intestine, and the efficiency of the sugar transporter SGLT1, that determine whether all the lactose is first cleaved and then the resulting glucose and galactose fully absorbed in the small intestine, before having a chance to reach the bacteria in the large intestine.

Intestinal lactase levels are low in the foetus, unlike sucrase. Lactase only appears in the foetus a few days before birth, reaching a peak some 3 days afterwards, just right for the baby to receive lactose from the mother's milk. After weaning, some 6–12 months later, lactase begins to decline. The rate of this decline varies considerably between ethnic groups[3,17,32]. In Chinese and Japanese, lactase decreases rapidly after 2–3 years of age, reaching

its nadir by the age of 5–10 years. In Asians the rate of decline is slightly slower, but still this group have lost some 75% of their lactase by their teens. In contrast, in the 8–10% of white Northern Europeans who lose lactase after weaning the rate of decline is slower, lactase not reaching its nadir until almost adulthood. The ultimate level of lactase also varies between ethnic groups (Table 1), being lowest in adult Chinese and Japanese, who retain just 5–10% they had as a suckling infant. Whereas in Europeans that lose their lactase after weaning, the eventual level maybe 30–50% they had as a baby. However it is not entirely clear what these numbers mean. Human lactase activities are measured from biopsies, and are expressed as U/mg intestinal weight or /mg protein, or as a ratio against sucrase-isomaltase. Let us suppose that these values are only 5% of values found in a suckling infant, *i.e.* 1/20. What matters is the total activity in the entire small intestine. The length and surface area, and thus the number of lactase expressing cells, of the small intestine of an adult will be much greater than when they were just a few days old. Since the size of the entire small intestine of an adult is likely to be some 20 times that of the suckling infant, then if the activity per unit weight is 1/20 that as a suckling infant then the total level of lactase throughout the small intestine will be the same. A suckling infant ingests perhaps 1 litre of milk a day, more than even most white Northern Europeans. This simple calculation shows that if an adult has lost 95% of their total lactase, the level per unit weight or protein would have to be just 1% of that in a suckling infant. This raises the question as to whether the concept of hypolactasia is flawed. These variations confuse the definition of phenotype in genetic studies, particularly if symptoms after a lactose load, rather than enzymatic activity, are used as the principle criterion. The small intestine is made up of three main segments. The duodenum connects from the stomach. This then leads to the jejunum, and then the longest section, the ileum, which then connects to the large intestine. Biopsies are often taken from the jejunum. But does someone who is highly sensitive to lactose have a major loss in the ileum? We need a PET or MRI indicator that can assess the total lactase in the whole of the small intestine.

There have been extensive attempts to discover a polymorphism, and thus a molecular mechanism, to explain loss of lactase on weaning. It is assumed that lactase persistence/non-persistence is a polymorphic trait, where the allele frequencies have been affected by selection, but where genetic drift has also occurred to influence haplotype frequency in any particular population. Haplotype is a

single genetic unit on one chromosome, *i.e.* one member of a pair of alleles. The unimodal distribution of lactase levels in infants moving to a trimodal distribution in adults, with the occurrence of lactose intolerance in monozygotic twins, support the case that lactase persistence is a dominant inherited trait, with the genes on both chromosomes expressing. Lactase persistence is most common in North West Europe, with highest levels being in the Swedes and Danes. The mean population level of lactase decreases moving south. A similar southerly decline is seen when comparing North and South India. Several ethnic groups and races, known to be lactase non-persistent, still have some milk in their diet. These include the Mongols and several groups in Africa – the Herero, Nuer and Dinka tribes. Cows are sometimes retained as a status symbol; *e.g.* the Dinkas and Hindus. But the 'milk' is often ingested as a fermented product such as yoghurt or cheese where lactose levels are much lower than in milk.

Several polymorphisms have been found in the introns and exons of the lactase gene and its promoter, but none consistently correlate with lactase persistence/non-persistence[17]. There are four common haplotypes world wide, designated A, B, C and U. Only A, B and C are found in Europe, A being found in $>80\%$ northern Europeans. The four haplotypes, A, B, C, and U are not related and have different distributions. The A haplotype has high frequencies only in the Northern European population, which has a high prevalence of lactase persistence. The U haplotype is virtually absent in the Indo-European population. The haplotypes appear to be in a large region of linkage disequilibrium, where there is evidence of genetic drift in evolution, but they do not help in identifying the true basis of lactase persistence. Both alleles from each chromosome express high levels of mRNA in homozygous lactase persistence. Those who are homozygous for lactase non-persistence express low levels from both chromosomes. Heterozygotes express high levels from the chromosome with the lactase persistent allele, and low levels of mRNA from the other chromosome. The key question therefore is: what is the cellular basis of this? Does each cell only express lactase from one of the chromosomes?

An apparent breakthough was reported by a Finnish group[33]. Two polymorphisms were found in introns of the helicase MCM6, 14 Kb upstream (on the reverse DNA strand, like lactase) from the lactase gene itself, C/T_{-13910} in intron 13 and G/A_{-22018} in intron 9, numbered from the ATG start codon of lactase gene. CC and GG homozygotes had the lowest level of lactase. Homozygote

TT/AA had full levels of lactase, with heterozygotes being in the middle. Encouragingly, there have now been several clinical studies, including our own[2], showing that these polymorphisms provide a useful addition to clinical management. There are therefore five possible genotypes: CC/GG, CC/GA, CT/GA, CT/AA, and TT/AA. In our initial analysis, 210 patients referred with unexplained gut and other problems were investigated. 14.5% were homozygous CC/GG, 39% were heterozygous CT/GA and 46.5% were homozygous TT/AA. One patient only was CC/GA, and responded as the CC/GG. All CC/GG were diagnosed as lactose intolerant, 83% of CT/GA and 73% of TT/AA. In the control group, with no history of gut or systemic symptoms, none were CC/GG, 13% were CT/GA and 87% TT/AA. Although there have been reports that these polymorphisms can regulate lactase expression *in vitro*[34], these data do not support the hypothesis that either of the two polymorphisms are mechanistically the cause of hypolactasia. Several lactose intolerant families were TT/AA, and both Finnish and Italian studies had individuals who were CC and lactase persistent, or TT who were lactase non-persistent. Also there appears to be no correlation between the expression of mRNA for MCM6 and lactase in the gut cells of individuals with hypolactasia or lactase persistence. There are two explanations for this:

1. The C/T and G/A polymorphisms are simply a closely linked marker to lactase persistence/non-persistence.
2. There is genetic heterogeneity causing lactase persistence/non-persistence: *i.e.* there is more than one mutation that causes lactase persistence/non-persistence.

Lactase is synthesised in specific cells that begin their life by division from stem cells in the cleft of the villus in the small intestine. The intestine is made up of rows and rows of finger like projections. These are called villi, and are small folds along the intestine, which are lined by cells. As the cells move up each villus, the lactase gene is switched on and the lactase product is processed so that it appears on the apical surface. The cells are scattered in a non-clonal manner. They exhibit the 'Rubicon' principle[35], *i.e.* the ultimate level of lactase in the entire small intestine depends on the number of cells expressing lactase, rather than the level of lactase itself in each cell. If you have only 10% of the lactase you had as a suckling infant you are likely to have only a small % of the cells expressing fully lactase that you had as a baby. The final level of lactase in the intestine would be further

reduced by down-regulation regulation of transcription. This implies a fascinating developmental mechanism, perhaps involving DNA methylation, since thousands of gut villi cells expressing lactase are replaced every day. As any mother will tell you, there is a very simple biological process that takes a baby off the breast – the appearance of teeth. Thus, an obvious candidate for switching lactase-cells off would be deciduous dental homeobox genes, such as bmp-4, msx-1 and -2, shh, dlx-1 and -2, and lef1[36]. We have also observed that children who have a parent diagnosed as lactose intolerant, seem to become sensitive to lactose as they get their secondary teeth, and can become fully lactose-sensitive after puberty.

How failure to digest lactose leads to symptoms

Symptoms occur when lactose, undigested in the small intestine, reaches bacteria in the large intestine. These bacteria metabolise lactose, producing gases that distend the gut, causing pain and flatus, and toxins that, when reabsorbed into the body, cause harmful effects on a range of tissues, including neurones, heart cells, other muscles, endocrine cells, and cells of the immune system.

There are two ways in which lactose can be prevented from being digested in the small intestine:

1. Insufficient lactase.
2. Insufficient monosaccharide uptake after lactose cleavage.

Insufficiency arises either because there is not enough protein, as in hypolactasia, or from inhibition by something in food. Lactase is inhibited competitively and non-competitively by a number of naturally occurring substances. But, none have yet been shown to be involved in lactose intolerance. However, the uptake of galactose and glucose, through the Na^+ dependent transporter SGLT1 at the apical surface of the enterocytes in small intestine, can be inhibited by several substances found in food. SGLT1 enables monosaccharides to be transported into cells against a concentration gradient, using the Na^+ gradient as an energy source. The glucose and galactose are then transported into the blood at the other side (basolateral) of the cell by another glucose transporter not dependent on sodium called GLUT2. Galactose is quickly metabolised by the liver, as it is toxic to the eye, and other cells. This pathway is inhibited by ethanol, hence the use of ethanol in early lactose tolerance tests using measurement of blood

galactose and/or glucose as an indicator. SGLT1 is inhibited by the tri- and tetra-saccharides, raffinose and stachyose[3,37]. These are found in beans, pulses, root vegetables such as parsnips, and chick peas used in the production of humus. These sugars cause gas and toxins in the large intestine because not only are they not broken down in the small intestine, but also they inhibit the uptake of glucose and galactose. So glucose from starch or lactose hydrolysis ends up in the large intestine[37].

Not all sugars are transported into the gut epithelial cells by SGLT1. Fructose uses another transporter, facilitated diffusion via GLUT5 that does not use the Na^+ gradient. Intracellular fructose, like glucose, is then transported into the blood using GLUT2 on the other side of the cell. GLUT5 can be overloaded, and may be inhibited by certain substances found in food and drinks. Fructose tastes sweeter than glucose or sucrose. Hence, evolution has produced it as the main sweetener in fruits such as apples and grapes. One of our patients became ill after drinking two glasses of home made apple juice. We estimated that she had drunk the equivalent of 20 apples! As with lactose, a fructose industry has grown up over the past few decades, adding it as corn-syrup to sweeten many foods and drinks. Could other compounds, natural or food additives, also inhibit SGLT1 or GLUT5. One candidate is β coumarin (Figure 2), the orange colour in orange juice, since several patients complain of a headache 2–3 h after drinking orange juice. Grapefruit contain a substance that interacts with Ca^{2+} channels.

The bacterial toxin hypothesis

Lactose itself, and galactose, could be toxic if absorbed into the blood stream. But the major cause of symptoms in food intolerance is the production of gases and toxins by gut bacteria. The large intestine contains some 10^{14} individual bacteria, 100 times the cells in the rest of our body. There are over 1000 different species. The level of oxygen in the large intestine is low, probably $<1\,\mu M$, 1/200 of that in air-saturated water. Thus, >90% of the bacteria there are anaerobes. At least 25% are *Bifidobacter*, with the rest being other strict anaerobes. Some, such as *Bacteroides*, are so sensitive to oxygen that they are very difficult to culture directly from gut samples, as they die immediately on exposure to the air. The remaining eubacteria are mainly facultative anaerobes. Less than 1/1000 of gut bacteria are aerobes. There are also archae-

Table 2 *Substances that can released by different bacteria and archaebacteria*

Product released	Example
Gases	Carbon dioxide, hydrogen, methane, hydrogen sulphide, oxygen, nitrogen, ammonia
Ions	Calcium, sodium, potassium, magnesium, manganese, iron
Metabolites	Alcohols, diols, aldehydes, short chain fatty acids, dimethyl hydrazine, amino acid degradation products, cyclic AMP
Vitamins	K, B12, thiamine, riboflavin
Pheremones	Lactones, cytokines
Small molecule toxins	Antibiotics, tetrodotoxin
Drugs	Many
Peptides	Toxins, enzymes
Nucleic acids	Competence factors, plasmids, bacteriophages (=viruses)
Polymers	Poly hydroxybutyrate

Any particular bacterium can only release some of these.

bacteria, responsible for methane production. And there can be yeasts such as *Candida* and fungi.

Bacteria release a wide range of substances (Table 2). In low oxygen, bacterial metabolism of lactose and other carbohydrates produces gases and a range of small organic molecules. It is absorption of these that cause the symptoms of lactose intolerance. In order to make ATP, anaerobic bacteria use substrate level phosphorylation instead of oxidative phosphorylation. If the NADH from this is not re-oxidised to NAD, then glycolysis will shut down. In exercising muscle, we do this by generating L-lactate:

$$H^+ + NADH + \text{pyruvate} \rightarrow NAD^+ + L-\text{lactate} \quad (3)$$

Anaerobic bacteria have evolved several other ingenious pathways in order to remove the H from NADH, through 'fermentation'[38] (Figure 5). Several gut bacteria generate D-lactate instead of L-lactate. Measurement of blood D-lactate can thus be a good indicator of bacterial activity, *e.g.* in stressed neonates. A major route for removing the H from NADH is through the generation of gases, the cause of flatus. The main gas is H_2, with some CH_4 from the archaebacteria. Many bacteria contain an inducible formate hydrogenase, discovered by Marjory Stephenson in the 1930's, converting formate into CO_2 and H_2:

$$HCO_2H \rightarrow CO_2 + H_2 \quad (4)$$

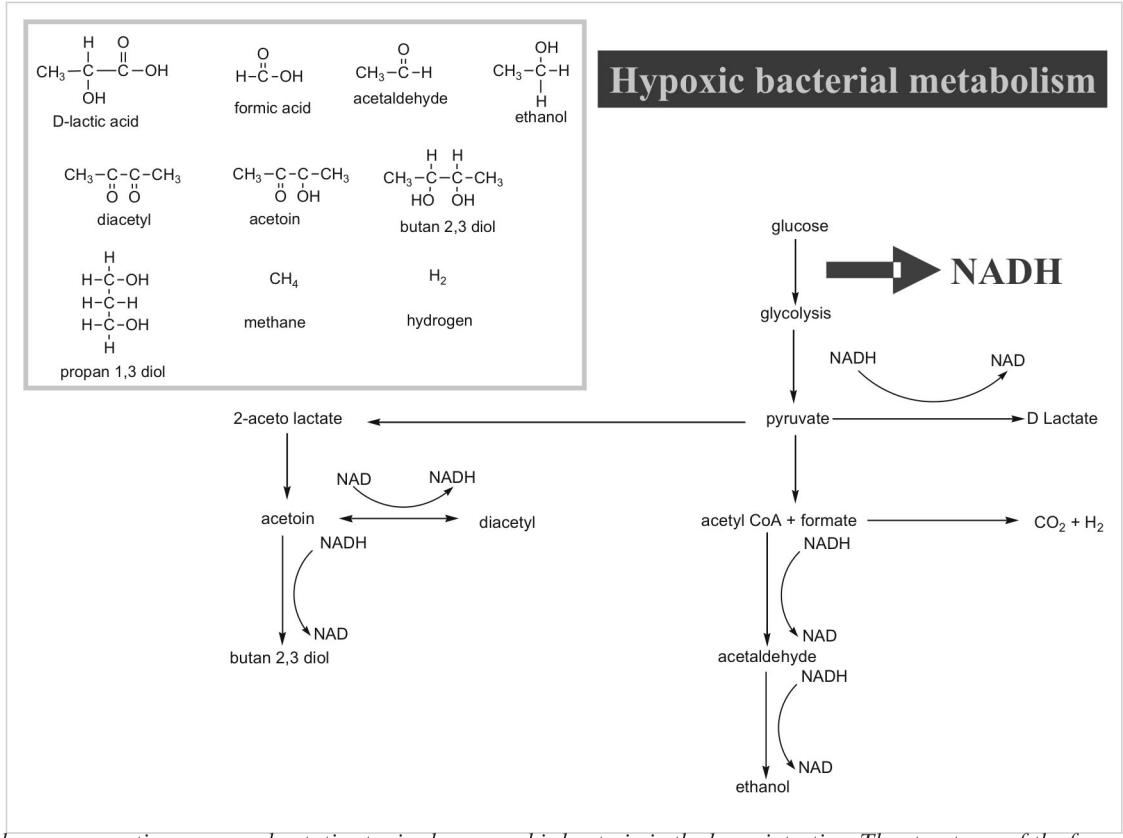

Fig. 5. *The pathways generating gases and putative toxins by anaerobic bacteria in the large intestine. The structures of the fermentation products and putative toxins are shown in the box.*

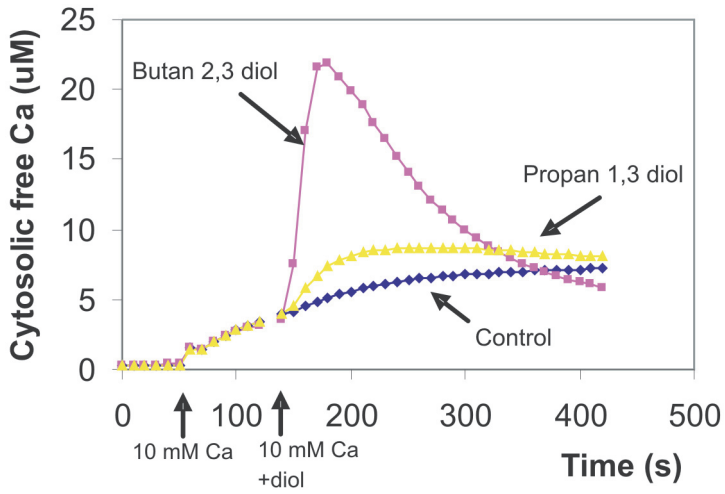

Fig. 6. *The effect of the bacterial fermentation products butane 2, 3 diol and propan 1, 3 diol on cytosolic free* Ca^{2+} *in* E. col. *JM109* E. coli *cells expressing the bioluminescent* Ca^{2+} *indicator aequorin were incubated in 25 mM HEPES, 125 mM NaCl, 1 mM* $MgCl_2$*, pH 7.5 for 1 min in a luminometer, and the luminescence counts recorded*$^{+}$*. 10 mM* Ca^{2+} *was then added for 2 min and then 100 mM meso butane 2, 3 diol or propane 1, 3 diol added for a further 5 min. Cytosolic free* Ca^{2+} *was then estimated by converting the luminescent counts to free* Ca^{2+} *using a standard curve. Results represent the mean =/− SEM from eight separate experiments. Temperature 21°C. (■) = 100 mM butane 2, 3 diol; (▲) = 100 mM propane 1, 3 diol; (●) = Control with no diol.*

The H_2 can then act as substrate for the methanogenic archaebacteria:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (5)$$

This explains why in some patients CH_4 is a useful clinical indicator, when lactose ingestion results in little or no H_2 in the breath. In this case, H_2 has been converted to methane. H_2 and CH_4 are therefore the main gases in flatus, with some H_2S from sulphurous bacteria. Absorption of these gases into the blood allows them to be detected in the breath.

In addition, there are several other pathways for removing the H from NADH, generating alcohols, diols, aldehydes, ketones and acids. These include acetaldehyde, acetoin, butan 2, 3 diol, dimethyl glyoxal, diacetyl, ethanol, formate, methane, propan 1, 3 diol and short chain fatty acids (Figure 5). Several of these have been detected in blood samples taken during a lactose tolerance test, indicating that colonic bacteria are actively metabolising lactose. Ironically, the one enzyme you don't want to see its

substrate is the β-galactosidase in bacteria, whose induction by lactose lead to the discovery of mRNA by Jacob and Monod, heralding the DNA revolution. Because once the β-galactosidase in the bacteria of the large intestine sees lactose, it metabolises it to gases and toxins.

A crucial group of putative toxins are the diols. Butane 2, 3 diol is a fermentation product of glucose, there being three naturally occurring stereoisomers: meso, 2R, 3R (−), 2S, 3S (+). Propan 1, 3 diol is a fermentation product of glycerol. Harden and Walpole[39] showed that fermentation products of *Aerobacterer aerogenes* differed from those produced by *E.coli*, consisting mainly of butane 2, 3 diol and acetoin. The production of acetoin, and its oxidation product diacetyl, is the basis of the Voges-Proskauser test widely used in bacteriology. Other bacteria capable of producing butane 2, 3 diol include: *Klebsiella*, *Enterobacter*, *Serratia*, *Bacillus*, *Lactobacillus*, and *Aeromonas*, all of which can be found in the human colon, though butane 2, 3 diol is not the only fermentation product. Three enzymes are required to produce butane 2, 3 diol from pyruvate: α-acetolactate synthase, α-acetolactate decarboxylase and acetion reductase. In *Enterobacter* and *Klebsiella*, butane 2, 3 diol production in culture requires an acid pH and the presence of acetate as a regulator.

The plasma concentration of butane 2, 3 diol in healthy humans or alcoholics is 10–100 μM. If the lactose in a glass of milk (approx. 10 g) were converted to butane 2, 3 diol, then the local concentration of this diol in the gut would be 100–200 mM. Butane 2, 3 diol, and propane 1, 3 diol, generate Ca^{2+} transients in *E. coli* (Figure 6). The role of cytosolic free Ca^{2+} as an intracellular signal is well established in eukaryotic cells[40]. However, the role of intracellular Ca^{2+} in bacteria is less well established[41]. Ca^{2+} transients and effects of diols on growth (Figure 7), suggest that sugar fermentation products may determine the balance of bacterial species in the colon. Changes in gene expression that lead to just a 10% decrease in generation time would, through Darwinian-Wallace selection, result in >90% of these bacteria dominating within 20 generation times, *i.e.* <24 h.

Other bacterial toxins include amino acid degradation products such as the phenol cresol, indoles and skatoles (Figure 2), or peptide and protein toxins. The bacterial toxins are primitive signalling molecules. They act on pathways that switch cells on or off in the nervous system, heart and muscles, and the immune system. Butane 2, 3 diol also appears to affect Ca^{2+} signalling in the cytosol and ER of tissue culture cells, and apoptosis (Trimby

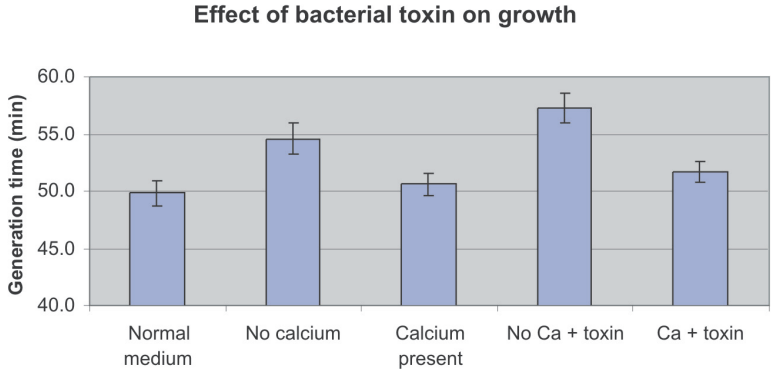

Fig. 7. *The effect of butane 2, 3 diol on bacterial cell growth. JM109 cells expressing aequorin were suspended in 25 mM HEPES, 125 mM NaCl, 1 mM $MgCl_2$, pH 7.5. 50 µl aliquots were added to 15 ml of growth medium LB (Luria Bertani) medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl pH 7.2) with carbenicillin (100 µg.ml^{-1}) and 5 mM EGTA with or without butane 2, 3 diol or 10 mM Ca with or without butane 2, 3 diol. The cells were then incubated at 37°C with vigorous shaking for up to 24 h and samples taken every hour to measure viability by their ability to grow as assessed by the absorbance at 600 nm. The generation times were then estimated. Colony counts confirmed the viability of the cells under all conditions. Results represent the mean +/− SEM of three determinations. Statistical significance: 5 mM EGTA + 100 mM butane 2, 3 diol versus 10 mM Ca^{2+} + 100 mM butane 2, 3 diol* $P = 0.0002$*; 5 mM EGTA versus 5 mM EGTA + 100 mM butane 2, 3 diol* $P = 0.008$*; 10 mM Ca^{2+} versus 10 mM Ca^{2+} + 100 mM butane 2, 3 diol* $P = 0.09$*. All other comparisons* $P > 0.10$.

and Campbell, unpublished). In a model invertebrate system – the water flea *Daphnia* – lactose induces heart arrhythmia[42], similar to that in 25% of our patients with lactose intolerance.

The idea that bacteria in the gut can release toxins is over 100 years old. Elie Metchnikoff (Figure 8) was a founder of modern immunology, discovering phagocytes for which he was awarded one of the earliest the Nobel Prizes with Ehrlich in 1902. However, his real intellectual 'baby' was the idea that gut bacteria produce toxins. He wrote; 'The large intestine must be regarded as one of the organs possessed by man and yet harmful to his health and his life. The large intestine is the reservoir of the waste of the digestive processes, and this waste stagnates long enough to putrefy. The products of putrifaction are harmful.' 'Bacterial putrefaction is the cause of all disease.' He even carried out experiments injecting cresol and other putative toxins into mice, showing they could be lethal[43,44].

Fig. 8. *Elie Metchnikoff (1845–1916), pioneer of the bacterial toxin hypothesis.*

A key issue is whether sugars such as lactose can induce gene expression and growth of toxin-producing bacteria, as opposed to those that simply produce gas. And also whether there are just one or two species of bacteria capable of producing large amounts of toxins, analogous to *Helicobacter* whose discovery revolutionised the treatment of stomach ulcers.

The science of clinically managing lactose intolerance

Irritable bowel syndrome (IBS), with unexplained gut problems – pain, distension, gas, tummy rumbling, diarrhoea or constipation – is the most common problem faced by gastroenterologists. But patients may also complain of non-gut (systemic) symptoms, including severe recurrent headaches, chronic fatigue, loss of concentration and a dizzy head, muscle and joint pain, allergies such as eczema, pruritis, urticaria, asthma, sinusitis, rhinitis and hay fever, heart palpitations, and increased micturition[1–3,45] (Table 3). It is these non-gut symptoms, and their irregular occurrence, that have confused diagnosis. A high percentage of these patients are intolerant to lactose[2]. In some cases this explains all their symptoms, while others have a wider food intolerance[8,9],

Table 3 *Gut and systemic symptoms of people with lactose intolerance*

Symptoms of lactose intolerance	No. of people with symptom (% of total with lactose intolerance)
A. Gut related	
Abdominal pain	100%
Gut distension	100%
Borborygmi (tummy rumbling)	100%
Flatulence (gas)	100%
Diarrhoea	70%
Constipation	30%
Nausea	78%
Vomiting	78%
B. Systemic	
Headache and light headedness	86%
Loss of concentration and poor short term memory	82%
Chronic severe tiredness	63%
Muscle pain	71%
Joint pain, and/or swelling and stiffness	71%
Allergies, such as:	40%
Eczema (skin rash)	
Pruritis (itchy skin)	
Rhinitis (runny nose)	
Sinusitis (stuffed up sinus)	
Asthma (wheezing and shortness of breath)	
Heart arrhythmia	24%
Mouth ulcers	30%
Increased frequency of micturition (weeing)	Less than 20%
Sore throat	Less than 20%

Systemic = around the body.

often to other carbohydrates such as fructose and starch in particular forms, and foods containing stachyose or raffinose (Figure 1). Their threshold to these non-lactose foods varies considerably, confusing diagnosis and treatment.

We have now analysed data from several hundred patients referred to our food intolerance clinic, the first in Wales. Our recommended diagnosis and management of lactose intolerance now is[2,3]:

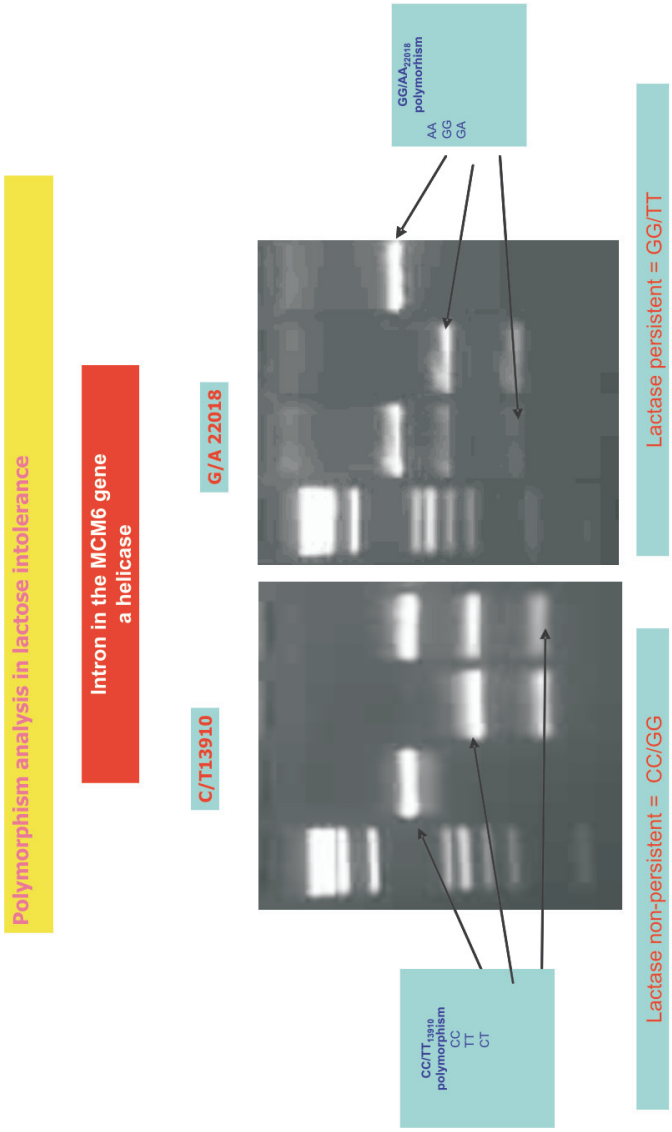

Fig. 9. *Polymorphism analysis in lactose intolerance. PCR was carried out on DNA isolated from buccal (mouthy swab) samples as described in ref. 2. Introduction of restriction sites enabled samples homozygous for CC/GG, heterozygous for CT/GA or homozygous for TT/AA to be distinguished.*

1. Buccal (swab inside the mouth) sample for C/T_{-13910} genetic analysis (Figure 9).
   a. If CC, immediately remove of all lactose from diet. If symptoms improve after one month diagnosis of lactose intolerance confirmed.
   b. If CT or TT carry out lactose tolerance test.
2. New recommended lactose tolerance test:
   a. 50 g (1 g/kg for children) dissolved lactose.
   b. Record breath hydrogen and methane for 6 h.
   c. Record all symptoms for 48 h.
   d. If the breath test is positive, *i.e.* H_2 rises to >20 ppm or CH_4 >5 ppm over the nadir, then change to a lactose free diet.
   e. Every patient followed up in 12 weeks for a definitive diagnosis.
   f. If the breath test is negative, but there is a significant increase in symptoms after the lactose load, the patient should undergo a supervised trial to determine their lactose threshold.
   g. Family studies should be carried out to determine other affected individuals.
   h. Hypolactasia caused by infections such as *Giardia* or rotavirus should be investigated if there is no evidence of family history.
3. Give advice on lactose free meals, and the danger of hidden lactose.
4. Follow up in 1 year.
5. Calcium and vitamin D status should be monitored, and advise on the use of probiotics.
6. Patient advised to keep a food diary to identify culprits if caught out.

Several hundred patients with unexplained gut and other symptoms have now been referred by GPs and consultants to our clinic. The patients were diagnosed using the new clinical procedure into those with lactose intolerance and those not lactose intolerant. Those diagnosed without lactose intolerant were all C/T or T/A. But there was no difference in the total number of symptoms reported using C/T_{13910} genotyping between the lactose intolerant and non-lactose intolerant groups. However, a major difference was found between these two groups when lactose was removed from the diet (Figure 10). 100% of CC/GG, 83.3% of CT/GA patients and 76.3% TT/AA were diagnosed as lactose intolerant. Thus for CC/GG a breath test is unnecessary. This is of considerable benefit, as many suffer badly from prolonged symptoms after the

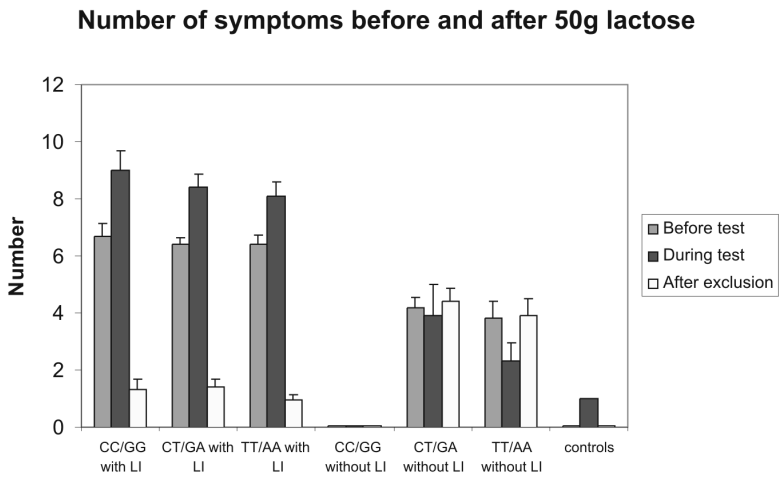

Fig. 10. *Number of symptoms before and after a lactose tolerance test. Individuals were given 50 g lactose (1 g/kg for children), and symptoms recorded with severity, using a standard clinical scale from 0–10, for 48 hours. Breath hydrogen and methane were also measured every 30 min for 6 hours. The number of symptoms was recorded prior to the test, during the test and after 1 month on a lactose free diet. These were plotted, after separating the patients into the polymorphism groups CC/GG, CT/GA and TT/AA. Control subjects were normal volunteers with no history of gut and other symptoms.*

50 g lactose load used in the lactose intolerance test. This major revision of the clinical management of lactose intolerance has not only benefited individual patients, but has resulted in huge savings for the NHS. Many of our patients were constantly seeing their GPs and specialists, and were taking a cohort of drugs. Most are now off all drug therapy and rarely have to see a doctor! Coming off lactose reduced the number of symptoms from an average of nine to one (Figure 10).

Probiotics are friendly bacteria such as *Lactobacillus* that can be taken with food and are claimed to help digest foods when someone has an intolerance. But the scientific evidence for long term benefits as opposed to short term placebo effects is weak. There is a suggestion that dietary intake of lactose prior to the test may have an inverse affect on the breath test[46]. This is consistent also with the increased sensitivity experienced by some patients when they eliminate lactose from their diets, while higher lactose intake prior to the breath test may reduce symptoms and gas score, when compared to patients who have a low lactose intake prior to the test (50 g lactose). This again highlights the limitations of the current breath test.

In our cohort of >250 patients, all referred with unexplained gut and other symptoms, the percentage of total symptoms (abdominal + systemic) reported during the lactose tolerance test was significantly higher in lactose intolerant individuals than those who had gut symptoms but turned out to be lactose tolerant (>40%, $P = 0.01$, $n = 130$). However no significant difference was observed when total symptoms were compared by C/T_{13910} lactase genotyping alone ($P = 0.1$). In contrast significant differences were observed between lactase genotype when symptoms were categorised to abdominal, neuromuscular, cardiac, oral and allergy in lactose intolerant and tolerant patient groups ($P = 0.01$). Palpitations (cardiac) symptoms were only observed in lactose intolerant patients, being highest in CT genotypes, a finding consistent with our observations that hyperlipidaemia is more prevalent in lactose intolerant CT genotypes. Oral symptoms such as mouth ulcers were significantly higher in intolerant patients when compared to tolerant, being highest in the CC genotype, with a low oral symptom prevalence in tolerant TT genotypes and no reports from CT genotype tolerant individuals. This is consistent with our hypothesis that intestinal bacterial toxins and high levels of hydrogen and methane gas lead to epithelial hypersensitivity and ulceration. Anal hypersensitivity is well described in irritable bowel syndrome but with unknown cause. Constipation was only reported in the lactose intolerant patient group. General allergy symptoms were also markedly higher in the intolerant patients when compared to tolerant. Abdominal symptoms were higher (>35%) in lactose intolerant patients compared to those with symptoms, but who turned out to be lactose tolerant, but not distinguishable by genotype. Similar findings were observed for neuromuscular symptoms, including muscle and joint pain (>65% higher in lactose intolerant).

The differences in type of symptom in referred patients eventually diagnosed as lactose intolerant emphasise the benefit of genotyping in the clinical management of this condition.

The problem of lactose in food

Dairy products, together with foods and drinks containing milk, are abundant in the supermarket. Some are obvious, others are not. Food labelling is poor. Many patients do not realise that if dried milk powder, condensed or evaporated milk is used, then this will add more lactose than the equivalent amount of milk (Table 4). Recipe books sold with home bread makers recommend adding

Table 4 *Lactose content of some foods and drinks*

Food or drink	Relative lactose content	Lactose as a % (*i.e.* g/100 g or 100 ml)
Dairy		
Lactose	Complete	100%
Whey	Very high	70%
Non-fat dry milk powder	Very high	50%
Cow's milk	High (47 g/litre)	5%
Goat's milk	High (44 g/litre)	4%
Reduced lactose milk	Low	1%
Lactose-free milk	Very low	0–0.5%
Sour milk	High	4%
Buttermilk	High	4%
Commercial yogurt	High	4%
True natural yogurt	Moderate to low	2%
Cheese	Moderate	
Feta	Quite high	4%
Diet cottage	Quite high	3%
Parmesan (hard block for fresh grating)	Low to moderate	1%
Parmesan (grated in packet)	Can be quite high	3%
Cheddar	Trace to low	0–2%
Camembert	Not very high	0–1%
Edam	Low	0–1%
Some cheese products	Can be high	5–10%
Cream	Moderate	4
Butter	Low	1
Clarified butter	Very low	Should be 0
Chocolate	High	?
Milk proteins (casein etc.)	Low, but can be present	0–2

Table 4 *Lactose content of some foods and drinks (continued)*

Food or drink	Relative lactose content	Lactose as a % (*i.e.* g/100 g or 100 ml)
Non-dairy – 'hidden'		
Processed meats such as sausages and salamis	Added, can be high	?
Breads and cake mixes	Added, can be high	?
Slimming bars	Added, can be high	?
Powdered sauces	Added, can be high	?
Reduced fat foods *e.g.* mayonnaise and biscuits	Added, can be high	?
Lager	Added, can be high	?
Powdered or artificial fruit juice	Added, can be high	?
Fresh meat from the butcher	None	0
Fresh fruit	None	0
Fresh vegetables	None	0
Eggs	None	0
Pure squeezed orange juice	None	0
Lactic acid (lactate)	None	0

The numbers in this table have been rounded up and thus are very approximate. ? = no accurate levels known. 1% is equivalent to about 20 ml milk, a typical amount in a cup of tea, 10% to 200 ml. A normal glass of milk contains about 200–250 ml, a block of butter weighs 250 g, and a block of cheese perhaps 250–500 g. A spoonful of Parmesan cheese, freshly grated = about 30 g, equivalent to 0.3 g lactose or 6 ml milk.

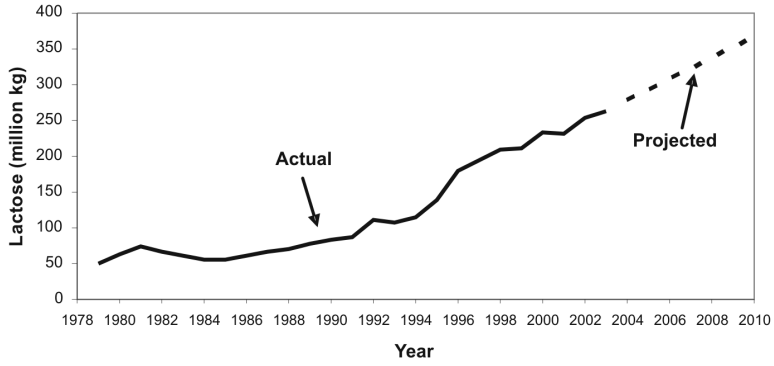

Fig. 11. *The production of lactose in the USA over the past 15 years and predicted.*

dried milk powder to many recipes. Many Asian restaurants are now using cream and evaporated milk instead of the classic ingredient in Asian cooking, coconut milk which is lactose free. Then there is the problem of pharmaceuticals. The major filler is usually lactose. And many fluids used clinically in enteral feeding contain lactose. Many people do not realise that products such as whey contain all the lactose in milk. In order to isolate casein, the major milk protein, the milk is first centrifuged and the cream skimmed off the top. The remaining fluid, the skimmed milk, is acidified to a pH of 4.7 so that the casein precipitates. The precipitate is removed leaving the supernatant – whey. This contains 20% of the original protein in the milk, and crucially all the lactose. Whey is added to many foods, but lactose itself may not be on the label. It is the whey that is used to make lactose itself, by evaporation to crystallisation.

A further major problem is that of 'hidden' lactose (Table 4). Lactose can be added to breads, cake mixes, sausages and processed meats, and even chicken and drinks, without being properly labelled[3]. Labelling regulations have changed in Europe since 2005. But many food manufacturers have been slow to respond to this. The US alone produces some 300 million kg of lactose per year (Figure 11). Everyone can tolerate some lactose. You would have to eat a kilogram of Parmesan cheese to be equivalent to a glass of milk. Sprinkling a teaspoon on pasta should therefore be no problem. But, the amount of 'hidden lactose' can be considerably more than this. We estimated that the amount of lactose in a slimming drink taken daily by one of our patients was equivalent to 1–2 litres of milk!

Darwin's illness revealed

'I have had a bad spell, vomiting every day for eleven days and some days after every meal'. So Charles Darwin (1809–1882) wrote in a letter to his friend Joseph Hooker in December 1863. Later he wrote to his father, a doctor himself, 'The sickness starts usually two hours after a meal'. In fact Darwin had already suffered chest pain and heart palpitations in December 1831 while staying in digs at Plymouth awaiting better weather for the Beagle to depart. He told no one until years afterwards for fear he would not be allowed on his 'trip of a lifetime'. For over 40 years Charles Darwin was frequently ill. He lived in the Kent village of Down(e) as a semi recluse because he was ill so much, sometimes for days on end. He failed to go to the famous Oxford debate in 1860 because he was in the middle of one of his attacks. He saw some twenty doctors, including his father, and tried dozens of remedies None really worked, though Darwin did seem to improve when he underwent Gully's water therapy at Malvern. The only time he got better was when, by chance, he came off milk.

Darwin's symptoms fit exactly systemic lactose intolerance[47] (Table 5). Darwin suffered from stomach ache, flatulence, headaches and a swimming head, vomiting, and chronic fatigue, joint pains, skin rashes and boils, mouth ulcers and heart palpitations. And he was often depressed. Many proposals have been put forward to explain his illness, including arsenic poisoning, Chagas' disease and psychosomatic disorders such as bereavement syndrome, because of the death of his mother at the age of 8. None match his symptoms. Six pieces of evidence support our hypothesis that Charles Darwin suffered from lactose intolerance:

1. Darwin's symptoms fit exactly those we have identified in systemic lactose intolerance.
2. The timing of his vomiting and gut pain was 2–3 hours after a meal, just as expected for lactose to reach the large intestine.
3. His wife Emma used milk and cream constantly in her recipes.
4. There was a clear history of illness in the Darwin family, in his children and on the Wedgwood side of the family.
5. Darwin did not suffer from his illness on the Beagle (1831–1836) where there was no fresh milk. He just had sea sickness and a fever in South America, probably typhoid.
6. Darwin only got better when, by chance, he came off milk.

Table 5 *Systemic lactose intolerance versus Darwin's illness*

Symptoms of systemic lactose intolerance	% people with lactose intolerance who have this symptom*	Darwin's description of his symptoms	Occurrence of Darwin's symptoms
Gut symptoms (pain, bloating, diarrhoea)	100%	Stomach ache	Common
Flatulence (farting)	100%	Flatulence (belching)	Common
Headache	86%	Headache	Common
Light headedness and loss of concentration	82%	Swimming head and difficulty to concentrate	Common
Nausea and vomiting	78%	Vomiting	Very common
Muscle and joint pain	71%	Rheumatic pain	Often
Tiredness and chronic fatigue	63%	Chronic fatigue and exhaustion	Very common
Allergy (eczema, hay fever, rhinitis, sinusitis)	40%	Skin rash and boils	Often
Mouth ulcers	30%	Mouth sores	Common
Heart palpitations	24%	Palpitations in the chest	Common
Depression	Common, but not quantified	Depression	Frequent

*Proportion of people diagnosed as lactose intolerant who have this particular symptom within 48 h of taking lactose. Darwin's occurrence is based on his notes and letters during periods of the attacks.

What Darwin missed

Charles Darwin not only missed the cause of his life-time illness, but he also missed **the** most important aspect of our own evolution[48]. In 'The Origin' one chapter (4 in the 1st edition of 1859, and 6 in the 6th edition of 1868) is entitled 'Difficulties on/of Theory'. These were not the famous difficulties highlighted by his opponents. Rather they were Darwin's difficulties. He wrote, *Natura non facit saltus(m)* – Nature takes no leaps. He saw no way to explain how small change by small change could lead to the **origin** of the electric organs of fishes, the luminous glands of fireflies and glow-worms, and even the eye. Yet he failed to highlight the most obvious Rubicon crossed by our evolutionary ancestors – the breast and its ability to produce milk. Even in 'The Descent of Man' this aspect of human biology has just a cursory mention. So does the **principle** of natural selection alone explain this unique feature in the evolution of mammals, and our own species? Which came first, lactose or lactase? How does a new protein such as lactase, or a process such as lactose production, originate and develop **before** it can respond to the forces of natural selection?

Lactose is restricted to the milk of terrestrial mammals, but cerebrosides (glycosyl ceramides) are present in the diet of all vertebrates. The origin of intestinal lactase is therefore likely to be its phlorizin, or rather its glycosyl ceramidase, activity. Of the common sugars found in plants, the order of sweetness is fructose > glucose = sucrose > lactose ($\beta > \alpha$). Milk is not sweet because lactose has 1/6 sweetness of sucrose. A non-sweet sugar would be much less prone to attracting insects to the breast. Then molecular biodiversity took over[49] – the evolution of the diversity of lactase levels within the human population.

Domestication of animals and agriculture, and cheese-making, began some 10,000 years ago[50]. Legend has it that an Arabian merchant was carrying a pouch made of sheep stomach, full of milk. The heat of the sun, together with the release of rennet from the stomach, caused the milk to separate into the solid curds and the liquid whey. Rennet contains the protease rennin (not to be confused with renin). This cleaves a glycopeptide from casein to form paracasein, which then binds Ca^{2+}, causing the protein to precipitate to form the curd. Dairying proper did not begin until 6,000–8,000 years ago, originating in the great civilisations of Babylon and Assyria from Mesopotamia[50]. The use of milk probably began with camels and goats. This was followed some 1,000–2,000 years later by the use of milk from sheep and cows.

Archaeological data from pots and other artefacts, together with ancient writings, seals and drawings, puts the origin of milk drinking even more recent, in Mesopotamia and Egypt 5,000 years ago, and in Africa 7,500 years ago.

10,000 years ago a huge geological change occurred that had a major influence on the current prevalence of lactase persistence in the white Northern Europeans. The last ice age ended, having begun in the Pleistocene Era 2.5 million years before, freeing Europe from ice. Given a generation time in humans of 20–30 years, and an origin of dairying some 6,000 years ago, this means that there have been only some 200–300 generations to select the 90% prevalence of lactase persistence in Northern Europeans today. In the nomads of Asia and Africa camel's milk, cheese and yoghurt are major components of their diet. Humans moving north into the plains of Europe would have needed a transportable, and continuous, food supply.

Three hypotheses have been proposed for the selective advantage of lactase persistence; *i.e.* keeping lactase after weaning and thus being lactose tolerant, rather than lactose intolerant:

1. A major food source for nomadic populations.
2. A source of water in desert zones.
3. A source of calcium in geographical areas where sunlight is poor.

But the real puzzle is, why do all mammals, including most humans, lose most of their lactase after weaning? Why not keep it all? Linnus Pauling argued that keeping a protein, such as lactase, would be energetically wasteful, when there was no dietary source of its main substrate. But there must be another selective advantage. Non-milk drinking communities have a very different diet from the first dairying groups of humans. In addition to fish and meat, the diet of Asians and Africans contains brown and white rice, soya, beans and pulses, exotic fruits such as bananas, oranges and lemons, spices and nuts. Most of these only became available in Europe after the 15th and 16th century voyages of explorers. Until then, the European diet consisted mainly of dairy products, animal and bird meat, eggs, a few natural fruits when in season such as apples and berries, wild herbs, and bread, once agriculture was in full swing. Many exotic spices and fruits may contain substances analogous to phlorizin, in that they may be hydrolysed by lactase to products that are potentially poisonous and pathogenic. So there would be a clear selective advantage of only keeping the minimum amount

of lactase, necessary to digest the small amount of glycosyl cerebrosides in the diet.

Attempts to develop mathematical models for lactase persistence/non-persistence[51], producing a population where >80% are lactase persistent, assume:

1. The ancestral state was lactase non-persistence; *i.e.* loss of lactase on weaning.
2. A mutation occurred about 10,000 years ago leading to lactase persistence.
3. Lactase persistence has a Darwin-Wallace selective advantage.
4. This selective advantage is reflected mathematically by a high selectivity coefficient.

A founder effect with genetic drift seems the most plausible explanation for the world-wide distribution of the four main alleles A, B, C and U, and the prevalence of lactase persistence or non-persistence in particular ethnic or genetic groups, with loss of certain haplotypes such as U outside Africa. A delay in weaning, concomitant with retention of lactase, has been proposed to have a selective advantage in monkeys, as the young would be protected longer and births of future siblings would be more spaced out. However this does not explain the selective advantage of retaining large levels of lactase in white Northern Europeans.

Many hypotheses about the evolution of lactase persistence/non-persistence, and mathematical models, are flawed because:

1. Natural selection works on the phenotype in populations, not the genes of individuals.
2. The mechanism of lactase persistence/non-persistence involves first a change in the number of cells expressing lactase[3,25,52], and only then regulation of the level of lactase within the cell.
3. There is a huge molecular biodiversity in the level of lactase within and between genetic and ethnic groups.
4. Natural selection does not take account of the reproduction time.
5. The numbers of individuals where selection was acting are too small to allow mathematical models that use probabilities, and assume populations of 'infinite' size, In the Galapagos finches, evolution of new species occurred through just a few 100 individuals in each generation.

Lactose intolerance illustrates the Rubicon principle[35], where a threshold has to be crossed before biological experience begins.

Five such Rubicons need to be explained in the evolution of lactase, and lactose intolerance:

- The origin of mammals, lactose and the cells of the mammary gland.
- The origin of lactase and the specialised cells in the small intestine.
- The switch that determines whether an intestinal cell expresses lactase or not.
- The level of lactose that generates significant gas and toxins when it reaches the bacteria in the large intestine.
- The threshold that determines the experience of a particular symptom.

Is milk bad for you?

There are reports claiming that, for adults, milk is beneficial, and may reduce, for example, heart disease[53]. Others claim that milk intake correlates with heart attacks, certain types of cancer and even Parkinson's disease[54]. Milk is highly nutritious, containing proteins, fats, salts, and vitamins. But can any potential harmful effects of milk be attributed to lactose? The key is mechanism – the bacterial toxin hypothesis. Only when studies correlating milk consumption with an end response, such as heart disease or cancer, identify a mechanism will there be clarity. A further reason for confusion is the lack of separating cohorts into ethnic groups, and genetically based on the C/T polymorphism[33]. Without this, any conclusions will be essentially meaningless. A further problem is that of biochemical individuality[35,49,55]. The study of lactose intolerance demonstrates the need for a new approach to epidemiology, where mechanism and individual molecular diversity within a population are taken into account.

The future

Science is about discovering how the Universe works, from the big bang to how bacteria naturally evolved to be resistant to antibiotics. Lactose intolerance highlights a molecular mechanism, forgotten for 100 years, that is likely to be of major importance in many unsolved diseases, such as the diabetic epidemic in Asians, reactive and rheumatoid arthritis, multiple sclerosis and some cancers. Lactose intolerance also illustrates the need for a new approach to how we teach medicine, moving away from box

ticking and the heavy reliance on drugs to an understanding of mechanism.

Coming off lactose has transformed our lives and those of our families, as well as the lives of several hundred patients. They now feel wonderful, with a massive reduction in drugs and visits to the doctors, and even coming off surgery lists. Three of our patients even became unexpectedly pregnant after coming off lactose. It is hardly surprising Darwin missed his own lactose intolerance. This condition was not recognised in the 19th century. But how did he miss this most obvious characteristic in our own evolution? The science of lactose intolerance can reveal the answer to the problem Darwin never really addressed, the true 'origin' rather that the 'development' of the human species[48,49].

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WEB SITES

1. www.lactose.co.uk, Conventional information about lactose intolerance and selling products.
2. www.lactoseintolerance.co.uk, Conventional information about lactose intolerance and milk allergies, with symptoms, treatments, and 'hidden' milk products.
3. www.lactoseintolerant.org, Conventional information only about lactose intolerance and milk allergies.
4. www.welstonpress.com, The only one at present that deals with systemic symptoms caused by lactose