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Faculty of Medicine

Department of Clinical Biochemistry

GUT BACTERIAL METABOLITES IN URINE OF PREMATURE BABIES

(effects of a probiotic-Lactobacillus GG)

by

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MB

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE
DEPARTMENT OF CLINICAL BIOCHEMISTRY
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GUT BACTERIAL METABOLITES IN URINE OF PREMATURE BABIES
effects of a probiotic-*Lactobacillus GG*

by Song Chen

The intestinal microflora in preterm infants nursed on a neonatal intensive care unit is different from that of more mature individuals. Because of their immaturity and treatment with broad spectrum antibiotics during their early life, these babies are at increased risk of intestinal bacterial colonisation with pathogens, which may cause serious illness. Colonisation with a non-pathogenic organism (probiotic) might protect them from bacterial infections arising from the gut. However, introduction of a foreign organism might have widespread effects on the metabolic activity of the gut bacteria. In a randomised double blind study, the probiotic *Lactobacillus GG* was fed for the first time to well, premature babies. As a part of this trial, the effects of feeding *Lactobacillus GG* on urinary excretion of three gut bacterial metabolites: phenol, ρ-cresol and 2,3-butanediol was investigated.

Methods using high performance liquid chromatography (HPLC) and gas chromatography (GC) were investigated for analysis of total urinary phenol and ρ-cresol from premature babies. An anion exchange extraction procedure with HPLC analysis worked well for aqueous standards but was not satisfactory for urinary analysis. A gas chromatographic method was set up and investigated, using diethyl ether to extract urinary phenol and ρ-cresol. These compounds were well resolved. Recoveries and analytical precision were good. The method was sensitive, simple and suitable for batch analyses. A β-glucuronidase/sulphatase preparation was used successfully for hydrolysis of conjugated urinary phenol and ρ-cresol.

During the first week of life, phenol was present in all urine samples from *control* babies, sometimes in high concentration. ρ-Cresol, in contrast, was detected in only one sample. Thereafter, phenol and ρ-cresol were found in the majority of samples, with higher concentrations of ρ-cresol than phenol. This pattern reflects the known sequence of bacterial colonisation of the intestine after birth. The major phenol producing organism was probably *Enterococcus*. *Clostridium sp* were associated with ρ-cresol production. *Lactobacillus GG* colonised 9 of 10 premature babies. Colonisation had little effect on the species, or number, of other faecal bacteria, and did not have significant impact on urinary phenol, or ρ-cresol excretion.

2,3-Butanediol was detected in only one urine sample from control babies in the first week of life. Thereafter, it was present in around two thirds of samples. Colonisation with *Lactobacillus GG* had no effect on urinary 2,3-butanediol excretion. The association between urinary 2,3-butanediol excretion and faecal growth of diol-producing bacteria was investigated. A diol-producing organism was grown from faeces on 53% of occasions when the diol was excreted in urine, and 32% occasions when it was not. The bacteria isolated were most often *Klebsiella sp* and *Enterobacter sp,* which are potential pathogens. On 6 occasions, no diol was detected in urine although diol-producing bacteria were present in faeces. Excretion of 2,3-butanediol in urine may indicate that there is abnormal gut colonisation with diol-producing micro-organisms. However, its absence from urine does not definitely exclude abnormal colonisation.

Antibiotic regimens, including erythromycin, flucloxacillin and netilmicin, were associated with decreased faecal anaerobes and significantly decreased excretion of ρ-cresol. There was no effect on urinary phenol or 2,3-butanediol excretion.

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CHAPTER ONE

INTRODUCTION

1.1 NORMAL MICROFLORA OF THE HUMAN GASTROINTESTINAL TRACT

The bacterial inhabitants of the human gastrointestinal tract constitute an enormously complex ecosystem that includes both aerobic and anaerobic microorganisms. It is estimated that the number of microbial species in the human intestine exceeds 400 [1]. Despite this, the stability of the flora is rather remarkable.

Bacteria from the oral cavity are washed with saliva into the stomach, where the vast majority are destroyed by gastric acid [2]. The small intestine constitutes a zone of transition between the sparsely populated stomach and the luxuriant bacterial flora of the colon in the adult bowel. The bacterial concentration is 10^3-10^4 colony forming units (cfu)/ml. The predominant species are aerobic and gram-positive [3,4]. In the distal ileum, gram-negative bacteria begin to outnumber gram-positive organisms. A significant change in the intestinal microflora in humans is seen below the ileocecal valve. Within the colon the number of bacteria increases sharply, the concentration being 10^{11} - 10^{12} cfu/ml. Nearly one third of faecal dry weight consists of viable bacteria [1,5]. Anaerobic bacteria outnumber aerobes by a factor of 10^2-10^4. The predominant isolates are *Bacteroides*, *Bifidobacterium* and *Eubacterium* [1-4,6-8]. Anaerobic gram-positive cocci (*peptococci* and *peptostreptococci*), *Clostridia, enterococci* and various species of enterobacteriaceae are also common [9].

1.2 MICROFLORA IN THE COLON OF NEWBORN INFANTS

The normal fetal bowel is sterile until shortly before birth, as long as the amniotic membrane remains intact. After birth, the neonate rapidly acquires commensal bacteria that colonise the skin and mucous membranes [10]. Colonisation of the gastrointestinal tract of newborn infants occurs within a few days of birth [11], mainly by facultative bacteria that can grow in the presence or absence of oxygen [10]. The initial colonisation in normal term babies is by *Escherichia coli* and certain types of

streptococci, which later decrease in numbers as lactobacilli and *Bacteroides* emerge as the dominant bacterial inhabitants [12]. Anaerobes colonise the neonatal gastrointestinal tract from the end of the first 1-2 weeks of life, and are greater in number and variety than aerobes or facultative species [10]. The predominant anaerobic organisms are *Bifidobacteria* and *Bacteroides.* The acquisition of intestinal flora in the neonatal period is different in the premature and critically ill babies nursed in neonatal intensive care units. Bell et al reported that these babies were significantly more likely to grow a single organism from stools compared with healthy babies [13]. It is suggested that factors such as prematurity, birth by caesarean section, parenteral antibiotic treatment and being nursed in an incubator may influence early colonisation in newborns [14].

1.3 FACTORS INFLUENCING COLONIC COLONISATION

There are a number of factors influencing colonisation in the colon. Host, microbial and environmental factors are all important.

1.3.1 Host factors influencing colonic colonisation

1.3.1.1 The colonic mucosa

The colonic mucosa, as other mucosal surfaces in humans, serves as an important interface between elements from the external environment and the internal milieu.

The intestinal surface has a protective role. Firstly, the mucosal surface comprises secreted mucus and the microvillus membrane (MVM) [15]. Together, these provide a major physical barrier to the attachment, uptake and penetration of harmful agents in the gut lumen, including bacteria and viruses and their products, as well as other potentially noxious substances. Secondly, the intestinal surface also serves as a selective inlet for beneficial substances such as nutrients and antibodies. The composition of carbohydrate side chains on glycoproteins, glycolipids in mucus and the microvillus membrane, may influence the specific adherence of bacteria and their toxins to the intestinal surface [15]. Adherence is a necessary prerequisite to bacterial colonisation and toxin penetration. Carbohydrate moieties on mucin represent binding sites for intestinal microorganisms. Changes in membrane composition also may determine whether bacteria bind to the cell.

The bacterial population in the colon forms thick layers which are embedded in the mucus gel. Many indigenous species are able to degrade mammalian mucus [16]. Yoshioka et al have shown the capacity of a variety of bacterial species present in the

colon to hydrolyse the carbohydrate side chains of mucin [17,18]. Monosaccharides produced are used in energy-producing reactions in fermentation processes. Glycoproteins may serve as an important carbon and energy source in all regions of the bowel, contributing to the stability of the flora even when the host is fasting or near-fasting [19]. The colonic mucosal cell may also derive some nutrition from macromolecular components of lysed microbial cells, and the products of the energy-yielding metabolism of such cells [13].

1.3.1.2 Antibodies and cell-mediated immunity

The second effective mechanism of protection in the intestinal barrier is by immunoglobulins and local cell-mediated immunity [15]. IgA in the intestine acts to modulate the further uptake of antigen by interfering with antigen attachment and uptake at the epithelial surface. The concentration of IgA in the stools of newborns is low [20]. Cell-mediated immunity is capable of responding to invading foreign antigens. However, the gut-associated lymphoid tissue develops slowly [21]. This may be related to the lack of intestinal microflora in fetal life. With advancing age, and increased exposure to microbial and dietary antigens, the relative proportion of B-lymphocytes with surface immunoglobulin becomes higher [15]. The ontogenic development of gut immunity of the neonate can be modified by a number of factors, including gestational age and nutritional state [21]. There is a consistent impairment of cellular immunity in low birth weight infants. The absolute numbers of T-lymphocytes in the intestine is reduced in small for gestational age infants [15].

1.3.1.3 Lactoferrin

Almost all bacteria require iron for growth. Because of their iron sequestering properties, the iron-free (apo) forms of the proteins, lactoferrin and transferrin, are able to impede iron utilisation by bacteria [22].

Lactoferrin is present in milk. It has a bacteriostatic effect and, in some cases, a bactericidal effect in vitro on a wide range of microorganisms, including gram-positive and gram-negative aerobic and anaerobic bacteria, and yeasts [23]. There may be additional mechanisms involved in the antibacterial action of lactoferrin: for example, blockade of microbial carbohydrate metabolism [24] or destabilisation of the bacterial cell wall, perhaps through binding of calcium and magnesium [25]. Lactoferrin may synergise with other anti-bacterial proteins present in milk, such as lysozyme [26]. Lysozyme modifies the bacterial cell walls, and lactoferrin then causes bacterial

agglutination. Antibodies may also enhance the bacteriostatic action of lactoferrin. It has frequently been suggested that the anti-microbial activity of lactoferrin plays a part in directing the intestinal colonisation of the newborn, particularly in preventing colonisation by pathogenic organisms. The conditions in the intestine of the newborn may favour the action of lactoferrin. However, proteolytic enzymes in the intestine may decrease its action [22].

1.3.1.4 Maturation of the infant affects colonisation in the colon

After birth, maturation of the intestinal barrier appears to occur along with the normal development of other organ systems [15]. Immaturity of the barrier may underlie susceptibility to certain diseases seen in the neonatal period such as infectious diarrhoea, necrotising enterocolitis (NEC) and some allergic diseases. It may also be implicated in the development of diseases appearing later in life, such as coeliac disease and inflammatory bowel disease [15]. It was reported that in newborn rats the molar ratio of carbohydrate to protein in mucus is low, compared with mature animals. The content of fucose and N-acetylgalactosamine was also decreased [27-29]. In immature human newborns, less ordered composition of mucus and incompletely developed MVM surface may, in part, account for abnormal colonisation of the gut and enhanced antigen penetration. These factors work in conjunction with the already compromised intestinal host defence by further increasing mucosal permeability to enteric bacteria [15]. The newborn, and especially the preterm infant, also has decreased gastric acidity and pancreatic function, and mucosal surface IgA is suboptimal [30,31]. There is, therefore, increased risk of abnormal colonisation of the small intestine in these babies.

1.3.1.5 Lactase deficiency affects colonisation of the colon

The disaccharide, lactose, is the major carbohydrate in breast and cows' milk, and is therefore an extremely important source of calories for babies. It is hydrolysed to glucose and galactose by the enzyme, lactase, a brush border enzyme of the small intestinal mucosa. The monosaccharides are then absorbed. Lactose digestion in the small intestine of the premature infant is thought to be incomplete [32-34]. In the fetus, mucosal enzyme activities rise gradually between 8 and 34 weeks' gestation and then rise rapidly to term [35]. A postnatal rise in lactase activity, after beginning oral feeding, has been reported in both preterm and full term newborns [36,37]. The percentage increase in intestinal lactase activity after milk feeding in the newborn is related directly to gestation. It is most pronounced in term infants and least in preterm infants [38]. It has

been suggested that the lactase activity of the newborn is equal to the immediate demands of milk feeding but that after five days, when the lactose load has risen appreciably, only full term infants have complete capacity to use this sugar [39]. The unabsorbed lactose passes from the small intestine into the colon. In spite of their deficiency of lactase, Kien et al found that premature infants (born at 28-32 weeks of gestation) excreted in faeces less than 15% of the dietary energy derived from lactose [40-42]. This is because unabsorbed lactose is fermented by colonic bacteria to a range of products: gases (hydrogen, carbon dioxide, methane), ethanol, organic acids (eg. lactate) and short-chain fatty acids. These are readily absorbed into the portal blood stream, and some are metabolised by the baby to provide energy [43,44].

1.3.2 Microbial factors influencing colonisation of the human colon

1.3.2.1 Microbial interactions

Competition between species Microbial interactions, especially in the densely populated colon, are of major importance in regulating the indigenous microflora [45-48]. Bacteria may interact to promote or prevent the growth of other species by several mechanisms. Facultative bacteria help to maintain a reduced environment in the colon by utilising oxygen that diffuses into the bowel lumen. In the absence of such organisms, many extremely oxygen sensitive anaerobic bacteria would not survive.

Bacteria produce a variety of substances that inhibit the growth of other species, or even control their own growth in an autoregulatory manner. Examples are colicines, which are bactericidal chemicals produced by strains of *Escherichia coli*, and short-chain fatty acids, such as acetic, propionic and butyric acid, the products of anaerobic and, to a lesser extent, facultative bacteria [49]. These acids inhibit bacterial proliferation, particularly in the reduced environment of the colon [50].

Competition for nutrients Microbial communities are associated with the epithelial surfaces of the colon [51]. The microbial layers are able to use unabsorbed nutrients in the host's diet which enter the colon, for example, starch and complex carbohydrates, as well as simple sugars [52].

1.3.2.2 Antibiotics

The colonic microflora may be altered by many antibiotics, depending on the intraluminal drug concentration and its antimicrobial spectrum [48,53-57]. On a short-term basis, orally administered antibiotics may substantially reduce the concentration of

colonic bacteria. This may be useful. For example, Bartlett et al found that short term oral neomycin and erythromycin treatment before bowel surgery, effectively reduced the concentration of aerobic and anaerobic bacteria in the colon [53].

1.3.3 Dietary factors

Infancy appears to be the only time in life when diet exerts a major impact on the gut flora [58]. The type of milk fed to newborn infants influences the physicochemical and microbiological properties of their faeces. This is apparent by the end of the first week of life when the predominant colonic bacteria, *E. Coli* and *Streptococcus*, produce a reduced environment, which is favourable to the appearance of anaerobes.

In breast-fed infants, *Bifidobacterium* reaches levels of 10^{10} - 10^{11} organisms/g faeces by 4 to 7 days of age. Yoshioka et al reported that *Bifidobacteria* was the predominant organism in the stools of breast-fed infants on day 6 of life, exceeding enterobacteria by a ratio of 1000:1. *Clostridia* and *Bacteroides* also began to appear at this time but the relative number of these organisms, and also of *E.coli* and streptococci soon declined rapidly [58]. The faeces of breast-fed babies had a low pH (5.1-5.4), high counts of *Bifidobacteria* and low counts of coliforms, *Bacteroides* and *Clostridia*. In addition there was a notable absence of volatile fatty acids, other than acetic acid, from these faeces. In contrast, enterobacteria were the predominant organisms in faeces of formula-fed infants, exceeding the *Bifidobacterium* by a ratio of 10:1. These organisms persisted beyond the neonatal period. Bottle-fed infants produced faeces with a high pH(6.4-8.2) and a wide variety of volatile fatty acids [59]. Breast-fed infants who received a formula milk supplement in the first week produced acid faeces, but the pH was higher than that of infants fed breast milk alone; *E.coli* and streptococci appeared in large number during the first 2 weeks but gradually fermentative organisms increased [59]. With the addition of solid foods to the diet of breast-fed infants, the flora became similar to that of formula-fed infants, with increased numbers of *E.coli*, *streptococci*, and *Clostridia*. Since these organisms were already present in large numbers in faeces of formula-fed infants, introduction of solids had little impact on their faecal flora. The conversion to an adult-type flora in which anaerobic bacteria outnumber aerobes, with predominance of *Bacteroides, Bifidobacterium* and *Eubacterium* [60], is complete by the second year of life [61].

1.3.4 The type of delivery

Long and Svenson demonstrated that vaginally delivered infants were colonised

with anaerobic bacteria earlier, and in greater in numbers, than infants delivered by caesarean section [62]. This suggested that significant contamination with these organisms occurred during passage through the birth canal.

1.4 METABOLIC ACTIVITY OF INTESTINAL MICROFLORA

1.4.1 Introduction

The human colon contains hundreds of species of microorganisms which have high metabolic activity. Degradation of carbohydrates under anaerobic conditions (fermentation) is a major activity of many species. The types of bacterial fermentation vary widely. There is a wide range of products [63]. Some are of benefit to the host but a few may cause illness if absorbed in large amounts.

1.4.2 Fermentation of carbohydrates by intestinal bacteria

A variety of bacteria ferment carbohydrates to products which include short-chain fatty acids (SCFA's), lactate, ethanol and gases (hydrogen, carbon dioxide and methane) [43]. The fermentation of carbohydrates is important physiologically to premature infants.

1.4.2.1 SCFA as the products of colonic fermentation of carbohydrate

The short chain fatty acids (also called the volatile fatty acids), are C_1 to C_7 organic acids (formate, acetate, propionate, butyrate, valerate, hexanoate, heptanoate) formed by anaerobic microbial fermentation of carbohydrates in the colon [43,44,64-66]. Since the SCFA are weak acids, with a pK of < 4.8, and the gastrointestinal pH is more nearly neutral, 90-99% of the SCFA are present in the gastrointestinal tract as anions rather than as free acids. Acetate, propionate and butyrate account for approximately 85% of formed SCFA [43,44]. In all mammals examined, acetate is present usually in higher concentrations than all other SCFA [44]. Propionate and butyrate also are present in large concentrations, although their amounts may vary with diet. Estimates of the molar ratios of acetate to propionate to butyrate in human have varied from about 75:15:10 to 40:40:20 [44]. In general, it appears that these SCFA are generated from glucose. Fermentation equations for human faeces have been estimated as follows: 56 glucose ($C_6H_{12}O_2$) $\rightarrow$ 74 acetate + 18 propionate + 8 butyrate + 55 CO_2 + 38 CH_4 + 17 H_2O. Overall, nearly 75% of the energy content of the carbohydrate is conserved in SCFA's. The remaining 25% is used by the microbes for growth, or lost as hydrogen and methane [44]. There are some differences in the pathways of formation of acetate, propionate, and butyrate. Fructose-1,6-diphoshosphate aldolase activity has been demonstrated in

many organisms studied by Macy (1979), with the exception of *Bifidobacterium* [43]. Thus, glucose oxidation to pyruvate is thought to occur via the Embden-Meyerhof-Parnas pathway (Figure 1-1)*. Acetate and ATP are produced when acetyl-CoA participates in further reactions involving phosphotransacetylase and acetate kinase. The formation of propionate from pyruvate is separate from that of acetate and butyrate and involves two major pathways. The first involves the formation of oxaloacetate and succinate, and the second involves the formation of acrylate. Both pathways are operative [44]. Butyrate is also produced by fermentation using pyruvate as substrate (Figure 1-2). The reactions involved in the formation of acetate and butyrate from pyruvate are interrelated, and all proceed through acetyl-CoA. Furthermore acetate and butyrate are interconvertible. The conversion of butyrate to acetate seems to be advantageous for microbial metabolism, since there is a net gain of ATP [43]. *Bifidobacteria* may convert glucose to acetate and glyceraldehyde-3-phosphate via the bifidum pathway (Figure 1-3), without involvement of hydrogenation and dehydrogenation reactions. The five-carbon SCFA, valerate, is formed by condensation of acetate and propionate [44].

1.4.2.2 Fermentation products of carbohydrates other than SCFA

Lactic acid is a common end-product of anaerobic fermentation of carbohydrate by gut bacteria. Organisms belonging to the species of lactobacilli produce the largest amounts. The microorganisms involved are highly saccharolytic. They derive their energy primarily from fermentation of carbohydrates [67], and lack most anabolic pathways. A low pH favours the growth of lactobacilli. In humans, high lactate concentrations are present only in very acid stools associated with defective carbohydrate digestion or malabsorption [68]. There are three **major** fermentation pathways involved in the conversion of carbohydrate to lactate.

1. Homofermentative pathway (Figure 1-4). Lactate is formed from glucose via pyruvate produced by the Embde-Meyerhof-Parnas pathway of anaerobic glycolysis. The pyruvate acts as a hydrogen acceptor, producing lactate.

2. Heterofermentative pathway (Figure 1-5). This involves conversion of glucose to the 5-carbon sugar, ribulose-5-phosphate. Glyceraldehyde-3-phosphate is formed and converted to lactate.

3. Bifidum pathway (Figure 1-3). In this pathway, glyceraldehyde-3-phosphate is

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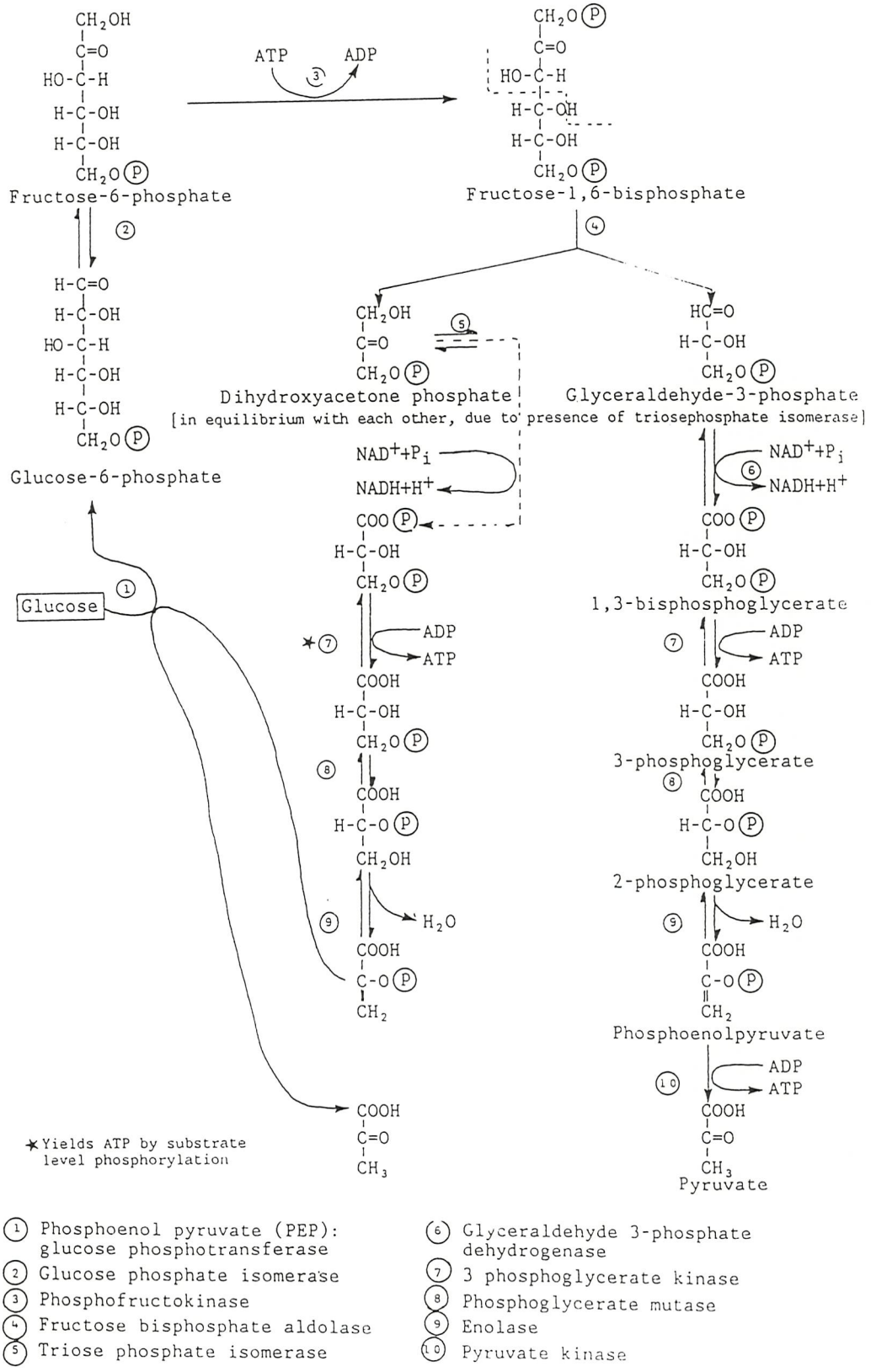
* Figures 1-1 to 1-8 were reproduced (with permission) from: Joanna Gale:(1989): Urinary D(-) and L(+) lactic acid excretion by neonates and children with gastrointestinal disorders. Fourth Year Medical Student Project, Department of Chemical Pathology, Southampton University Medical School

Figure 1-1

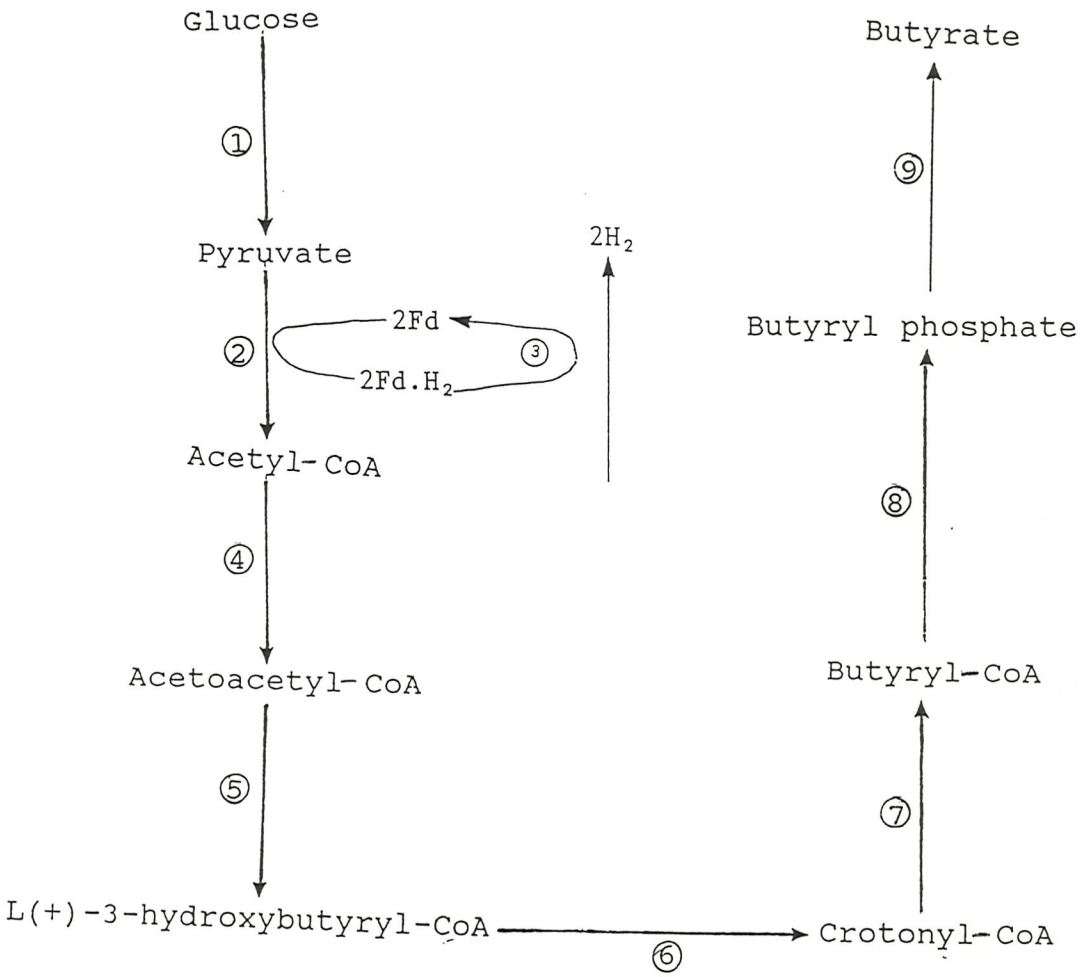
The Embden-Meyerhof-Parnas Pathway of Anaerobic Glycolysis



① Phosphoenol pyruvate (PEP): glucose phosphotransferase
② Glucose phosphate isomerase
③ Phosphofructokinase
④ Fructose bisphosphate aldolase
⑤ Triose phosphate isomerase
⑥ Glyceraldehyde 3-phosphate dehydrogenase
⑦ 3 phosphoglycerate kinase
⑧ Phosphoglycerate mutase
⑨ Enolase
⑩ Pyruvate kinase

Figure 1-2

Carbohydrate Fermentation Resulting in Butyrate Production



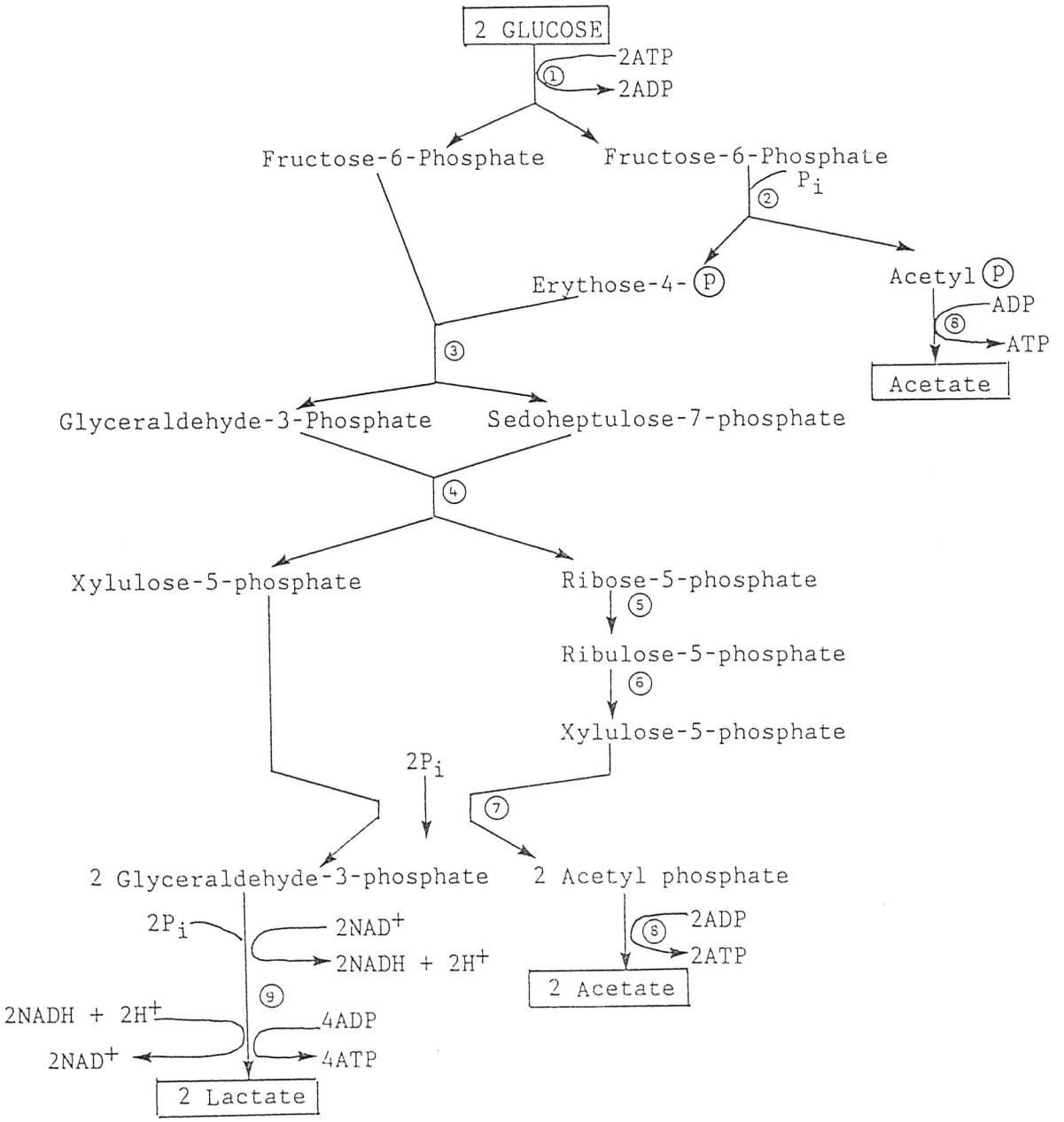
① Phosphototransferase system, and Embden-Meyerhof-Parnas pathway (shown in Figure 1-1)
② Pyruvate Ferredoxin oxidoreductase
③ Hydrogenase
④ Acetyl-CoA acetyl transferase (thiolase)
⑤ L(+)-3-hydroxybutyryl-CoA dehydrogenase
⑥ L-3-hydroxyacyl-CoA hydrolase (crotonase)
⑦ Butyryl-CoA-dehydrogenase
⑧ Phosphotransbutyrylase
⑨ Butyrate kinase

Abbreviation:-

Fd = Ferredoxin

Figure 1-3

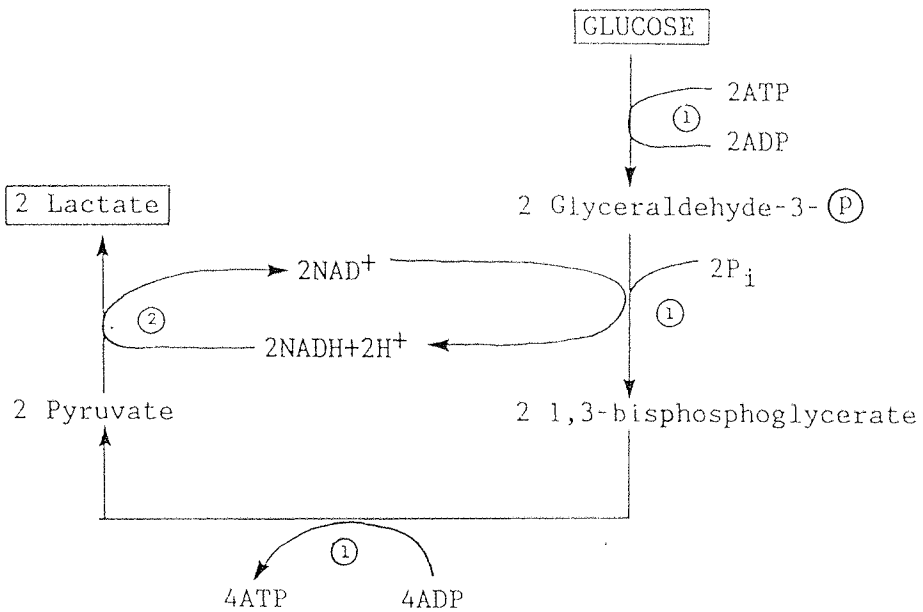
The Bifidum Pathway



① Hexokinase and Glucose-6-phosphate isomerase
② Fructose-6-phosphate phosphoketolase
③ Transaldolase
④ Transketolase
⑤ Ribose-5-phosphate isomerase
⑥ Ribulose-5-phosphate 3-epimerase
⑦ Xylulose-5-phosphate phosphoketolase
⑧ Acetate kinase
⑨ Enzymes as in Homofermentative Pathway (shown in Figure 1-4)

Figure 1-4

The Homofermentative Pathway

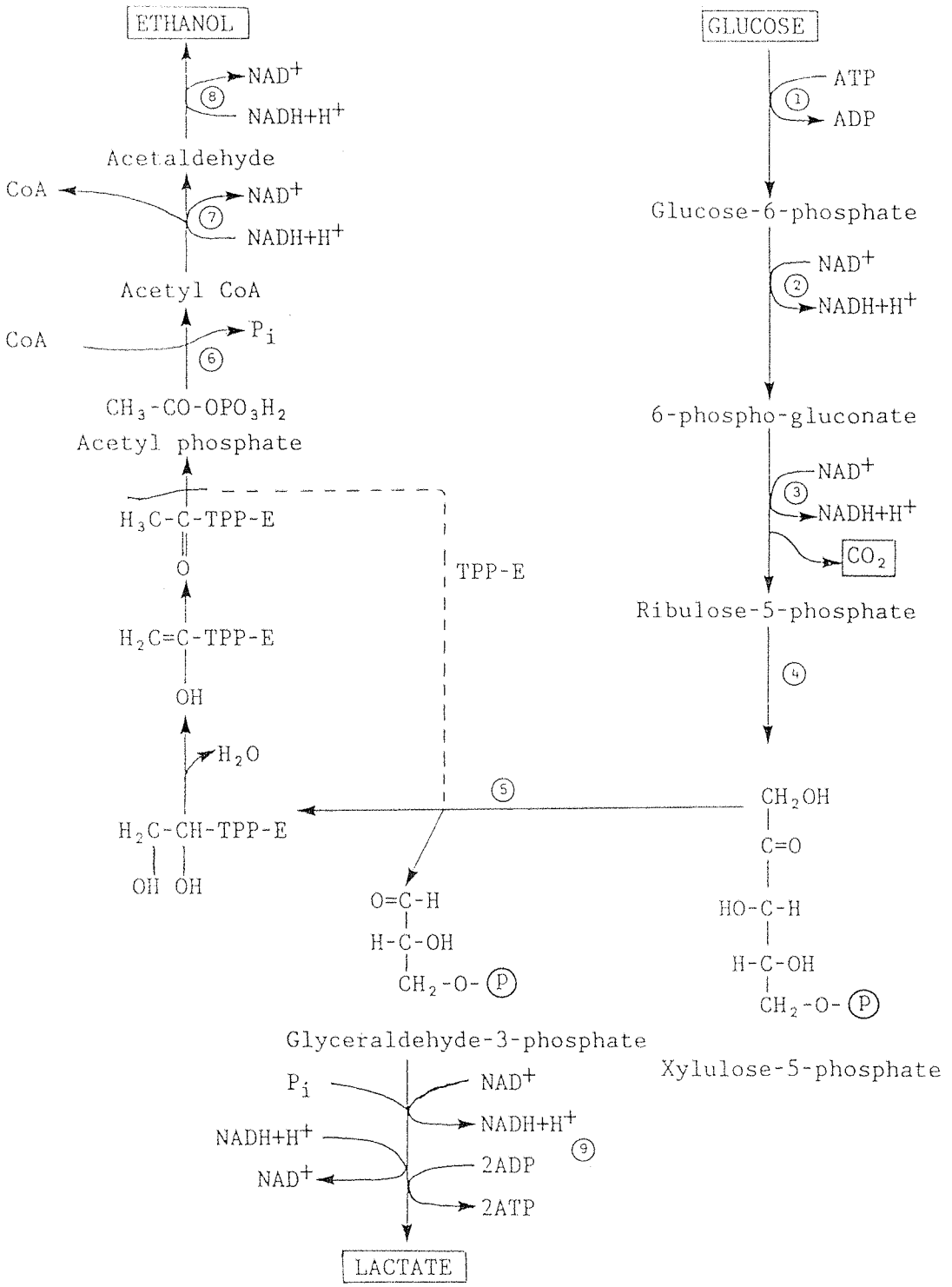


① Enzymes of Embden-Meyerhof-Parnas Pathway

② Lactate dehydrogenase

Figure 1-5

The Heterofermentative Pathway



① Hexokinase
② Glucose-6-Ⓟ dehydrogenase
③ 6-phosphogluconate dehydrogenase
④ Ribulose-5-Ⓟ 3-epimerase
⑤ Phosphoketolase
⑥ Phosphotransacetylase
⑦ Acetaldehyde dehydrogenase
⑧ Alcohol dehydrogenase
⑨ Enzymes as in Homofermentative Pathway (shown in Figure 1-4)

Abbreviations:-

CoA = Coenzyme A; TPP-E = Thiamine pyrophosphate enzyme complex

produced and converted to lactate by means of glyceraldehyde-3-phosphate and lactate dehydrogenase. In addition, enterobacteria, such as *Escherichia* sp., *Salmonella* sp., and *Shigella* sp., ferment sugars to a mixture of products including lactic, acetic and formic acids via mixed acid fermentation pathways shown in Figure 1-6. Some species of enterobacteriaceae, such as *Klebsiella, Enterobacter, Serratia*, and *Erwinia* ferment carbohydrates also via mixed acid fermentation pathways, but produce less acid and more gases.

2,3-Butanediol and its precursor acetoin Fermentation of glucose to acetoin and 2,3-butanediol is a major metabolic pathway of yeasts and of some enterobacteria (*Klebsilla* sp. and *Enterobacter* and *Serratia*). The bacterial pathway involves the intermediate α-acetolactate (Figure 1-7) and occurs at pH below 6.0. Significant endogenous production of these compounds probably only occurs infrequently and under abnormal metabolic conditions:

(a) heavy gut colonisation with acetoin-producing bacteria. These opportunitic organisms readily colonise the gut after antibiotic treatment.

(b) Increased amounts of lactose or glucose in the colon (as in lactase-deficient premature babies). These provide nutrient for acetoin-producing organisms.

(c) pH below 6.0 within the colon.

Other fermentative pathways Under certain conditions a number of butyrate-producing *Clostridia* species, such as *C. acetobutylicum* and *C. tetanomorphum*, produce n-butanol and acetone instead of butyrate (Figure 1-8).

1.4.3 Branched chain SCFA as the products of protein degradation

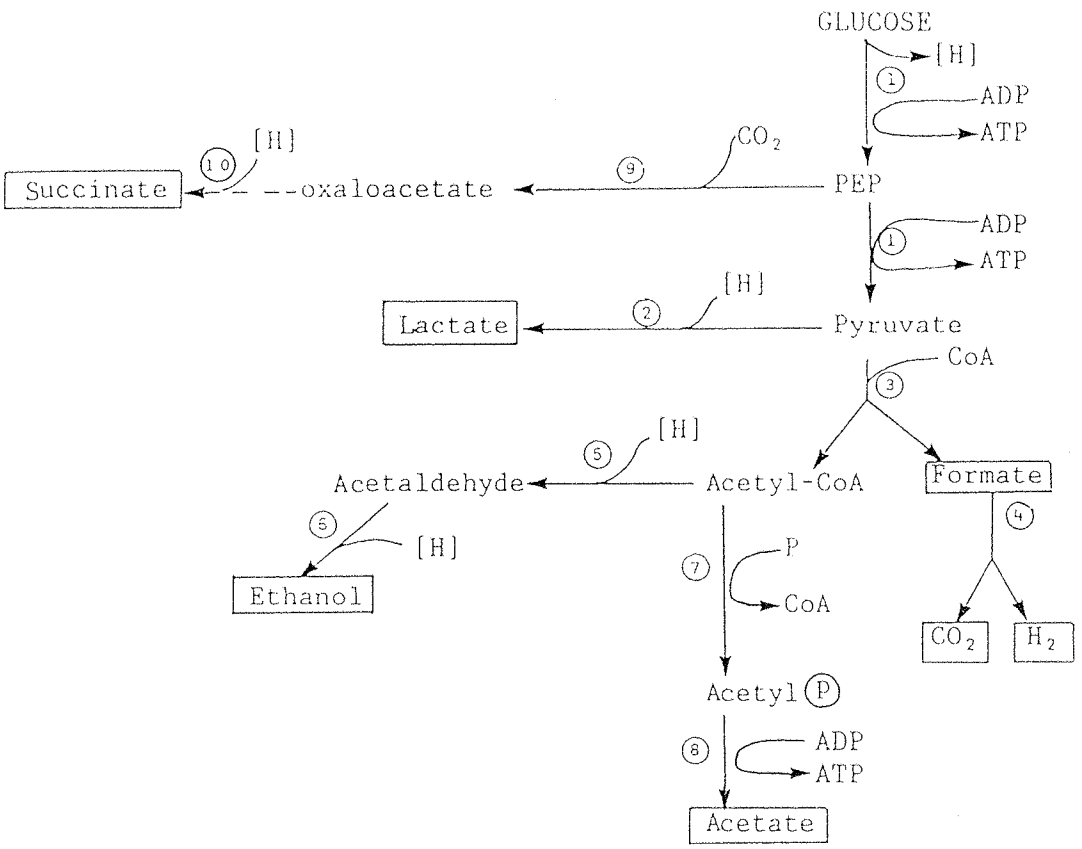
A wide range of human colonic bacteria have the ability to produce branched short-chain fatty acids as end products of metabolism of protein and amino acids [69,70]. Proteolysis is the first step in the utilisation of protein by bacteria. The large oligopeptides formed initially are degraded into small peptides and amino acids. These can be assimilated directly into microbial protein or fermented, with the production of branched short chain fatty acids and ammonia. The branched SCFA are principally isobutyric, isovaleric and 2-methylbutyric acids produced from the branched-chain amino acids valine, leucine and isoleucine [44,69].

1.4.4 FATE OF GUT BACTERIAL FERMENTATION PRODUCTS

1.4.4.1 Absorption and utilisation of SCFA in man

Figure 1-6

The 'Mixed Acid Fermentation' Pathway



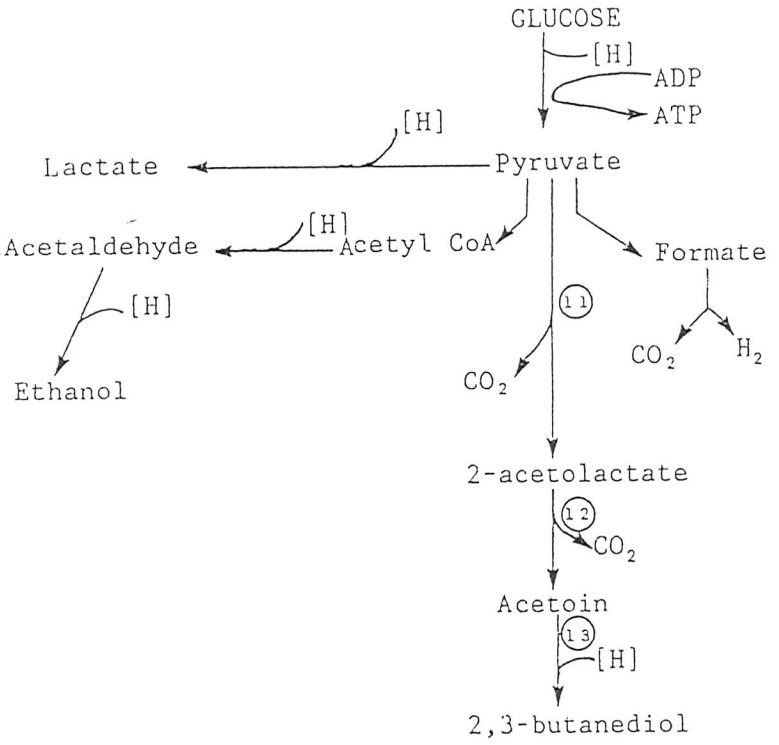
(1) Enzymes of Embden-Meyerhof-Parnas Pathway (shown in Figure 1-1)
(2) Lactate dehydrogenase
(3) Pyruvate Formate Lyase
(4) Formate Hydrogen Lyase
(5) Acetaldehyde dehydrogenase
(6) Alcohol dehydrogenase
(7) Phosphotransacetylase
(8) Acetate kinase
(9) PEP carboxykinase
(10) Malate dehydrogenase, Fumarase, and Fumarate reductase

Abbreviation:-

PEP = phosphoenol pyruvate

Figure 1-7

Carbohydrate Fermentation Resulting in 2,3-Butanediol Formation



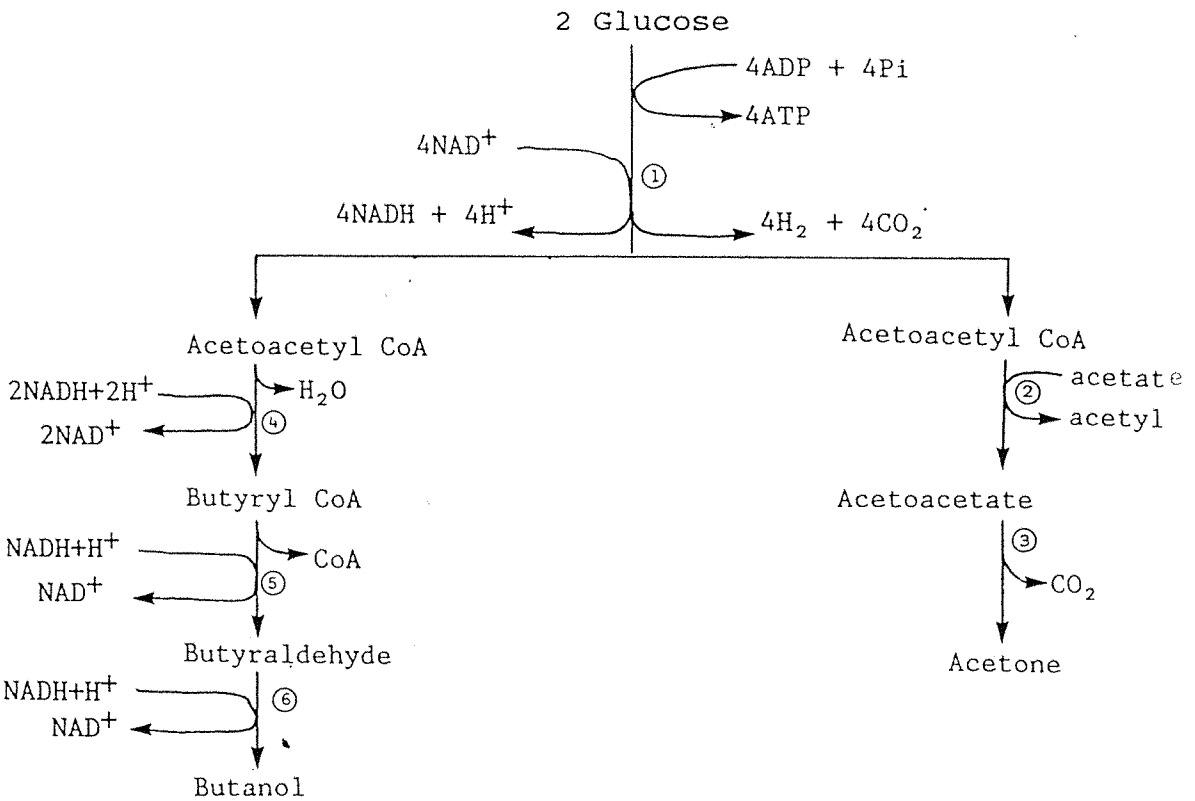
(11) 2-acetolactate synthase

(12) 2-acetolactate decarboxylase

(13) 2,3-butanediol dehydrogenase

Figure 1-8

Carbohydrate Fermentation Resulting in Butanol and Acetone Formation



① Reactions as shown in Figure 1-1

② Acetoacetyl CoA : acetate coenzyme A transferase

③ Acetoacetate decarboxylase

④ L(+)-3 hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase

⑤ Butyraldehyde dehydrogenase

⑥ Butanol dehydrogenase

SCFA, which are relatively high in caloric content, are readily absorbed by the intestinal mucosa and metabolised by the intestinal epithelium and liver. SCFA absorption is generally considered to be passive and increases linearly with increasing concentration gradient. Recent investigations have elucidated several important characteristics. First, absorption occurs in both the ionised and nonionised forms. Second, transport from the lumen is invariably associated with the luminal accumulation of bicarbonate ion. Third, SCFA are trophic to the intestinal mucosa, and stimulate sodium and water absorption [43,65].

All of the SCFA are readily and effectively used as energy sources for maintenance, growth, and lipogenesis. Ruppin et al have suggested that in adult man the energy available for metabolism from SCFA may be as high as 540 kcal/day, or 22% of the energy requirement [43]. The energy contributed by SCFA may be of greater importance for premature infants [71]. This has been deduced by measuring hydrogen excretion in the breath of premature babies. MacLean and Fink reported that between one half and two thirds of ingested carbohydrate was passed into the colon of newborn infants [72]. Modler et al found that the percentage of hydrogen absorbed per gram of carbohydrate passed into the colon was significantly higher in the newborn than in the adult. They recalculated MacLean and Fink's data, estimating that between 15% and 20% of the carbohydrate ingested as lactose passed into the colon [71]. These carbohydrates are fermented by colonic bacteria to products including SCFA. Butyrate is preferentially utilized over glucose, ketones, glutamine and other SCFA as an energy source for the colonic epithelial cells [43]. Once absorbed, butyrate is thought to be chiefly oxidized in the cells of the colonic mucosa. Butyrate that has not been metabolised, and the majority of all other SCFA, are transported to the liver via the portal vein. There is evidence that acetate and butyrate are precursors of acetyl-CoA and that they may have metabolic roles as substrates for energy production and cytosolic lipogenesis. Peripheral tissues, primarily adipose tissue and skeletal muscle, utilize about 80% of the total acetate entering the blood [44]. Propionate may be a substrate for gluconeogenesis.

1.4.4.2 Faecal Excretion of SCFA

SCFA are the main anions in the faeces of the adult human, accounting for about 50% of the total anions [44]. A number of factors influence the SCFA faecal excretion, including the amount and composition of available fermentable material, the composition and activity of the microflora, net SCFA absorption from the gastrointestinal tract, and transit time.

1.4.4.3 Urinary excretion of absorbed products of carbohydrate fermentation

2,3-Butanediol and its precursor acetoin have been detected in infants' urine [73]. These were babies nursed on a neonatal unit because of prematurity or neonatal problems. They had been treated with broad spectrum antibiotics. Increased 2,3-butanediol excretion did not occur immediately after birth but was delayed until the second week or later. The excretion was not observed continually, but occurred episodically in milk-fed infants. The probable explanation was that opportunistic 2,3-butanediol-producing bacteria were the first organisms to colonise the gut after discontinuation of antibiotics. They were later displaced as the normal gut flora became re-established. Production of 2,3-butanediol indicated that the pH of the gut lumen was below 6.0. Absorption of this metabolite (and its precursor, acetoin) may have occurred readily through the immature colonic mucosa of pre-term infants. The 2,3-butanediol is not metabolised after absorption, and is excreted by the kidneys. On some occasions when the diol was detected, *Klebsiella* sp. or *Enterobacter* sp. were isolated from the faeces. These prelimary observations suggested that urinary excretion of 2,3-butanediol could prove to be a useful biochemical marker for gut colonisation with species of organisms which are potential pathogens for preterm babies.

D-lactate produced by gut fermentation may also be detected in the urine. Whereas L(+) lactate produced by intestinal bacterial fermentation can be utilised by the host after absorption, D(-) lactate is not used significantly. It is excreted by the kidneys, at a very low plasma threshold [74]. In a few individuals with seriously impaired carbohydrate absorption (for example gut resection), abnormal colonisation of the gut with D-lactate producing lactobacilli has led to grossly increased blood and urinary levels of D-lactate. These patients became acutely ill with a metabolic acidosis.

1.4.5 Metabolism of phenylanine and tyrosine in the colon

1.4.5.1 Volatile phenols produced by metabolism of phenylalanine and tyrosine in the colon

Volatile phenols (Vph), mainly ρ-cresol and phenol, are found frequently as constituents of urine. Most are products of bacterial metabolism occurring in the intestinal tract. The amount of Vph in the urine depends upon the protein content of food, protein digestion [75]. and absorption, and on the prevailing species of gut bacteria The level of Vph was lowered by oral antibacterial agents, even down to zero,

confirming the belief that Vph in urine originates from the activity of intestinal microorganisms. Bakke et al have demonstrated that ρ-cresol and phenol were the only simple phenols produced from tyrosine during anaerobic incubation with rat caecal contents [100]. Addition of neomycin sulphate nearly abolished their formation. When tyrosine and phenylalanine are incompletely absorbed in the small intestine, they are subjected to bacterial degradation in the colon [76]. In the human intestine, various biochemical processes may be involved in the bacterial degradation of tyrosine: decarboxylation, deamination, dehydrogenation, demethylation, hydroxylation and dehydroxylation. It was thought that the degradation of tyrosine by intestinal microorganisms took place through deamination to phloretic acid, followed by decarboxylation to 4-ethylphenol, oxidation to 4-hydroxyphenylacetic acid, decarboxylation to ρ-cresol, oxidation to 4-hydroxybenzoic acid and finally decarboxylation to yield phenol [75] (Figure 1-9). However, Duran et al suggested that this route was probably not used, since the intermediate product, 4-ethylphenol, has not been found in the urine of patients with gastrointestinal disorders containing excessive large amounts of bacterial metabolites of tyrosine and phenylalanine [76]. They proposed two possible alternative routes: (a) tyrosine → 4-coumaric acid → 4-hydroxybenzoic acid → phenol (b) tyrosine → tyramine → 4-hydroxyphenylacetic → ρ-cresol → 4-hydroxybenzoic acid → phenol (Figure 1-9).

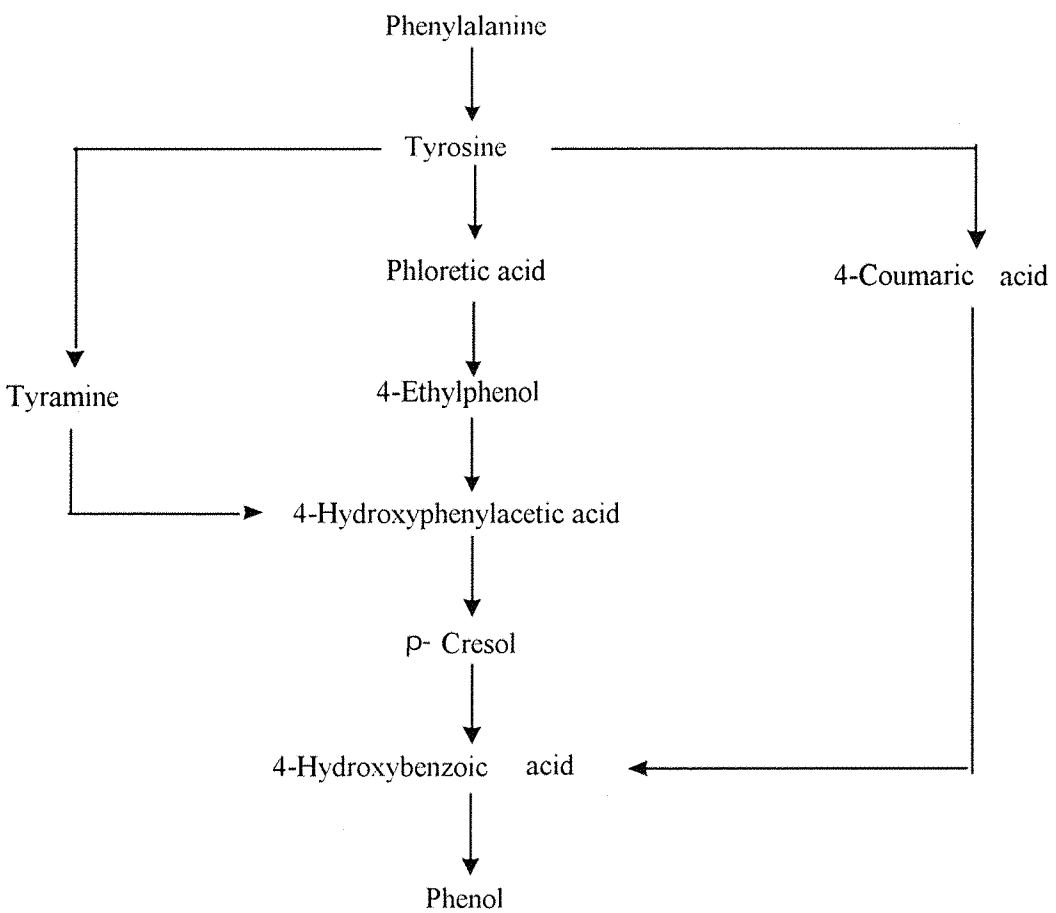
Generally, phenol is believed to be produced by facultative anaerobic bacteria, while ρ-cresol is produced by strict anaerobes. In the normal adult large bowel, the anaerobic organisms outnumber the aerobes by a factor of about 10^2-10^3. In vitro studies, the yield of ρ-cresol by the anaerobic organisms was about equal to that of phenol by the aerobes and so, on a priori grounds, a high ratio of ρ-cresol:phenol might be expected in urine. However, Bone et al reported that ρ-cresol concentrations were only about 4 times greater than phenol in adult urine [77]. They proposed a number of possible explanations:

1. The in vitro results do not reflect what happens in vivo: in the normal gut the aerobes might be at least 10 times as active as anaerobes in the production of volatile phenols.

2. Phenol is more efficiently absorbed from the intestine than ρ-cresol. If this were so, an overwhelming preponderance of ρ-cresol might be expected in the faeces.

3. There is a concentration gradient of substrate along the colonised region of the gut with the highest concentrations being in that part of the gut where the aerobes nearly approach numerical equality with anaerobes.

Figure 1-9 Bacterial production of phenol and ρ-cresol



Their results seemed to support the third explanation. Others, however found that ρ-cresol accounted for a higher proportion (around 90% of urinary volatile phenol) [75,76].

1.4.5.2 Products of tyrosine metabolism other than volatile phenols

In studies of the degradation of tyrosine by aerobic and anaerobic microorganisms, a wide variety of metabolites including several phenolic acids, has been identified in addition to ρ-cresol and phenol [78]. Unabsorbed tyrosine and phenylalanine can be decarboxylated by *Steptococcus faecalis* to tyramine and phenylethylamine. Some of these compounds are absorbed and oxidised to 4-hydroxy-phenylacetic acid and 4-hydroxyphenyllactic acid in the liver. Products arising from tyrosine include 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid (conjugated with glycine in the liver after absorption, and excreted in urine as 4-hydroxyhippuric acid), 4-hydroxylphenylpropionic acid and 4-hydroxyphenylhydracrylic acid. Products from phenylalanine are mainly benzoic and phenylacetic acids, which are conjugated in the liver to form hippurate and phenylacetylglutamine, respectively (Figure 1-10)*.

1.4.5.3 Urinary excretion of gut bacterial metabolites of tyrosine and phenylalanine

The absorbed gut bacterial metabolites of tyrosine and phenylalanine are excreted mainly in urine. Faecal excretion of these compounds was less than their urinary elimination [76]. Each day, 50-100 mg of volatile phenols are excreted normally in human urine mainly conjugated with sulphuric or glucuronic acids. Urinary concentrations of unconjugated phenol and ρ-cresol are very low in comparison. Increased urinary excretion was found in patients with gastrointestinal disorders [76]. Generally the toxicity of conjugated phenols is considered to be small, but it has to be questioned whether a constant passage of fairly large amounts of these compounds through the body is completely harmless. Other metabolites, phenolic acids, and conjugates of phenylacetic, benzoic and 4-hydroxybenzoic acids, phenylacetylglutamine, hippurate and 4-hydroxyhippurate are detected in urine. Van der Heiden et al (1971) reported that the urinary excretion of metabolites of phenylalanine and tyrosine was increased markedly in a patient with cystic fibrosis who had severely impaired amino acid absorption [78]. Later they found high amounts of one or more of the metabolites in other patients with cystic fibrosis, or other gastrointestinal disorders including coeliac disease, intestinal resection and unclassified enteritis.

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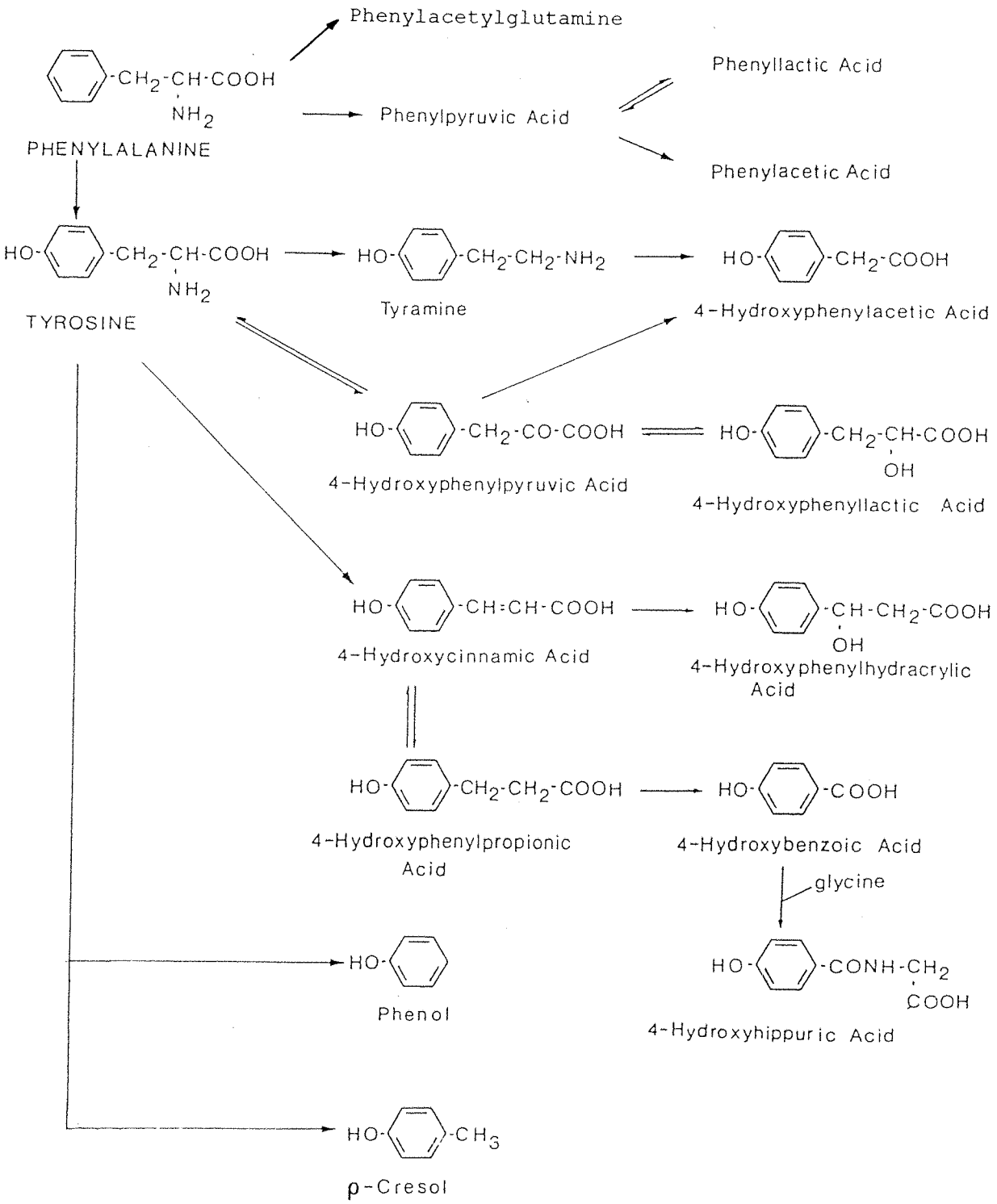
*.Figurue 1-10 was reproduced (with permission) from : F.E.Harris (1989): Urinary imidazoles of normal clidren,premature babies and chldren with intestinal disorders. Four Year Medical Student Project, Department of Chemical Pathology, Southampton University.

Figure 1-10 BACTERIAL DEGRADATION PATHWAYS OF PHENYLALANINE AND TYROSINE



1.4.5.4 Factors influencing the urinary excretion of bacterial metabolites of tyrosine and phenylalanine

A number of factors influence intestinal microbial metabolism. These, in turn, may influence urinary excretion of tyrosine and phenylalanine metabolites.:

(a) factors affecting the types and amounts of nutrients available for microbial use:

1. diet composition
2. rates of nutrient passage through the intestine, and absorption from the lumen
3. secretions of the host into the digestive tract

(b) microbial metabolic capacity, as reflected in the rates of nutrient metabolism and assimilation

(c). metabolic pathways and metabolic characteristics of the microbes present

1.5 PROBIOTIC THERAPY TO MODIFY THE GUT BACTERIAL FLORA

1.5.1 Introduction

The bacterial flora of the bowel of premature newborn infants nursed in neonatal intensive care units differs from that of normal full-term infants [13]. It has been suggested that lactobacilli form an important part of the faecal flora of newborn infants [14,79,80]. A number of factors such as prematurity, birth by caesarean section, parenteral antibiotic treatment, and being nursed in an incubator, may deprive infants of colonisation with lactobacilli during the first few days, and even weeks of life. Preterm infants are at increased risk of infection from bacteria encountered in the hospital environment. Bacteria such as coagulase-negative staphylococci and enterobacteriaceae may contribute to the pathogenesis of neonatal necrotising enterocolitis [81-83]. Prolonged broad spectrum antibiotic treatment is used frequently by doctors to prevent, or overcome, infectious diseases in the lungs or other systems during the first weeks of life. This may cause imbalance of the intestinal microflora. There have been many attempts to modify the infants' intestinal flora by adaptation of the components of artificial milk formulae and by the administration of probiotics [84]. A probiotic can be defined as 'a live microbial feed supplement which beneficially affects the host animal by improving intestinal microbial balance'. It has been suggested that induced colonisation of preterm infants with a probiotic may produce bacteriological, metabolic and clinical benefits for these infants [85]. There are several reports about studies of the effects of

orally administered lactobacilli and *Bifidobacteria* to subjects with diarrhoea. The results were variable [86-91]. However, there is no convincing evidence that the administration of such organisms leads to a measurable long-term improvement in the well-being of normal babies, or is associated with a consistent pattern of bacterial colonisation in the lower intestine. A non-pathogenic *E.coli* (lactose-fermenting strain) was found to be capable of colonising the bowel of premature infants [84]. However, some strains of *E. coli* cause sepsis in newborn infants, and the safety and possible benefits of administering probiotics to preterm babies require careful evaluation.

1.5.2 Use of the probiotic *Lactobacillus GG* in man

Lactic acid bacteria are supposed to have many beneficial effects on human health. *Lactobacillus GG* is a strain of *Lactobacillus casei* which was isolated from the faeces of a healthy man [86,92]. It was shown to be resistant to low pH and bile acids, adhesive to human ileal cells and to colonise the human bowel. This bacterium is thought to be able to elaborate antimicrobial substances such as short chain fatty acids, lactocidin, bacteriocin, acidolin and microcin, which have potent inhibitory activity against a wide range of bacteria [93-95]. These would facilitate colonisation in the intestinal tract by suppression of other members of the microflora.

Many hospitalized patients are given antibiotic treatment. This is often accompanied by diarrhoea and other gastrointestinal side effects. The pathogenesis of antibiotic associated diarrhoea has been related to quantitative changes in the intestinal and faecal microflora [96]. *Lactobacillus GG*, therefore, has been recommended and used for the prevention of antibiotic therapy associated diarrhoea and gastrointestinal side effects. Salminen et al (1989) reported that a good colonisation with *Lactobacillus GG* was obtained during erythromycin and ampicillin therapy, and was only slightly lower during penicillin administration. They suggested that *Lactobacillus GG* may offer one means of balancing the intestinal microflora during prolonged antibiotic therapy [97]. Bennett et al, from the United States, have successfully used a lyophilized preparation of *Lactobacillus GG* to treat five adult patients with recurrent *Clostridium* difficile diarrhoea. This probiotic was also given to treat acute diarrhoea and to prevent antibiotic associated diarrhoea and travellers' diarrhoea in adults [98].

Although *Lactobacillus GG* has not been reported to have been given to newborn infants, it has been used to treat relatively young infants suffering from acute gastroenteritis with no reported adverse effects [84]. If *Lactobacillus GG* can be implanted in the preterm infants' bowel, and induce colonisation with other

non-pathogenic bacteria, it might bring bacteriological, metabolic and clinical benefits for these infants. This study, the only reported one so far, showed that *Lactobacillus GG* could colonise the colon of premature infants. However, there was little change in the large bowel reservoir of potential pathogens [99].

1.6 AIMS OF STUDY

The aims of this study were:

(1) To review the literature concerning two aspects of metabolism of intestinal microflora in man and premature babies: carbohydrate fermentation and metabolism of aromatic amino acids.

(2) To select a method which might be suitable for analysis of urinary phenol and p-cresol of premature babies.

(3) To set up, optimise and validate the chosen procedure.

(4) To learn an established method for urinary 2,3-butanediol analysis.

(5) As part of a controlled study of premature babies, to investigated the effects of feeding a probiotic-*Lactobacillus GG*, on urinary excretion of the gut bacterial metabolites phenol, ρ-cresol and 2,3-butanediol.

CHAPTER TWO

ANALYTICAL INSTRUMENTS, MATERIALS AND METHODS

2.1 ANALYTICAL INSTRUMENTS

2.1.1 HPLC apparatus

The apparatus comprised a Bio-Rad Soft Start HPLC Pump with Rheodyne injector (Bio-Rad Labs; Watford, U.K.), Bio-Rad Column heater, LDC Spectromonitor III adjustable wavelength ultraviolet (UV) Detector. The HPLC was connected to a Trio2+ computing integator (Triovector Systems International,Sandy, U.K.). The integrator attenuation setting was 32 and chart speed 0.5 cm/min. The HPLC column was a Bio-Rad Aminex short fermentation, strong cationic exchange column, 150x7.8 mm, protected by a guard column cartridge HPX-85H, 40mmx4.6 mm.

2.1.2 Gas Chromatographic equipment

The gas chromatography was an HP 5890 Series II model with flame ionisation detector (Hewlett Packard, Bracknell, U.K.). The chromatograph was connected to the Trio monitor system, also used for HPLC (see above), and to a chart recorder. The integrator attenuation setting was 32 and chart speed 0.5 cm/min. The GC column was a megabore fused silica column (25m x 0.53mm I.D.) coated with a 1.0 μm film of polyethylene glycol BP-20 (SGE Ltd, Milton, Keynes, U.K.).

2.2 MATERIALS

Analytical reagents: all organic acid standards, phenol, ρ-cresol and β-glucuronidase HP-type 2 (from Helix pomatia) were obtained from Sigma Chemical Co. Ltd., Poole, U.K. 3-(4-hydroxyphenyl) propionic acid 50 μmol/L in Tris buffer (PH 7.0) was used as internal standard (I.S.) for HPLC. 2-Ethylbutyric acid (3.93 mmol/L) was used as internal standard for GC. Hydrochloric acid, sulphuric acid, Tris (hydroxymethyl) methylamine (TRIS), ethylacetate, and diethyl ether, all of Analar grade, were purchased from BDH Ltd., Poole, U.K. Stock 0.5 mol/L sulphuric acid was diluted with deionised distilled water to 5 mmol/L and degassed with helium for use as the HPLC mobile phase. 1 ml strong anion-exchange columns (trimethylamino-propyl bonded to silica, chloride form), for HPLC sample preparation, were obtained from

Jones Chromatography Ltd. Glamorgan, U.K. (Bond Elut columns). The Analytichem 10-port Vac-Elut System was purchased from Jones Chromatography Ltd. U.K. The water used for all procedures was deionised, double distilled and filtered through 0.22 μm Millipore filters (Waters Chromatography Division, Harrow, U.K.). Filtration was performed in two stages under vacuum to avoid problems with blockages which occurred when 0.22 μm filters were used alone. The first step of filtration (0.45 μm filters) was carried out in large batches each week (5 litres). Aliquots were filtered through 0.22 μm filters on the day of use.

2.3 URINE SAMPLE COLLECTION

Random urine samples from babies were collected into sterile polythene bags (Argyle pediatric urine collector bags, Sherwood Medical, USA). Urine was frozen immediately without preservative and stored at -20°C.

2.4 ANALYTICAL METHODS AND METHOD VALIDATION

2.4.1 Determination of urine creatinine

The urine creatinine concentration was determined using an alkaline picrate kinetic method and a Multistat Centrifugal Analyser (IL Laboratories, U.K.). This method is in routine use in the laboratory of Chemical Pathology, Southampton General Hospital.

2.4.2 Hydrolysis of urine with β-Glucuronidase

2.4.2.1 Introduction

Phenol and ρ-cresol absorbed from the gut are conjugated extensively (more than 90%) with glucuronide or sulphate in the liver. These, and unconjugated compounds, are excreted in urine. Enzyme hydrolysis has been used to release free phenol and ρ-cresol from the conjugates for analysis [100, 101].

β-glucuronidase HP-type 2 was used for this study. It contained 10,000 β-glucuronidase units and 1,140 sulphatase units per ml. One unit of β-glucuronidase will liberate 1.0 microgram of phenolphthalein from phenolphthalein glucuronide and one unit of sulphatase will hydrolyse 1.0 micromole of ρ-nitrocatechol sulphate per hour at pH 5.0 at 37°C.

The manufacturers recommended sodium acetate buffer (pH 5.0) for hydrolysis of urine with β-glucuronidase.

2.4.2.2 Investigation of the optimal incubating time for β-glucuronidase hydrolysis

of conjugated urinary phenol and ρ-cresol

Sodium acetate buffer was prepared at pH 5.0 and at a concentration of 0.2 mol/L.

One urine sample, (creatinine 2.9 mmol/L), was used for determination of the optimal time of incubation for urine hydrolysis with β-glucuronidase. The urine pH was adjusted to 5.0 with 5M hydrochloric acid. A volume of urine equivalent to 1 μmol creatinine was taken, and mixed with 200 μl sodium acetate buffer. 20 μl of β-glucuronidase was added. Three aliquots of this urine sample were incubated at 37°C for 18, 21 and 24 hours, respectively. Each was extracted using the procedure for gas chromatography (GC) described on page 49-50. The extracts were concentrated to 250 μl by evaporation under nitrogen, 1.5 μl was analysed by GC.

Phenol and ρ-cresol concentrations did not differ significantly for incubation times of 18, 21 and 24 hours (Table 2-1; Figure 2-1). Consequently, 18 hours incubation was selected for urine sample analysis.

2.4.2.3 Investigation of the optimal amount of β-glucuronidase for urinary phenol and ρ-cresol hydrolysis

Three urine samples were analysed to investigate the optimal amount of β-glucuronidase for hydrolysis of conjugated phenol and ρ-cresol.

Urine was adjusted to approximately pH 5.0 with 5M hydrochloric acid and 5M sodium hydroxide. 500 μl of each urine sample was taken into four tubes A, B, C and D. The urine was mixed with 200 μl of sodium acetate buffer (0.2M, pH 5.0). 0 to 30 μl of β-glucuronidase was added to the four urine-buffer mixtures:

Mixture	A	B	C	D
Urine volume	500μl	500μl	500μl	500μl
Sodium acetate buffer	200μl	200μl	200μl	200μl
β-glucuronidase: volume	0	10μl	20μl	30μl
β-glucuronidase (units)	(0)	(1070)	(2140)	(3210)
sulphatase (units)	(0)	(11.4)	(22.8)	(34.2)

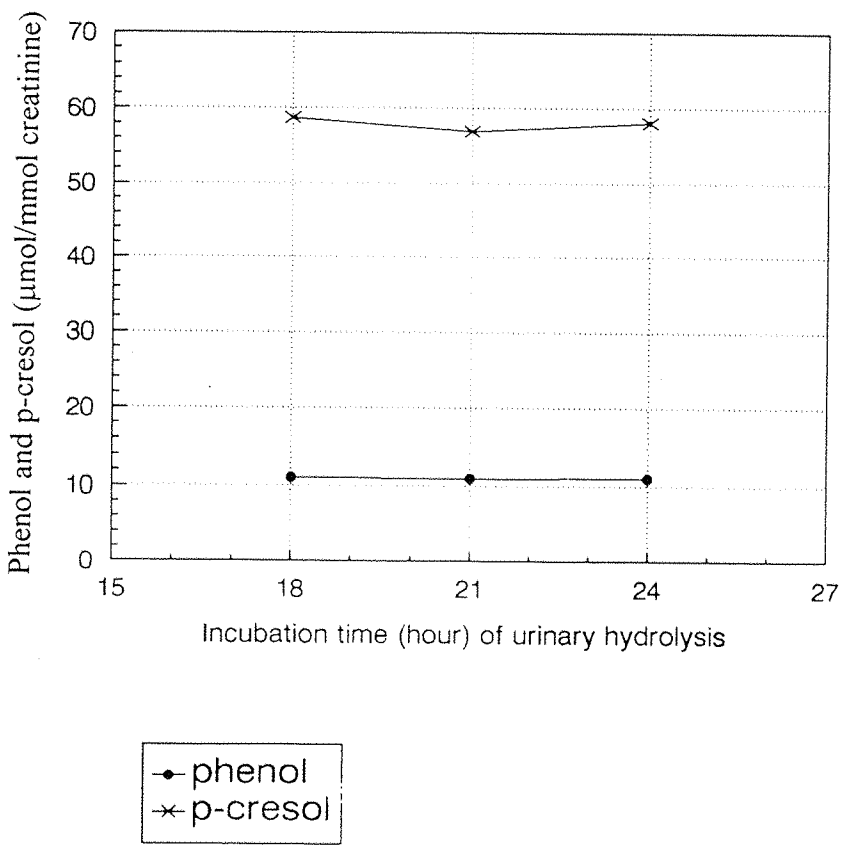
The mixtures were incubated at 37°C for 18 hours.

The hydrolysates of one urine sample (creatinine 6.9 mmol/L) were extracted with the modified SAX column extraction procedure and analysed by HPLC (Page 44) (Table 2-2; Figure 2-2). The hydrolysates of the other two urine samples (urine creatinine 22.7 mmol/L, and 26.5 mmol/L, respectively) were extracted with the GC extraction procedure and analysed by GC (Table 2-3; Figure 2-3).

Table 2-1 Experiment to determine the optimal incubation time of urinary hydrolysis with β-glucuronidase, at 37°C, pH 5.0

Incubation time (hour)	Phenol (μmol/mmol creatinine)	ρ-Cresol (μmol/mmol creatinine)
18	11.0	58.7
21	10.9	56.9
24	11.0	58.1

Figure 2-1 Phenol and p-cresol released by β-glucuronidase hydrolysis with increasing incubation time *



* analysis was with the GC procedure

Table 2-2 Experiment to determine the optimal volume of β-glucuronidase for urine hydrolysis: one urine sample analysed by HPLC

β-glucuro-nidase (μl)	phenol, ρ-cresol and phenolic acids (μmol/mmol creatinine)				
	phenol	ρ-cresol	hippuric acid	4-hydroxy-phenyl-acetic acid	4-hydroxy-benzoic acid
0	2.2	ND	96.8	48.8	3.5
10	22.9	139.3	96.8	37.5	12.6
20	23.6	137.8	94.2	48.5	13.1
30	19.3	98.5	59.7	42.82	ND

ND = not detected

Figure 2-2 Phenolic acids released by hydrolysis with increasing amounts of β-glucuronidase, analysed by HPLC

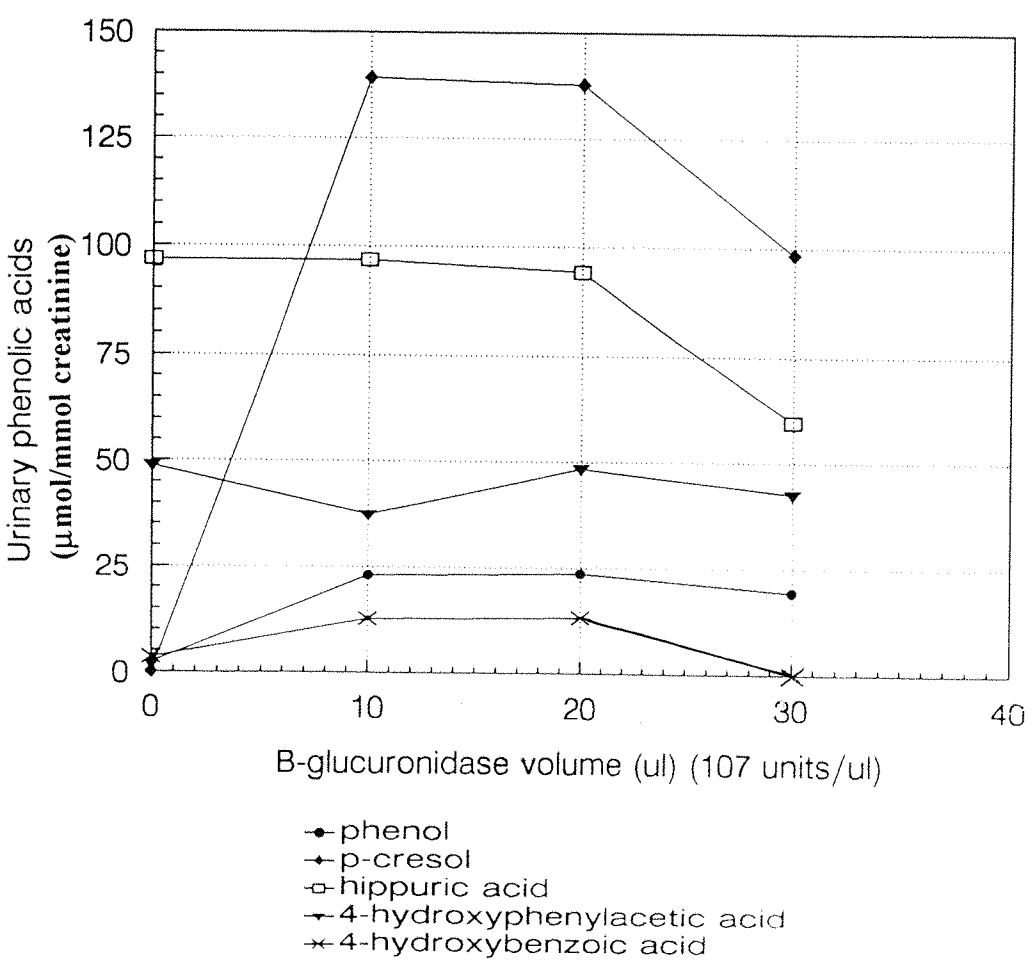


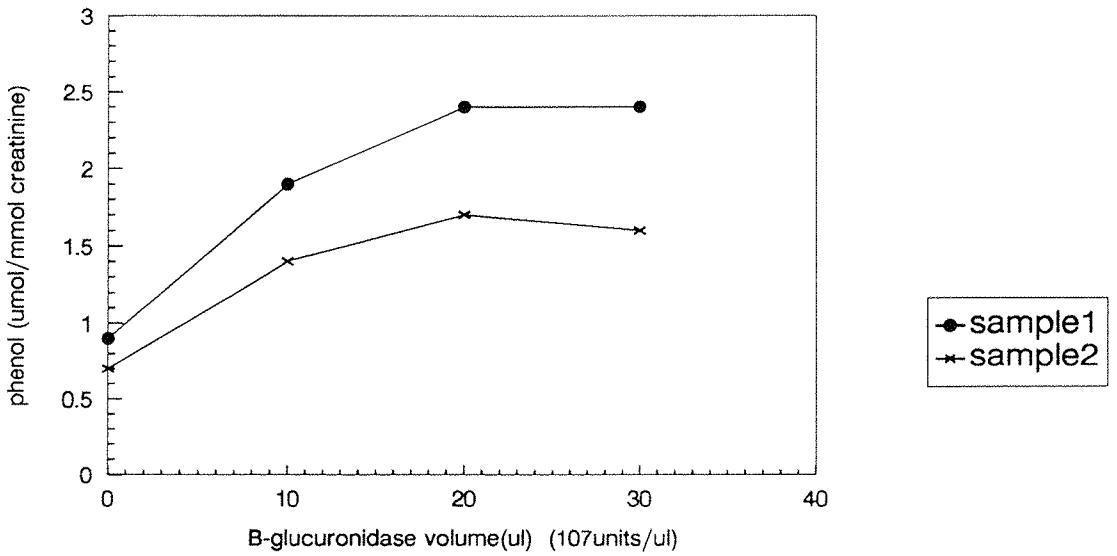
Table 2-3 Experiment to determine the optimal volume of β-glucuronidase for urinary hydrolysis: two adult urine samples analysed by GC

β-glucuronidase (μl)	phenol (μmol/mmol creatinine)		ρ-cresol (μmol/mmol creatinine)	
	sample 1	sample 2	sample 1	sample 2
0	0.9	0.7	2.9	12.4
10	1.9	1.4	6.0	30.1
20	2.4	1.7	7.6	34.1
30	2.4	1.6	7.7	34.5

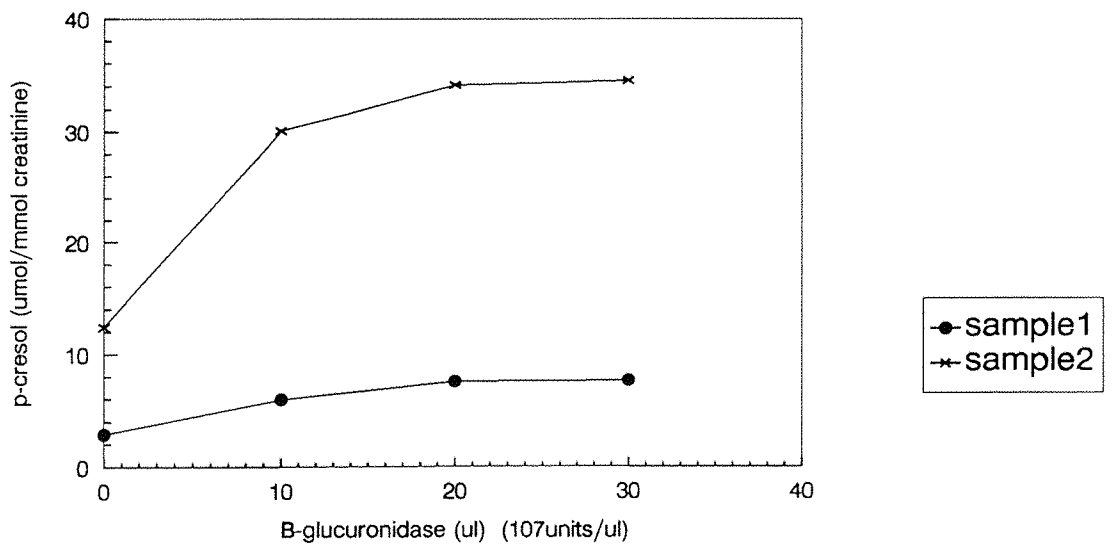
Each urine was incubated at 37°C for 18 hours with β-glucuronidase (107 units/μl).

Figure 2-3

(a) Phenol released by hydrolysis with increasing amounts of β-glucuronidase, analysed by GC



(b) ρ-cresol released by hydrolysis with increasing amounts of β-glucuronidase, analysed by GC



The yields of free phenol and ρ-cresol increased by adding β-glucuronidase. There were no significant differences between mixtures incubated with 20 μl and 30 μl of β-glucuronidase. 20 μl of β-glucuronidase was selected for hydrolysis of urinary phenol and ρ-cresol for all urine samples. Concentrations of two other urinary phenolic acids (hippuric acid and 4-hydroxyphenylacetic acid) were not increased by incubation with enzyme. A small increase in 4-hydroxybenzoic acid was noted, using HPLC (Table 2-2; Figure 2-2).

2.4.2.4 The selected β-glucuronidase hydrolysis procedure for urine samples

Urine samples were thoroughly thawed and mixed. For samples with creatinine of 4 mmol/L or less, a urine volume was taken which contained 1 μmol of creatinine:

$$\text{Urine volume (ul)} = \frac{1\text{x}1000}{\text{Urine creatinine concentration (mmol / L)}}$$

When the urinary creatinine exceeded 4 mmol/L, 250 μl neat urine was taken.

The volume of sodium acetate buffer and β-glucuronidase used were adjusted to the urinary volume, proportionately:

$$\frac{\text{Sodium acetate buffer 200ul} + \beta\text{-glucuronidase 20ul}}{\text{Urine volume 500ul}}$$

The samples were incubated at 37°C for 18 hours.

2.4.3 High-performance liquid chromatography

2.4.3.1 Introduction

High-performance liquid chromatography (HPLC) using polymeric strong cation-exchange columns eluted with dilute acid, combined with ultraviolet (UV) detection, has been proposed as a screening procedure for organic aciduria using prefractionated urine or plasma samples [102,103]. This technique has the ability to detect compounds of high polarity and glucuronide conjugates. Standard and unconjugated phenol and ρ-cresol were detected. Chong et al [102] used a short HPLC column (HPX-85H, 150x7.8mm, 8% cross-linked sulphonated divinylbenzenestyrene co-polymer in the hydrogen form) at 60°C, to analyse organic acids in urine and plasma. Diagnostically important compounds eluted within 18 minutes and were well resolved. Their method was evaluated for analysis of unconjugated phenol, ρ-cresol and other bacterial phenolic acids in urine.

2.4.3.2 Preparation of standard solutions

Stock standard solutions of phenol and phenolic acids (Standard A and Standard B) were prepared at 10 mmol/L.

Standard A:	Standard B :
4-hydroxyphenyllactic acid	4-hydroxyphenylacetic acid
hippuric acid	phenol
4-hydroxybenzoic acid	ρ-cresol
benzoic acid	

2.4.3.3 HPLC analytical procedure

The procedure adopted was that of Rumsby et al [103], as modified by Chong et al [102]. 25 μl of extract was injected on to the HPLC guard column. The mobile phase was 5 mmol/L sulphuric acid degassed with helium, and flow rate was 0.8 ml/min. The column temperature was 60°C. The eluent was monitored at detector wavelengths of 210 nm or 280 nm. The integrator attenuation setting was 32 and chart speed 0.5 cm/min.

2.4.3.4 Retention times and relative retention times of standard phenol, ρ-cresol and phenolic acids using HPLC

Stock standard solutions were diluted. 200 μl of prediluted standard solutions A and B were mixed with 200 μl of 3-(4-hydroxyphenyl)propionic acid as I.S.(50 μmol/L in Tris buffer pH 7.0). 25 μl of mixture was injected onto the HPLC column. The analysis was carried out 20 times, respectively.

The retention times relative to the internal standard (RRT's) and the coefficient of variation (CV%) were calculated using the following formulae:

For any compound X

$$RRTx = \frac{\text{Retention time of X}}{\text{Retention time of IS}}$$

$$CV\% = \frac{\text{Standard deviation of set of results}}{\text{Mean of set of results}} \text{ X } 100.$$

The mean retention times and relative retention times are shown in Table 2-4, together with the coefficients of variation, indicative of within batch precision. A further 8 acids were analysed only once. Their RRT's are included in Table 2-4.

Table 2-4 Retention times and relative retention times of standard phenolic acids analysed by HPLC

Phenolic acid	RT (min)	SD (min)	CV%	RRT	SD	CV%
4-hydroxy-3-methoxymandelic	7.90			0.49		
*4-hydroxy-phenylacetic	** 8.46	0.02	0.24	***0.54	0.003	0.5
2-hydroxy-phenylacetic	10.48			0.66		
*4-hydroxy-phenyllactic	**11.21	0.06	0.54	***0.70	0.002	0.3
phenyllactic	12.42			0.78		
4-hydroxy-3-methoxy-phenylacetic	12.57			0.79		
*hippuric	**13.89	0.04	0.30	***0.87	0.009	1.0
*I.S	**16.04	0.06	0.40	***1.00		
*phenol	**18.59	0.06	0.54	***1.16	0.005	0.4
2-hydroxy-hippuric	20.75			1.18		
*4-hydroxy-benzoic	**20.76	0.10	0.48	***1.30	0.002	0.1
*benzoic	**27.82	0.11	0.38	***1.74	0.001	0.3
2-hydroxy-benzoic	28.72			1.81		
*p-cresol	**30.79	0.15	0.50	***1.93	0.003	0.2
m-cresol	18.43			1.16		
o-cresol	29.40			1.79		

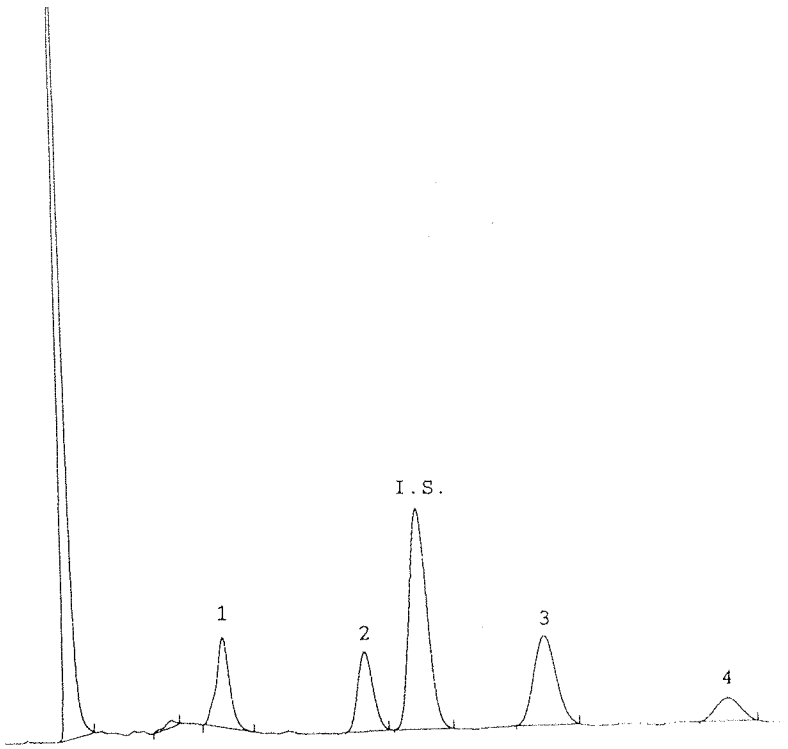
HPLC: conditions described on Page 35

RT: retention time, RRT: relative retention time

* analysed 20 times, other acids analysed once only

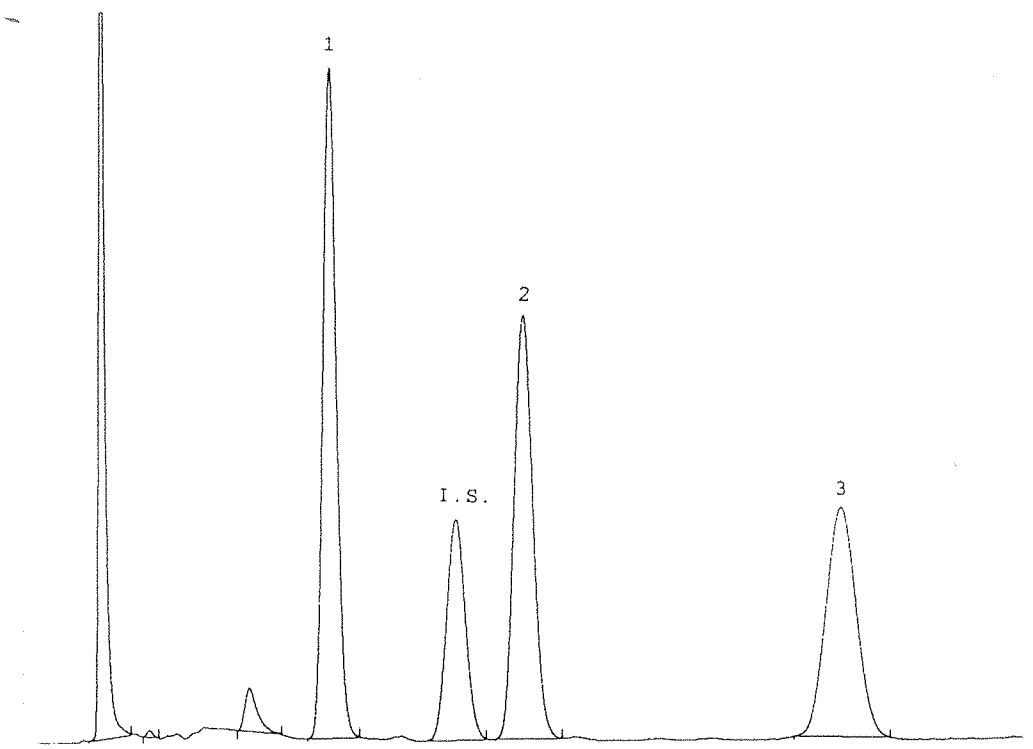
** mean RT, *** mean RRT

Figure 2-4



(a) Standard mixture A (200 μmol/L) analysed by HPLC at 210nm

I.S. 3-4-(hydroxyphenyl)propionic acid
1. 4-hydroxyphenyllactic acid 2. hippuric acid
3. 4-hydroxybenzoic acid 4. benzoic acid



(b) Standard mixture B (400 μmol/L) analysed by HPLC at 210nm

I.S : 3-4-(hydroxyphenyl)propionic acid
1. 4-hydroxyphenylacetic acid
2. phenol 3. p-cresol

The five standard phenolic acids, phenol and ρ-cresol eluted within 32 minutes and were well resolved (Figure 2-4). Precision was good with coefficients of variation ranging from 0.24 to 0.54 for retention times, and 0.1 to 1.0 for relative retention times.

2.4.3.5 Relative response factors

The response factors relative to the internal standard (RRF's) and the coefficients of variation (CV%) were calculated using the following formulae:

For any compound X

$$RRFx = \frac{\text{Area of compound X}}{\text{Area of IS peak}} \times \frac{\text{Concentration of IS}}{\text{Concentation of X}}$$

Prediluted standard solutions A and B each containing 50 μmol/L of IS, were analysed by the HPLC procedure, and detected at two different wavelengths, 210nm and 280nm. Each experiment was carried out 10 times and the RRF's calculated. Mean values for RRF's and coefficients of variation are shown in Table 2-5 (a) and (b).

All seven standard phenolic compounds, and the I.S., were detectable at 210 nm and 280 nm. However the absorbance of phenol at 210 nm was greater than that at 280 nm. This is reflected in the response factors relative to the I.S. (0.95 at 210 nm, 0.68 at 280 nm). The RRF of ρ-cresol at 210 nm was acceptable (0.83) and on the whole, the other phenolic acids had good absorbance at 210 nm. Since phenol and ρ-cresol were the major acids to be detected in this study, 210 nm UV wavelengths was selected for use.

2.4.3.6 Linearity and minimum detection limits of phenol, ρ-cresol and phenolic acids analysed by HPLC with detection at 210nm

Linearity and sensitivity of the HPLC procedure was tested for phenol, ρ-cresol and five phenolic acids in standard solutions. Stock solutions A and B were diluted serially to 1 μmol/L. 25μl of diluted standard solutions was analysed by HPLC, starting at the lowest concentration and working upwards to 400 μmol/L for Standard A and 500 μmol/L for Standard B. These concentrations are more than 5 times the upper limits reported for normal neonates [76]. The measured concentrations were plotted against true concentrations (Figure 2-5). The analysis was linear for all compounds. Table 2-6 shows the ranges of linearity and lower detection limits for the different compounds. Sensitivity was good for phenol (1 μmol/L) and ρ-cresol (4 μmol/L). The method was linear up to 5 00μmol/L for both compounds.

Table 2-5 (a) Response factors of organic acids relative to internal standard 3-(4-hydroxyphenylpropionic acid) at detector wavelength 210nm

Organic acids	RRF (mean) n=10	SD	CV(%)	Observed range
4-hydroxy-phenyllactic	1.08	0.06	5.6	0.97-1.15
hippuric	1.36	0.06	4.4	1.29-1.47
4-hydroxy-benzoic	2.19	0.12	5.4	2.03-2.37
benzoic	0.73	0.04	4.8	0.69-0.79
4-hydroxy-phenylacetic	1.05	0.07	7.1	0.95-1.16
phenol	0.95	0.06	6.1	0.83-1.02
ρ-cresol	0.83	0.05	5.8	0.75-0.89

RRF: response factor relative to internal standard

Table 2-5 (b) Response factors of organic acids relative to internal standard 3-(4-hydroxyphenylpropionic acid) at detector wavelength 280nm

Organic acids	RRF(mean) n=10	SD	CV(%)	Observed range
4-hydroxy-phenyllactic	0.99	0.035	3.5	0.95-1.05
hippuric	0.35	0.025	7.1	0.30-0.38
4-hydroxy-benzoic	3.58	0.130	3.6	3.40-3.80
4-hydroxy-phenylacetic	1.03	0.093	9.0	0.91-1.09
phenol	0.68	0.067	9.8	0.61-0.84
ρ-cresol	1.13	0.093	8.3	1.05-1.15
benzoic	0.67	0.029	4.3	0.64-0.72

RRF: response factor relative to internal standard

Figure 2-5 Linearity of standard phenolic acids analysed by HPLC

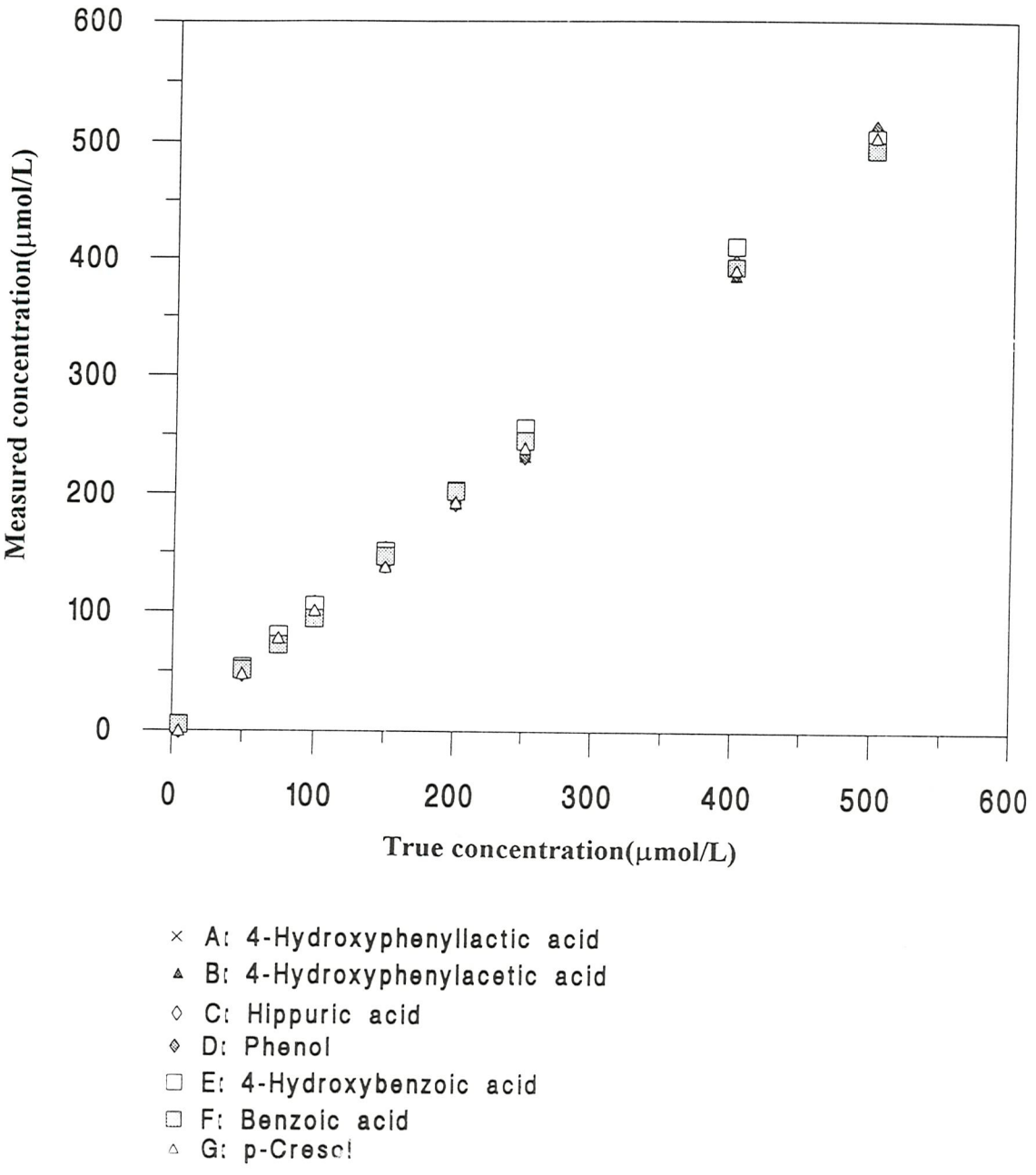


Table 2-6

Linearity for phenol, ρ-cresol and 5 other phenolic acids analysed by HPLC with 210nm detection

Phenolic acids	Range of linearity (μmol/L)
Standard A:	
4-hydroxyphenyllactic acid	5 - 400
hippuric acid	5 - 400
4-hydroxybenzoic acid	5 - 400
benzoic acid	50 - 400
Standard B:	
4-hydroxyphenylacetic acid	1 - 500
phenol	1 - 500
ρ-cresol	4 - 500

2.4.3.7 Investigation of extraction procedures for phenol, ρ-cresol and phenolic acids from urine for HPLC

(1) Unextracted urine samples analysed by HPLC

Six hydrolysed urine samples were prediluted to creatinine 0.1 mmol/L. 200 µl of each was mixed with 200 µl of I.S. and 25 µl was analysed by HPLC without using a preliminary extraction procedure. The chromatograms were not satisfactory, with only small peaks of phenol and ρ-cresol dominated by large amounts of other compounds (Figure 2-6).

It was concluded that a preliminary extraction procedure was essential. In addition, the small peaks of phenol and ρ-cresol found indicated that more urine would need to be injected into the HPLC system, to obtain quantitative data.

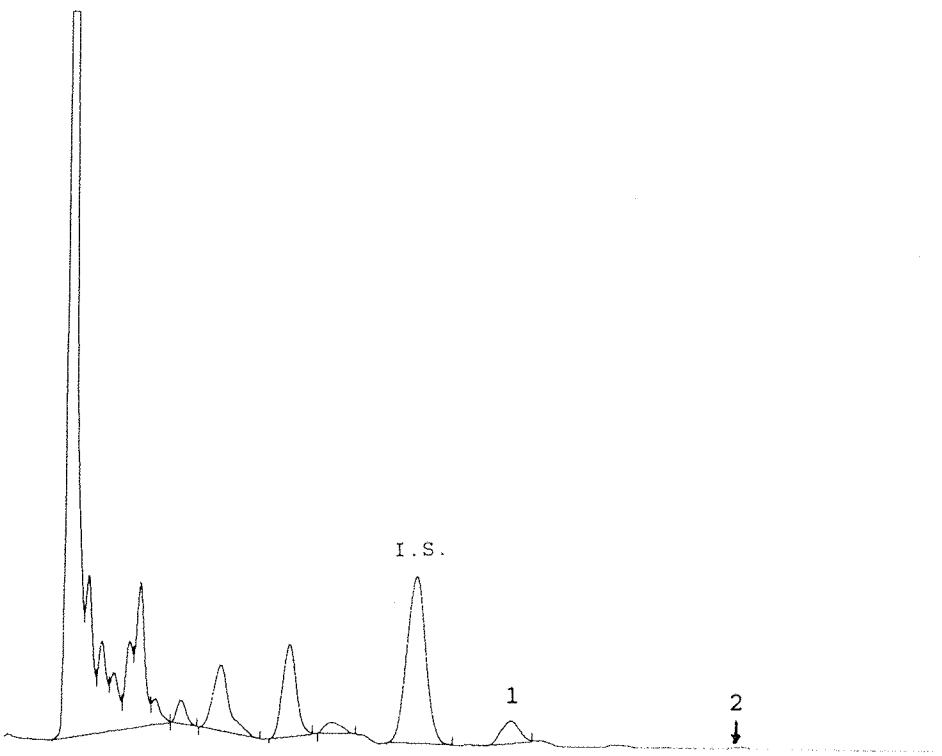
(2) Anion-exchange column (SAX column) extraction

Anion-exchange was considered an excellent extraction method for organic acid analysis of urine by GC. Anion exchange Bond Elut SAX columns (Supelchem, Sawbridgeworth, U.K.) have been applied successfully to urinary organic acid analysis by HPLC by Rumsby et al [103]. Their method was investigated as a means of extracting phenol and ρ-cresol. Urine, diluted with water to a creatinine concentration of 0.1 mmol/L, is added to Tris buffer containing I.S. (PH 7.0). This is applied to SAX columns, which are then washed with water, and organic acids are eluted with 0.5M sulphuric acid.

(3) SAX column extraction procedure

200 µl of pre-diluted urine or standard phenolic acid solution A or B was mixed with 200 µl of internal standard [3-(4-hydroxyphenyl)propionic acid]. 200 µl of this mixture was applied to a prepared SAX column (the method of column preparation is the same as of regeneration described below), and was eluted slowly, using the "Vac-Elut" apparatus under low pressure (less than 1 mmHg). The column was then washed with 2 ml water. The first eluate and wash were discarded. 250 µl of sulphuric acid (0.5M) was applied to the column and the eluate collected into an Eppendorf tube (3 ml size). The column was dried under pressure (15-20 mmHg) for 2 minutes and regenerated for next use: 1 ml of 0.5 mol/L hydrochloric acid, 1 ml of methanol, and two washes of 1 ml of water were applied sequentially under low suction pressure (less than 5 mmHg). The columns were dried for 2 minutes under increased pressure (15-20 mmHg). One SAX column can be regenerated and reused up to 20 times for urine analysis [103].

Figure 2-6 Diluted urine (creatinine 0.1 mmol/L) analysed by HPLC without extraction



I.S : 3-4-(hydroxyphenyl)propionic acid
1. phenol 2. ρ-cresol

(4) Recovery of phenol, ρ-cresol and phenolic acids using the SAX column extraction procedure

Aqueous standard A was diluted to 375.1 μmol/L, standard B to 380.3 μmol/L. 200 μl of each solution was extracted using SAX columns and 25 μl of the extract analysed by HPLC with detection at 210nm. Each extraction and HPLC analysis was carried out six times. In addition, 25 μl of each unextracted standard mixture was analysed by HPLC three times. Percentage recoveries of each compound were calculated from the integrated peak areas:

$$\text{Recovery (\%)} = \frac{\text{Area of peak of extracted acid}}{\text{Area of peak of unextracted acid}} \text{ X } 100$$

The mean recoveries for the 5 phenolic acids were acceptable ranging from 71.7% to 98.5% (Table 2-7). However, phenol and ρ-cresol were not detected in the sulphuric acid eluate. Preliminary observation showed that these compounds were eluted from the SAX columns in the water wash.

(5) Modification of the SAX column extraction procedure

The method of SAX column extraction was modified: after applying diluted standard mixture (containing I.S.) to the column, both the water wash and sulphuric acid eluates were saved. As I.S. was not eluted with water, 100 μl of 3-(4-hydroxyphenyl)propionic acid (I.S) was added to 100 μl of water wash as external standard and 25 μl was analysed by HPLC. 25 μl of sulphuric acid eluate was also injected onto the HPLC column.

Unhydrolysed urine was diluted to 0.1 mmol/L creatinine and 20 mmol/L standard phenol and ρ-cresol solutions were added to give final concentrations of 0.2, and 0.5 mmol/L. 200 μl of I.S was added to 200 μl of each mixture and the samples were applied to a SAX column. Water washes and sulphuric acid eluate were collected. 200 μl of I.S. was added to 200 μl of water wash and 25 μl was injected onto the HPLC. 25 μl of sulphuric acid eluate was also analysed by HPLC. Each experiment was carried out six times.

Unextracted standard solutions were analysed by HPLC in parallel. The unspiked diluted urine (0.1 mmol/L creatinine) was analysed by HPLC to obtain baseline concentrations. The yields of phenol/ρ-cresol in the water wash and sulphuric acid eluate were added together in order to calculate the recovered amounts. The mean percentage recoveries of phenol and ρ-cresol were good: 99.0% and 98.0% for phenol at the two concentrations; 98.9% and 95.8% for ρ-cresol, and precision was acceptable (Table 2-8).

Table 2-7 Recovery of standard phenolic acids, extracted with SAX columns and analysed by HPLC with detection at 210nm (n=6)

Phenolic acids	Expected $\frac{pk_{area}}{IS_{area}}$	Recovered $\frac{pk_{area}}{IS_{area}}$	SD	CV(%)	Recovery (%)
*4-hydroxy-phenyllactic	19.86	19.34	1.36	7.0	97.4
*hippuric	25.27	20.47	1.29	6.0	81.0
*4-hydroxy-benzoic	41.96	38.77	1.29	3.3	92.4
*benzoic	13.39	9.598	0.77	8.0	71.7
#4-hydroxy-phenylacetic	25.5	25.12	0.95	3.8	98.5
#phenol	18.3	ND			
#p-cresol	16.3	ND			

ND : not detected

* mixture of each standard acid of concentration 375.1 μmol/L

mixture of each standard acid of concentration 380.3 μmol/L

2.4.3.8 HPLC analysis of urine samples with the modified SAX column extraction procedure

Because of the very small peaks of phenol and ρ-cresol observed when urine was prediluted to 0.1 mmol/L creatinine for analysis (page 42), six urine samples were prediluted to creatinine 0.8 mmol/L. 200 µl of each was mixed with 200 µl of I.S., and 200 µl of the mixture was applied to a SAX column. Both the water washes and sulphuric acid eluates were collected and analysed by HPLC as described above. Phenol and ρ-cresol were detectable, however, the chromatograms showed gross overload (Figure 2-7). The SAX column procedure was abandoned as an unsuitable extraction procedure for urinary phenol and ρ-cresol.

2.4.3.9 Other methods of sample preparation for HPLC analysis of phenol and ρ-cresol

Solvent extraction is used widely to isolated organic acids from biological fluids for analysis by GC. This method was explored for extraction of urinary phenol and ρ-cresol for analysis by HPLC. A volume of hydrolysed urine equivalent to 0.1 µmol of creatinine was taken. 200 µl of I.S. was added to the urine and the mixture was extracted with 2 x 2 ml of ethyl acetate followed by 2 x 2 ml of diethyl ether. The extract was dried completely under nitrogen at 40°C, and 250 µl of 0.5M sulphuric acid was added to the residue. 25 µl was injected into the HPLC. The yields of phenol and ρ-cresol were very poor. This was attributed to losses of phenol and ρ-cresol by evaporation during drying, since these compounds are volatile.

Other columns investigated for urinary extraction for HPLC Bond Elut SCX columns (Varian, European Technical Centre, Cambridge, UK), (strong cation exchange columns), were investigated for phenol and ρ-cresol extraction. Standard phenol and ρ-cresol (0.5 mmol/L) was applied to the column with the same procedure used for SAX columns (page 42). Phenol and ρ-cresol were not detected in the water wash or sulphuric acid eluate by HPLC. Use of an alkaline eluting buffer was not investigated

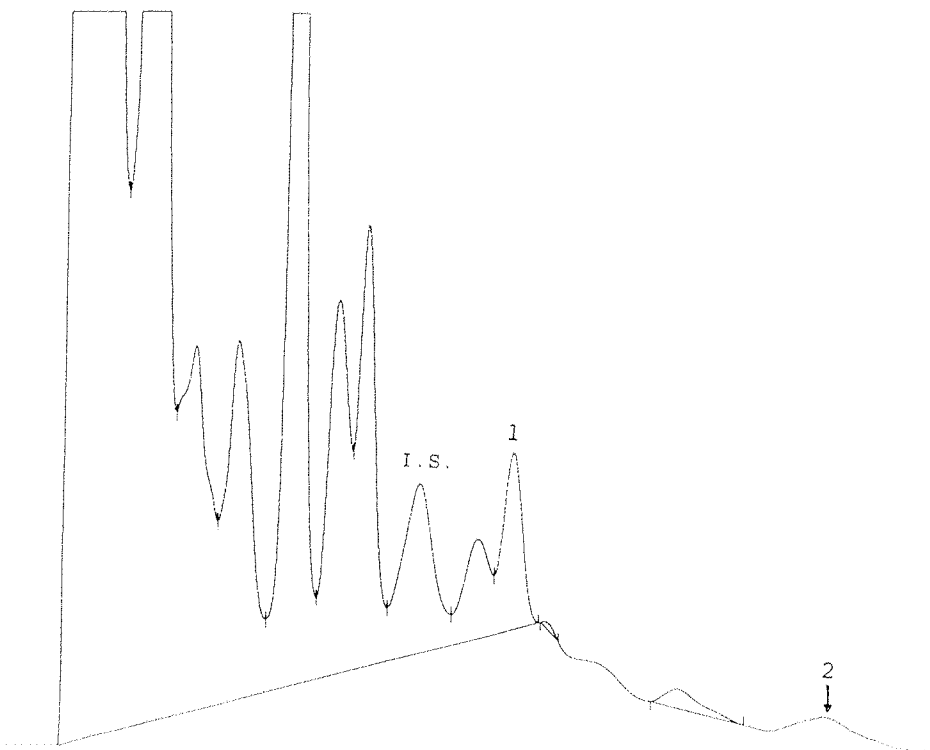
Cellulose columns (Celute MX tube Jones Company, U.K.) were investigated. Standard solutions of phenol and ρ-cresol were diluted to 500 µmol/L. 500 µl of standard was mixed with 500 µl of IS. 400 µl was applied to the column and eluted for 10 minutes without any pressure. The column was then eluted without pressure with 3 x 1 ml of isopropanol/dichloromethane (10:90 v/v). The eluates were collected together and evaporated to dryness under nitrogen at 40°C. 250 µl of 0.5M sulphuric acid was added to the residue and 25 µl injected into the HPLC. Phenol and ρ-cresol were not detected.

Table 2-8 Recoveries of standard phenol and ρ-cresol extracted from urine with the modified SAX procedure and analysed by HPLC

Expected Conc. (mmol/L)	phenol (n=6)			
	Mean recovered Conc. (mmol/L)	SD	CV(%)	Mean recovery(%)
0.105	0.104	0.003	2.9	99.0
0.405	0.397	0.021	5.3	98.0

Expected Conc. (mmol/L)	ρ-cresol (n=6)			
	Mean recovered Conc. (mmol/L)	SD	CV(%)	Mean recovery(%)
0.089	0.088	0.003	3.4	98.9
0.334	0.320	0.021	6.6	95.8

Figure 2-7 Diluted urine (creatinine 0.8 mmol/L extracted with the modified SAX column extration procedure and analysed by HPLC(water wash)



I.S : 3-4-(hydroxyphenyl)propionic acid
1. phenol 2. p-cresol

2.4.3.10 Unsuitability of the HPLC procedures for detecting urinary phenol and ρ-cresol

From the experiments above, it was concluded that the combined extraction and HPLC procedure investigated was unsatisfactory for analysis of urinary phenol and ρ-cresol. The method appeared promising when tested with standards, and small peaks of phenol and ρ-cresol were detected if relatively large amounts of urine were extracted and analysed. However, the chromatography was poor, because large amounts of other compounds were also extracted and overloaded the column. The procedure was, therefore, abandoned.

2.4.4 Gas Chromatography techniques

2.4.4.1 Introduction

Gas chromatography (GC) is a chromatographic method in which compounds are separated on the basis of their different partition between a stationary liquid and mobile gaseous phase.

GC techniques have been used to determine phenols in biological samples after preliminary extraction [75,76]. This method was investigated using a solvent extraction procedure

2.4.4.2 GC conditions

A method for measuring volatile fatty acids in faeces was already established in the laboratory, using a polyethylene glycol BP-20 column [104]. The same GC conditions were adopted with minor modifications, for phenol and ρ-cresol analysis.

The GC oven temperature programme was adjusted to give a good separation of phenol and ρ-cresol:

Initial Temperature	50°C
Initial Time	0.5 minutes
Ramp 1	40°C/min to 150°C for 0.1 minute
Ramp 2	4°C to 215°C for 1 minute

Helium was using as carrier gas (flow rate of 3 ml/min), and as the detector make-up gas (flow rate 30 ml/min). The split injector (1:7 split ratio) and detector were maintained at 200 °C.

2.4.4.3 Sample extraction for GC

Standard solution or hydrolysed urine samples containing 30 μl of I.S.(3.93 mmol/L of 2-ethylbutyric acid) with addition of water to 1 ml if the volume was less than

1 ml, was acidified with 5M hydrochloric acid (100 µl of 5M HCl to 500 µl of standard solution or urine). The mixture was extracted with 2 x 3 ml of diethyl ether. The ether extracts were combined together and concentrated approximately to volume of 250 µl under a gentle nitrogen gas steam at temperature 37°C. 1.5 µl of concentrated extract was injected into the gas chromatograph.

2.4.4.4 Retention times (RTs) and relative retention times (RRTs) of standard phenol and ρ-cresol

A standard of phenol and ρ-cresol standard solution (200 µmol/L in water) containing 30 µl 2-ethylbutyric acid (3.93 mmol/L) as I.S. was extracted with 2 x 2ml of diethyl ether. The combined ether extracts were concentrated to volume 250 µl and 1.5 µl analysed by GC 20 times respectively. Narrow, well resolved, peaks were obtained (Figure 2-8). The mean retention time for I.S. was 7.30 minutes, for phenol was 10.87 minutes and for ρ-cresol, 12.19 minutes, and relative retention times were 1.52 for phenol and 1.71 for ρ-cresol. The precision was good (Table 2-9).

2.4.4.5 Relative response factors (RRFs) of phenol and ρ-cresol analysed by GC

A stock standard mixture of phenol and ρ-cresol was prepared in water at a concentration of 20 mmol/L. 4 µmol/L, 0.6 mmol/L, 0.8 mmol/L and 1 mmol/L of standard 1 ml containing 30 µl of I.S. were extracted with ether and analysed by GC. Each experiment was carried out six times.

RRFs of phenol and ρ-cresol are shown in Table 2-10 including coefficient of variation (CV%). The mean RRFs ranged from 0.95 to 1.07 for phenol at four concentrations and from 0.92 to 1.11 for ρ-cresol.

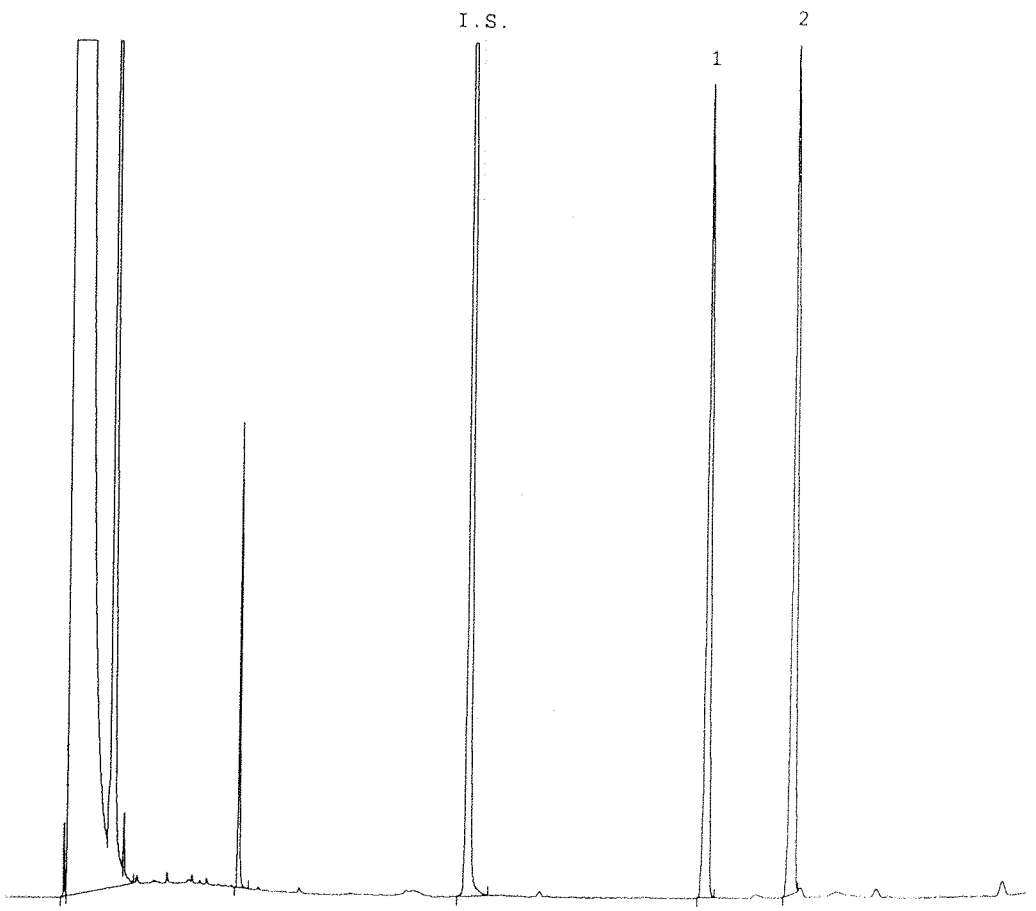
2.4.4.6 Linearity and detection limits of phenol and ρ-cresol with the GC procedure

Linearity of the GC procedure was tested for phenol and ρ-cresol. Serial dilutions of the 20 mmol/L stock standard mixture of phenol and ρ-cresol were prepared over the range 2 µmol/L to 1 mmol/L. 1 ml of each dilution was extracted as above and analysed by GC, starting at the lowest concentration and working up to 1 mmol/L, which is about 10 times the upper limit per µmol of creatinine reported for normal neonates [76]. The method was sensitive to 4 µmol/L of both phenol and ρ-cresol, and was linear to 1 mmol/L for both compounds (Figure 2-9).

2.4.4.7 Recovery of phenol and ρ-cresol from urine

Standard phenol and ρ-cresol was added to 500 µl of urine, and diluted with

Figure 2-8 Standard phenol and ρ-cresol extracted with ether and analysed by GC



I.S. 2-ethylbutyric acid

1. phenol 2. ρ-cresol

Table 2-9 Retention times and relative retention times of standard phenol and ρ-cresol (n=20) analysed by GC

	Mean RT (min)	SD	CV(%)	Mean RRT	SD	CV(%)
IS	7.13	0.026	0.37			
phenol	10.87	0.033	0.30	1.52	0.0047	0.31
ρ-cresol	12.19	0.034	0.28	1.71	0.0048	0.28

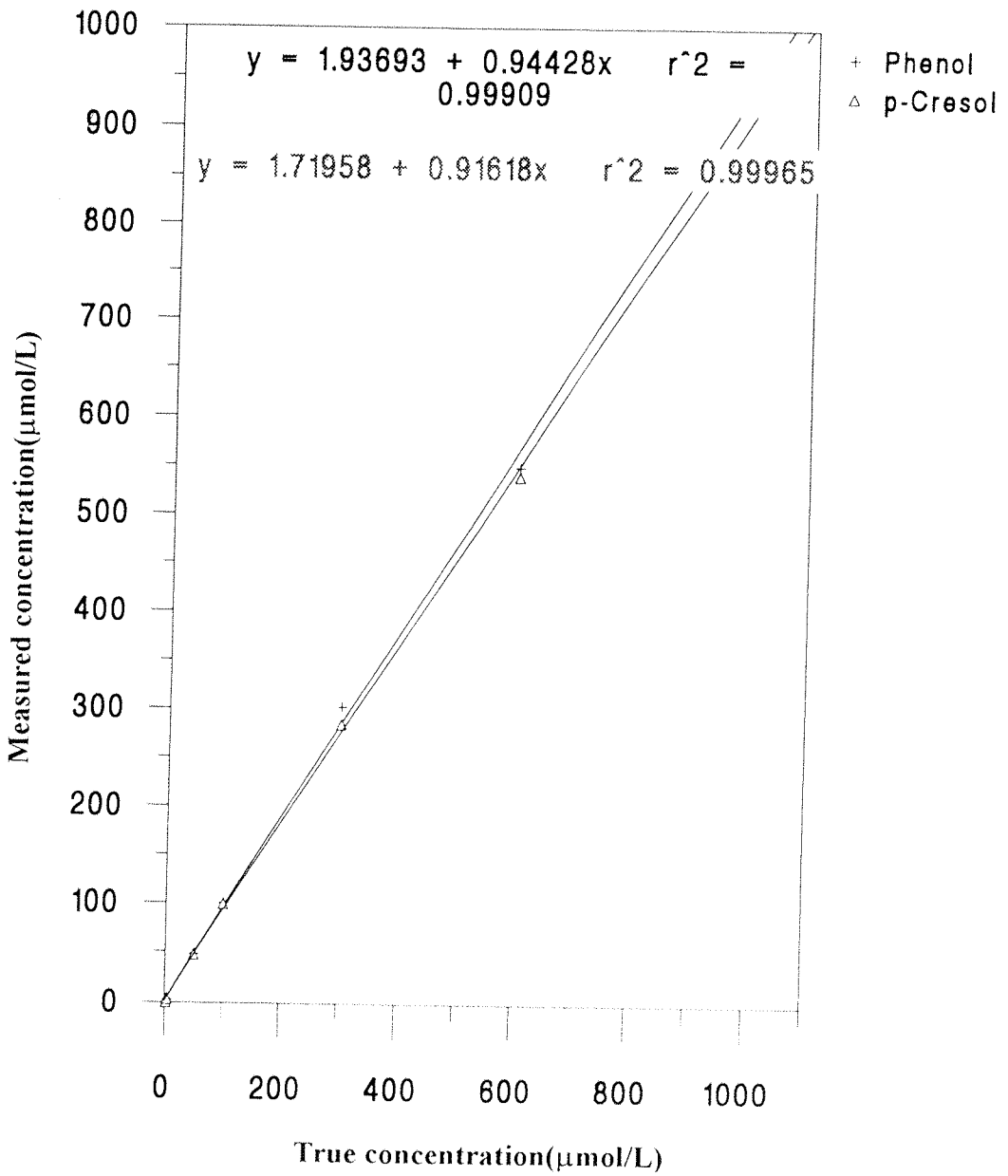
IS: internal standard 2-ethylbutyric acid
RT: retention time
RRT: relative retention time

Table 2-10 Relative response factors of standard phenol and ρ-cresol in four concentrations (n=6) analysed by GC

concen-tration (mmol/L)	phenol			ρ-cresol		
	mean RRF	SD	CV(%)	mean RRF	SD	CV(%)
$4x10^{-3}$	1.06	0.02	1.89	1.04	0.03	2.88
0.1	0.95	0.02	2.11	0.92	0.07	7.6
0.6	1.07	0.02	1.87	1.07	0.01	0.93
1.0	1.07	0.03	2.80	1.11	0.02	1.80

RRF: relative response factors

Figure 2-9 Linearity of standard phenol and ρ-cresol analysed by GC



water to a total volume of 1 ml to determine recoveries. The final concentrations of phenol and ρ-cresol were 4 μmol/L, 0.6 mmol/L, 0.8 mmol/L and 1 mmol/L in the 1ml diluted urines. 30 μl of I.S was added to the mixtures, which were acidified by 5M HCl, then extracted with 2 x 2 ml of diethyl ether. The combined extracts were concentrated to a volume of 250 μl and 1.5 μl was analysed by GC. Each extraction and GC analysis was carried out six times. In addition unspiked urine was extracted and analysed twice to obtain baseline concentrations.

The mean recoveries and coefficients of variation, indicating within batch precision are shown in Table 2-11. The mean recoveries ranged from 89.3% to 106.0% for phenol and from 92.6% to 109.0% for ρ-cresol. Analytical precision was acceptable for phenol (CV 0.23 - 6.10%) and for ρ-cresol at concentrations of 0.1 μmol/L and above (0.25-6.40%).

2.4.4.8 Between-batch precision of phenol and ρ-cresol

Urine samples of 1 ml were spiked with standard phenol and ρ-cresol solution at four different concentrations. The spiked samples were analysed by GC consecutively for six days to determined between-batch precision. Precision was acceptable for both phenol and ρ-cresol (Table 2-12).

The ether extraction and GC procedures were considered satisfactory and were used for the clinical studies. Urine samples were first hydrolysed with β-glucuronidase. Figure 2-10 outlines the complete analytical procedure. The concentrations of phenol and ρ-cresol were calculated by reference to IS (2-ethylbutyric acid). Two examples of gas chromatograms of urine from premature babies are shown in Figure 2-12 and 2-13.

2.4.5 GC analysis of urine 2,3-butanediol

The procedure was that of Mills and Walker , who investigated 2,3-butanediol in premature babies [73]. Figure 2-11 outlines the complete analytical procedure.

2.4.5.1 GC instrumentation

Capillary gas chromatography was carried out using a Pye 204 instrument (Pye Unicam, Cambridge, U.K.) equipped with a flame ionisation detector. This was connected to the same Trio monitoring system which was used for HPLC (see above), and to a chart recorder. The integrator attenuation setting was 32 and chart speed 0.5 cm/min. The compounds were separated on a non-polar BPI fused silica capillary column (Scientific Glass Engineering, Milton Keynes, U.K.) (25 m x 0.33 mm i.d.) using helium as carrier gas. The temperature programme was 50°C for 5 minutes then 6°C/min to 260°C (held for 10 minutes). The injector and detector were maintained at 250°C. 1.5 μl was injected.

Table 2-11 Recoveries of standard phenol and ρ-cresol from urine (n=6) and within batch precision (CV%): ether extraction and GC analysis

Expected Conc. (mmol/L)	phenol			
	Mean Recovered Conc.(mmol/L)	SD	CV(%)	Mean Recovery(%)
$4x10^{-3}$	$3.57x10^{-3}$	$1.3x10^{-4}$	3.60	89.3
0.1	0.106	0.0065	6.10	106.0
0.6	0.566	0.0013	0.23	94.3
1.0	0.926	0.0160	1.73	92.6

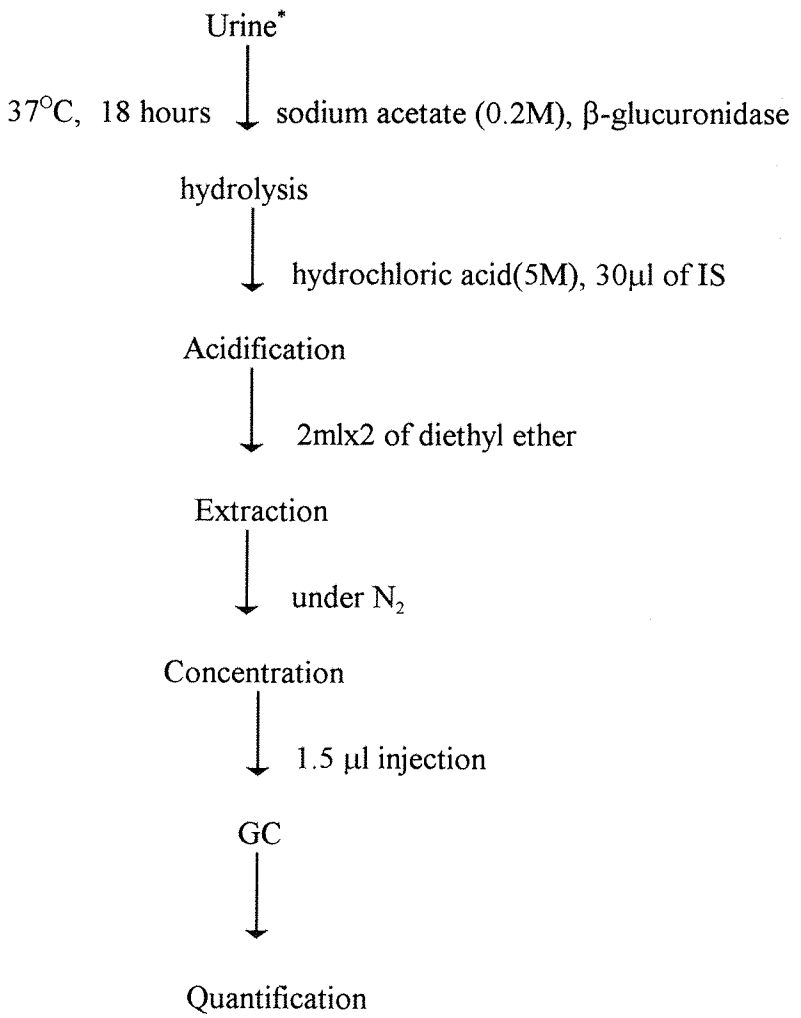
Expected Conc. (mmol/L)	ρ-cresol			
	Mean Recovered Conc.(mmol/L)	SD	CV(%)	Mean Recovery(%)
$4x10^{-3}$	$3.85x10^{-3}$	$4.6x10^{-4}$	12.00	96.3
0.1	0.109	0.0070	6.40	109.0
0.6	0.556	0.0014	0.25	92.6
1.0	0.898	0.0170	1.89	89.8

Table 2-12 Recovery of standard phenol and ρ-cresol from urine and between batch precision (CV%) (n=6) by GC

Expected Conc. (mmol/L)	phenol			
	Mean Recovered Conc.(mmol/L)	SD	CV(%)	Mean Recovery(%)
$4x10^{-3}$	$3.5x10^{-3}$	$8.9x10^{-5}$	2.5	87.5
0.1	0.100	0.0084	8.4	100.0
0.6	0.550	0.0260	4.7	91.7
1.0	0.952	0.0929	9.7	95.2

Expected Conc. (mmol/L)	ρ-cresol			
	Mean Recovered Conc.(mmol/L)	SD	CV(%)	Mean Recovery(%)
$4x10^{-3}$	$3.7x10^{-3}$	$2.5x10^{-4}$	6.8	92.5
0.1	0.099	0.0084	8.5	99.0
0.6	0.540	0.032	5.9	90.0
1.0	0.922	0.099	10.8	92.2

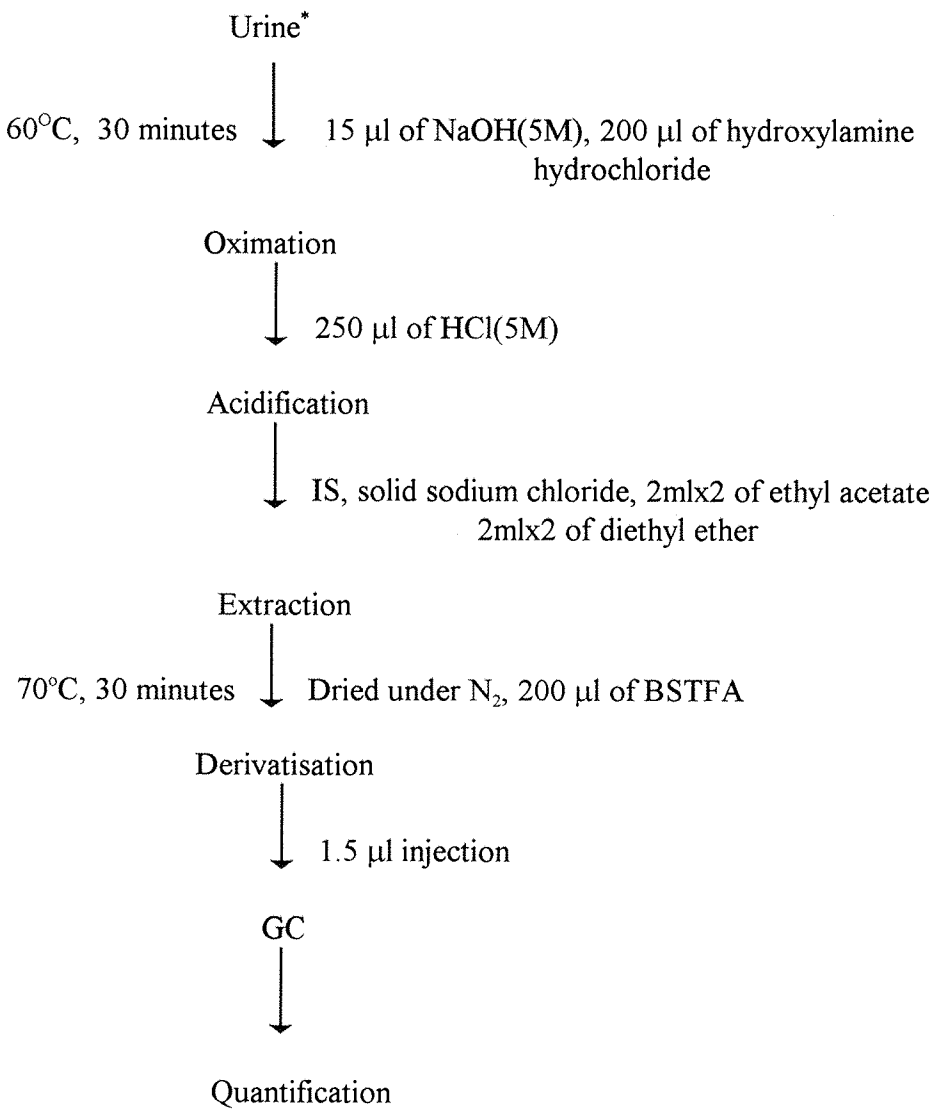
Figure2-10 Analytical procedure for analysis of urinary phenol and ρ-cresol



* urine volume according to creatinine: see p34

IS (internal standard): 0.118μm per tube of 2-ethylbutyric acid

Figure 2-11 The procedure of sample preparation for GC analysis of urinary 2,3-butanediol



* urine volume according to creatinine: see p59

IS (internal standard): 20ng per tube of tetracosane and of 3-phenylbutyric acid

2.4.5.2 Procedure

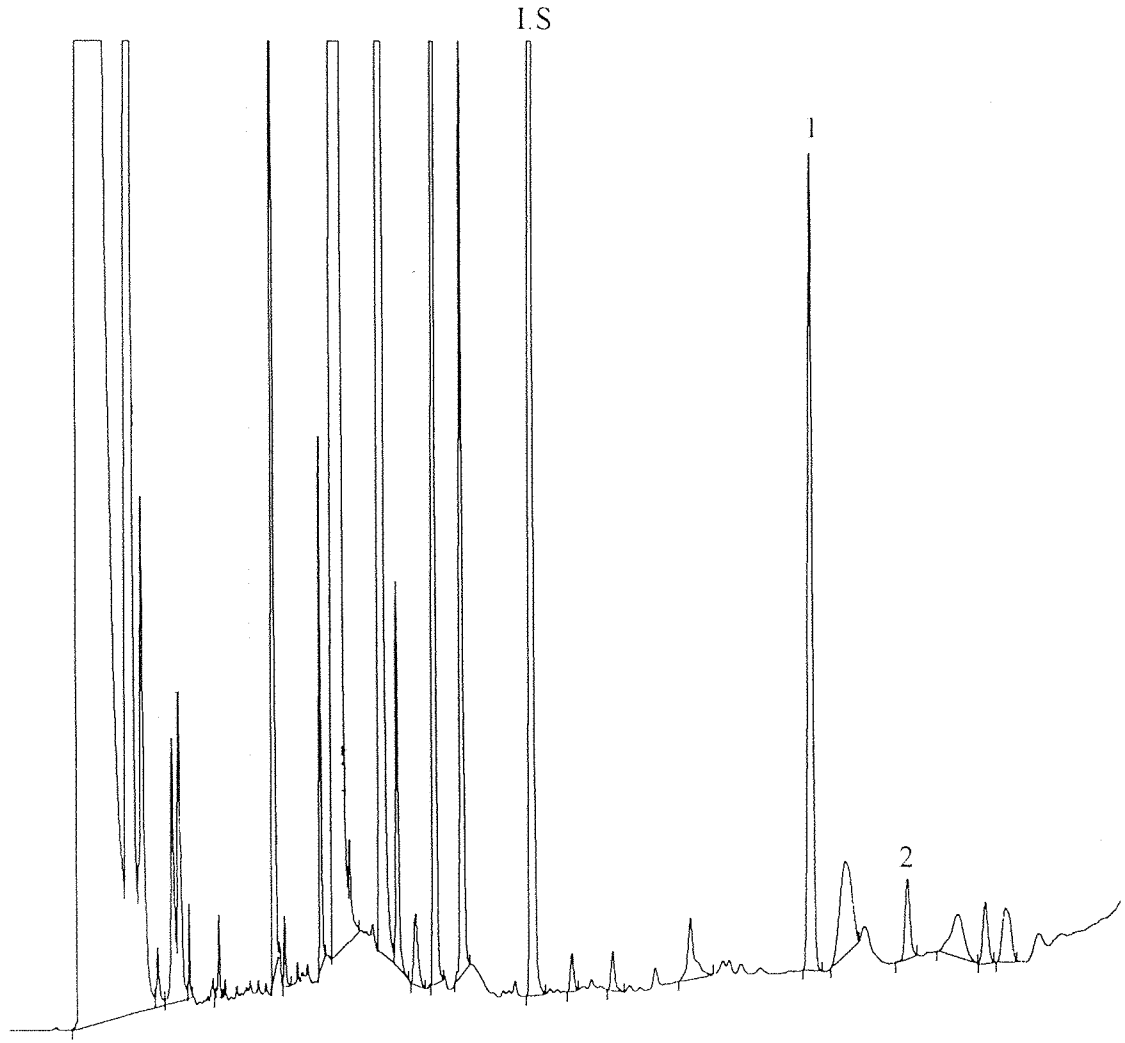
Up to a creatinine concentration of 8 mmol/L, a volume of urine was taken equivalent to 1 μmol of creatinine:

$$\text{Urine volume (ul)} = \frac{1 \times 1000}{\text{Urine creatinine concentration (mmol / L)}}$$

For urine stronger than this, 125 μl was taken. 750 μl of water was added if the volume of urine required was less than 1 ml. The urine was adjusted to pH greater than 10.0 using 5M sodium hydroxide and oximated by heating with 250 μl of 75g/L hydroxylamine hydrochloride solution at 60°C for 30 minutes. The mixture was acidified with 250 μl of 5M hydrochloric acid. 20 μl of 1 mg/ml of C_{24} alkane (tetracosane) and 3-phenylbutyric acid in cyclohexane solution was added as internal standard (I.S.). The sample was saturated with sodium chloride. 2 ml of ethyl acetate was added and mixed vigorously for 1 minute (whirlimix). After centrifugation, the solvent was transferred to a clean glass tube. The urine was extracted as above, with another 2 ml of ethyl acetate , followed by 2 x 2 ml diethyl ether. The combined extracts were dried completely under a gentle stream of nitrogen at 40°C. Organic acids were derivatised by addition of 200μl of BSTFA [bis(trimethylsilyl)-trifluoroacetamide] and heating at 70°C for 30 minutes. 1.5μl was analysed by GC. The concentration of 2,3-butanediol was calculated by reference to tetracosane IS. Figure 2-14 shows a gas chromatogram of urine from a premature baby.

Figure 2-12 Baby No 7

Phenol and ρ-cresol excreted in urine analysed by GC

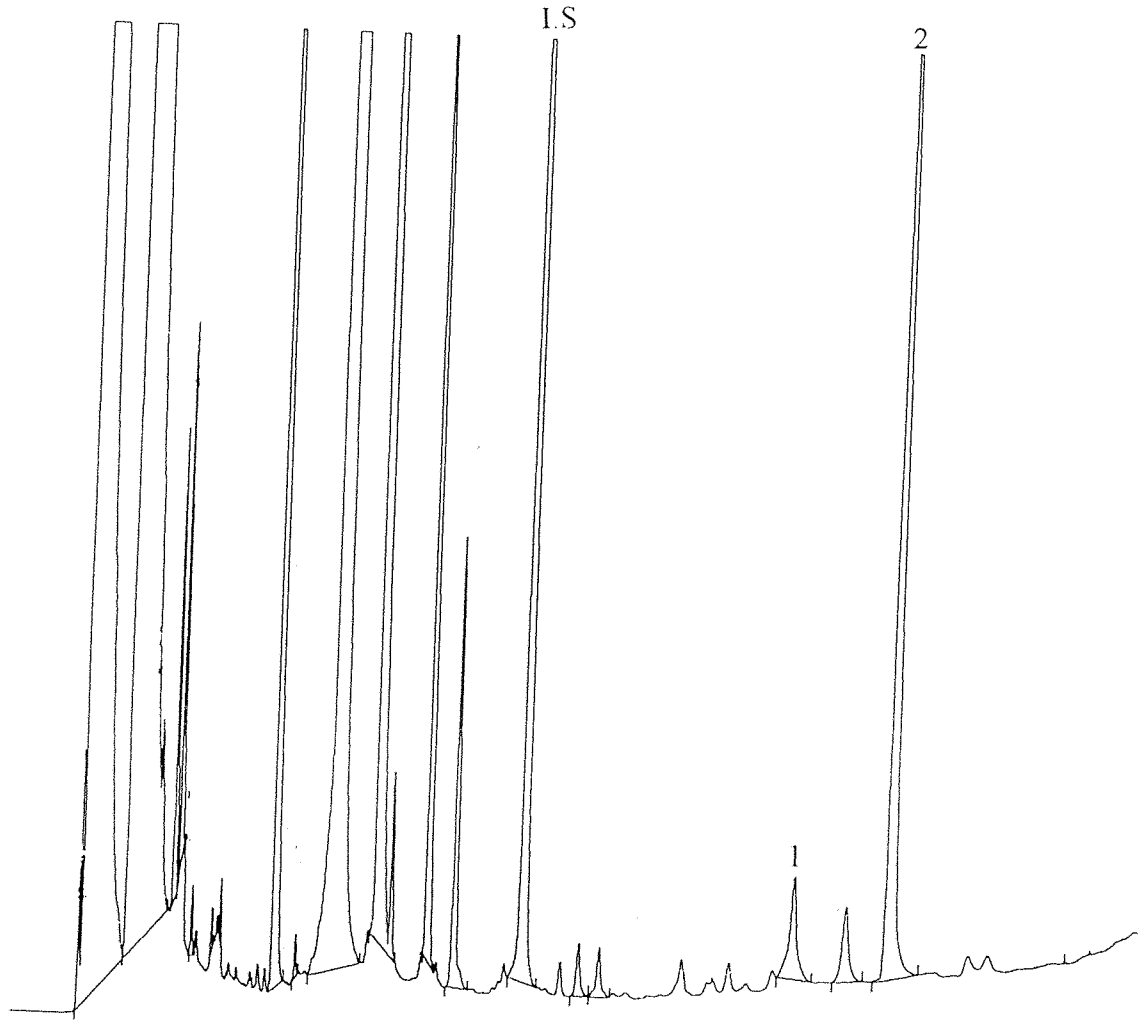


IS 2-ethylbutyric acid

1. Phenol 2. ρ-Cresol

Figure 2-13 Baby No 20

Phenol and ρ-cresol excreted in urine analysed by GC

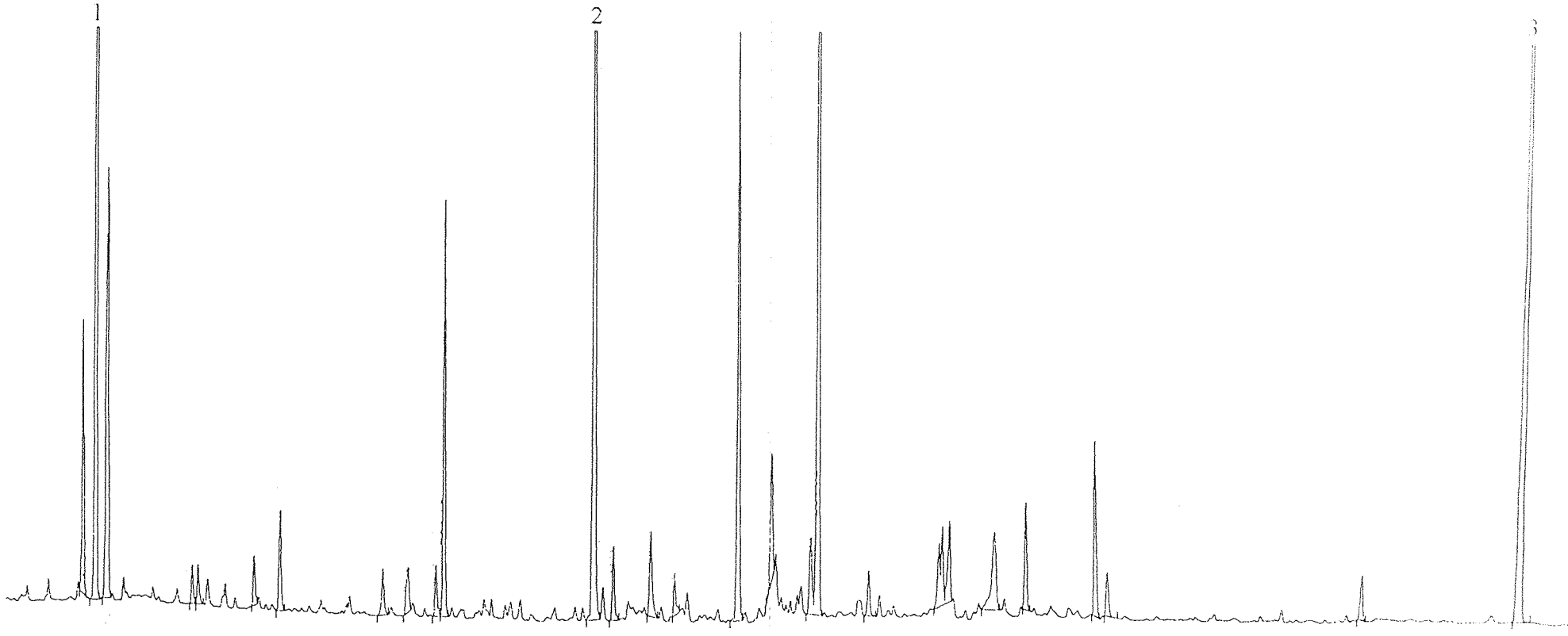


IS 2-ethylbutyric acid

1. Phenol 2. ρ-Cresol

Figure 2-14 Baby No 20

2,3-Butanediol excreted in urine analysed by GC



1. 2,3-Butanediol 2. 3-Phenylbutyric acid 3. Tetracosane

CHAPTER THREE

CLINICAL STUDIES AND ANALYTICAL RESULTS
EFFECTS OF FEEDING PREMATURE INFANTS WITH *LACTOBACILLUS GG*

This was a randomised double blind study to examine the effects of feeding *Lactobacillus GG* to premature babies. The aims were: to determine whether or not the probiotic *Lactobacillus GG* could colonise the bowel and, if so, whether colonisation reduced the reservoir of potential pathogens; to find out whether there was any effect on clinical progress and outcome; to find out whether colonisation influenced gut bacterial metabolism; specifically, carbohydrate fermentation and production of volatile phenols. The study was interdisciplinary, the clinical leg being supervised by Dr M A Hall (Consultant Paediatrician), Microbiology by Dr M R Millar (Consultant Microbiologist) and the biochemistry by Dr V Walker (Consultant chemical pathologist). My project was part of the biochemical investigation and was to measure gut bacterial metabolites in urine (phenol, ρ-cresol and 2,3-butanediol).

3.1 BABIES STUDIED

Twenty preterm babies with gestational age (GA) of 33 weeks or less were studied, who were admitted to the neonatal unit of Princess Anne Hospital, Southampton, from 1st September 1991 to 31st January 1992. With informed parental consent, they were randomised to two groups: Group A received standard milk feeds with *Lactobacillus GG* added in a dose 10^8 colony forming units (cfu), twice daily for 14 days , starting with the first feed. Group B received standard milk feeds without *Lactobacillus GG*. Other care, including antibiotic treatment, followed routine procedures. Oral feeding and *Lactobacillus GG* supplements started on days 1 to 3. Seventeen study babies (eight Group A, nine Group B) received breast milk initially until 3 to 10 days of age (median 6 days), when formula milk feeds were supplemented. The three other babies were fed a standard formula milk initially. A low birth weight formula milk (SMA, Wyeth) was introduced from age 5 to 19 days (median 10) in 16 cases, eight in each group. Other care followed routine practice. Antibiotics given were: none (two Group A, three Group B); cefotaxime only, age 1-5 days, (four Group A, five Group B); flucloxacillin with netilmicin (four Group A); erythromycin (one Group B); and multiple (one GroupB). The clinical features of the two groups are shown in Table 3-1. All the infants were studied for their full stay in the neonatal unit. Every infant

Table 3-1 Clinical details of the study infants

	Group A (n=10)	Group B (n=10)
Birth weight (gm) mean (range)	1425 (800-2560)	1457 (830-2150)
Gestation (week) mean (range)	30.0 (26-33)	30.1 (24-33)
Mode of delivery		
VD	4	8
LSCS	6	2
PROM (>24 hours)	2	2
Duration of hospital stay (day) median (range)	51.1 (23-136)	42.8 (19-114)

PROM: prolonged rupture of membranes

VD: vaginal delivery

LSCS: lower segment caesarean section

was examined daily by a physician who was actively involved in the care of the infants on the neonatal unit, but who was not aware of the study randomisation schedule. The following clinical details were recorded daily: general well being, any signs of abdominal distension, vomiting or regurgitation, feed intolerance, the incidence of perineal rash, the frequency and consistency of stools, the number of suppositories used and the fluid intake. The exact type and amount of fluid was recorded and the total caloric intake was then calculated. The weight of each infant was recorded three times weekly. Other clinical parameters evaluated included any clinical or laboratory evidence of sepsis, antibiotic treatment or any other concomitant medication, oxygen and ventilatory requirements; the duration of hospital stay was also calculated for each group [105].

3.2 SAMPLES

3.2.1 Milk

Samples supplemented with *Lactobacillus GG* were collected daily for culture on selective and non-selective media, to ensure that they were not contaminated with other bacteria or with yeasts.

3.2.2 Faeces for microbiological studies

Samples were collected each day and sent in a Gaspak Pouch (BBL Microbiology Systems, Cockeysville, USA) to the microbiology laboratory for quantitative studies. Samples were labelled with the names of infants but the randomisation group was not given to the laboratory. Faecal samples were stored at -70°C in glycerol citrate broths, usually within 24 hours of collection. The maximum time that elapsed before samples were frozen was 72 hours. Details of the methods used for bacterial culture and identification have been reported [105].

3.2.3 Urine for biochemical analysis

A random sample of urine (minimum volume 3 ml), was collected into a bag on, or within 48 hours of, the first day of feeding and 7, 14, 21, 28 and 35 days from this. Additional samples were also sometimes available for study. Samples were frozen immediately and stored at -20°C, without preservative.

3.2.4 Urinary phenol and p-cresol of healthy adults

As an extension of the validation studies for the analysis of urinary phenol and p-cresol by gas chromatography, urine was analysed from 27 healthy adults. The ranges found were compared with published data.

Subjects studied 27 healthy adults were studied, who were attending the Renal Stones Clinic of Chemical Pathology, Southampton General Hospital. There were 19 men and 8 women, aged 20 to 70 years (median 37 years). None was known to have a gastrointestinal disorder, and all had normal renal function, as assesed by creatinine clearance studies. A fasting random urine sample was collected into a sterile container when patients came to the Pathology Department for metabolic investigations for their renal stones. Samples were stored at -20°C without preservative.

The results for these adults are listed in Appendix 1 and summarised in Table 3-2.

3.3 RESULTS OF THE *LACTOBACILLUS GG* STUDY

3.3.1 Clinical and microbiological results

The clinical and microbiological findings have been reported [105]. In summary, nine of the 10 Group A babies were colonised by *Lactobacillus GG* (see appendix 2). The numbers of *Lactobacillus GG* isolated from faeces, and the proportion of infants with bowel colonisation, declined after feed supplementation was discontinued. However, samples available at five weeks after starting feeds, and three weeks after discontinuing supplementation, still showed bowel colonisation with *Lactobacillus GG* in numbers ranging from 5.8 to 10.0 $\log_{10}$ cfu/g dry weight. The mean numbers (mean $\log_{10}$cfu/g dry weight) of *Lactobacillus GG* were 9.16 at seven days after the start of feeding, 9.04 at 14 days, 6.7 at 21 days, 7.23 at 28 days and 6.3 at 35 days.

The other microorganisms isolated from stool samples included enterobacteriaceae (*E.coli*, *Klebsiella* sp., *Enterobacter* sp., *Serratia* sp., *Citrobacter* sp., *Proteus* sp. and *Acinetobacter* sp.), staphylococci (coagulase-negative and *Staphylococcus aureus*), yeasts, *Enterococcus* sp., anaerobes (*Bacteroides* sp., *Clostridium* sp., *Veillonella* sp., *Bacillus* sp., *Bifidobacterium* sp.) and other unidentified anaerobic or micro-aerophilic Gram-positive bacilli. The proportion of infants colonised by the different bacterial species at each post-feeding week was similar in the two groups. There were no significant differences between the two groups with regard to the numbers of enterobacteriaceae, coagulase-negative staphylococci, *Enterococcus* sp. and anaerobes at any post-feeding week. Enterobacteriaceae were first isolated at a mean of 7.75 post feeding days in group A compared with 8.0 days in Group B.

There were no significant differences between the two groups for any of the

Table 3-2 Urinary phenol and ρ-cresol (μmol/mmol creatinine) of control adults

Adult controls	Phenol		ρ- Cresol		Ratio $\frac{\rho\text{- cresol}}{\text{phenol}}$
	No.*	Median (range)+	No.*	Median (range)+	Median (range)+
Men	19/19	2.8 (tr.-8.7)	18/19	22.4 (2.5-106.4)	6.9 (1.0-27.8)
Women	8/8	2.8 (2.4-4.5)	8/8	20.4 (tr.-75.9)	8.8 (4.2-25.3)

* Number of all samples that were positive

\+ Median (range) of positive samples only

clinical parameters recorded. Mean (SD) weight gain was 21.55 (9.22) g/kg per day for Group A and 22.40 (7.91) for Group B. Daily fluid and caloric intakes per kg were similar. Although the infants in Group A did appear to spend more time on the neonatal unit (Table 3-1), this was not significantly different using the Mann-Whitney U test. Intravenous antibiotic therapy was administered to eight of the ten infants who were given feed supplementation with *Lactobacillus GG* and to seven of the infants in Group B. There were no cases of proven sepsis in either of the two groups at any time, although one of the infants in each group was found to have perineal candida infection which required treatment with topical and oral nystatin. There were no differences between the two groups in the numbers of infants ventilated, the duration of ventilation, frequency of oxygen therapy and the incidence of chronic lung disease.

3.3.2 Analytical results

3.3.2.1 Urinary phenol and ρ-cresol excreted by control babies (Group B)

The microbiological studies showed that treatment with the antibiotic cefotaxime during the first five days of life, did not cause significant changes in faecal bacterial counts after 6 days of age, when compared with those of babies who did not receive antibiotic. From Table 3-3, it is evident that there was no demonstrable effect on urinary phenol and ρ-cresol. (Urine samples of baby N 10 were not analysed because the volumes were too small. For individual results of all other babies' samples, see Appendix 3)

Data was, therefore, pooled for the three babies (N 13, N 19, N 20) who received no antibiotics and five (N 11, N 14, N 16, N 17, N 18) who had cefotaxime only (Table 3-4). Figure 3-1 shows the time course of urinary phenol and ρ-cresol excretion for baby N. 20. Results for the two other babies treated with erythromycin (days 16-28; N 15) and multiple antibiotics (days 1-28; N 12) have been excluded, as these antibiotic regimens were associated with decreased counts of faecal bacteria. During the first week, phenol was present in all samples, with concentrations ranging from 11.1 to 61.4 μmol/mmol creatinine (median 22.9). ρ-Cresol was present in only 4 of 8 samples (50%), the concentrations in these ranging from 4.1-6.7 μmol/mmol creatinine (median 4.9). The ratio of ρ-cresol to phenol ranged from 0 to 4.5 (median 0.2). Thereafter ρ-cresol was present in the majority of samples, sometimes at high concentration. Concentrations of phenol were generally highest during the first week. The lower concentration in the second week were maintained during the remaining weeks. The ratios of urinary ρ-cresol/phenol were highest in the third and fifth weeks.

Table 3-3 Urinary phenol (μmol/mmol creatinine) of eight babies from Group B: three who received no antibiotics; five who received cefotaxime only in the first 5 days of life

Age (days)	No antibiotics		Cefotaxime only	
	No.*	Median(range)+	No.*	Median(range)+
0-7	4/4	16.5(11.1-45.4)	4/4	37.9(10.2-61.4)
8-14	1/1	tr.	4/5	4.5(tr.-4.7)
15-21	3/3	tr., 5.0, 22.8	2/3	1.7, 5.8
22-28	1/1	9.2	1/1	4.2
29-35	2/2	5.6, 64.0	1/1	5.0
>35	No samples		2/3	4.7, 8.1

* Number of all samples that were positive

\+ Median (range) of positive samples only

Urinary p-cresol (μmol/mmol creatinine) of eight babies from Group B: three who received no antibiotics; five who received cefotaxime only in the first 5 days of life

Age (days)	No antibiotics		Cefotaxime only	
	No.*	Median(range)+	No.*	Median(range)+
0-7	3/4	4.5 (4.1-5.2)	1/4	6.7
8-14	1/1	tr.	4/5	6.6 (tr-8.7)
15-21	3/3	tr., 132.0, 145.0	2/3	4.0, 132.0
22-28	1/1	100.0	1/1	4.5
29-35	2/2	6.2, 113.0	1/1	245.0
>35	No samples		2/3	4.2, 7.0

* Number of all samples that were positive

\+ Median (range) of positive samples only

Table 3-4 Urinary phenol and ρ-cresol (μmol/mmol creatinine) of eight babies from Group B: three who received no antibiotics; five who received cefotaxime only in the first 5 days of life

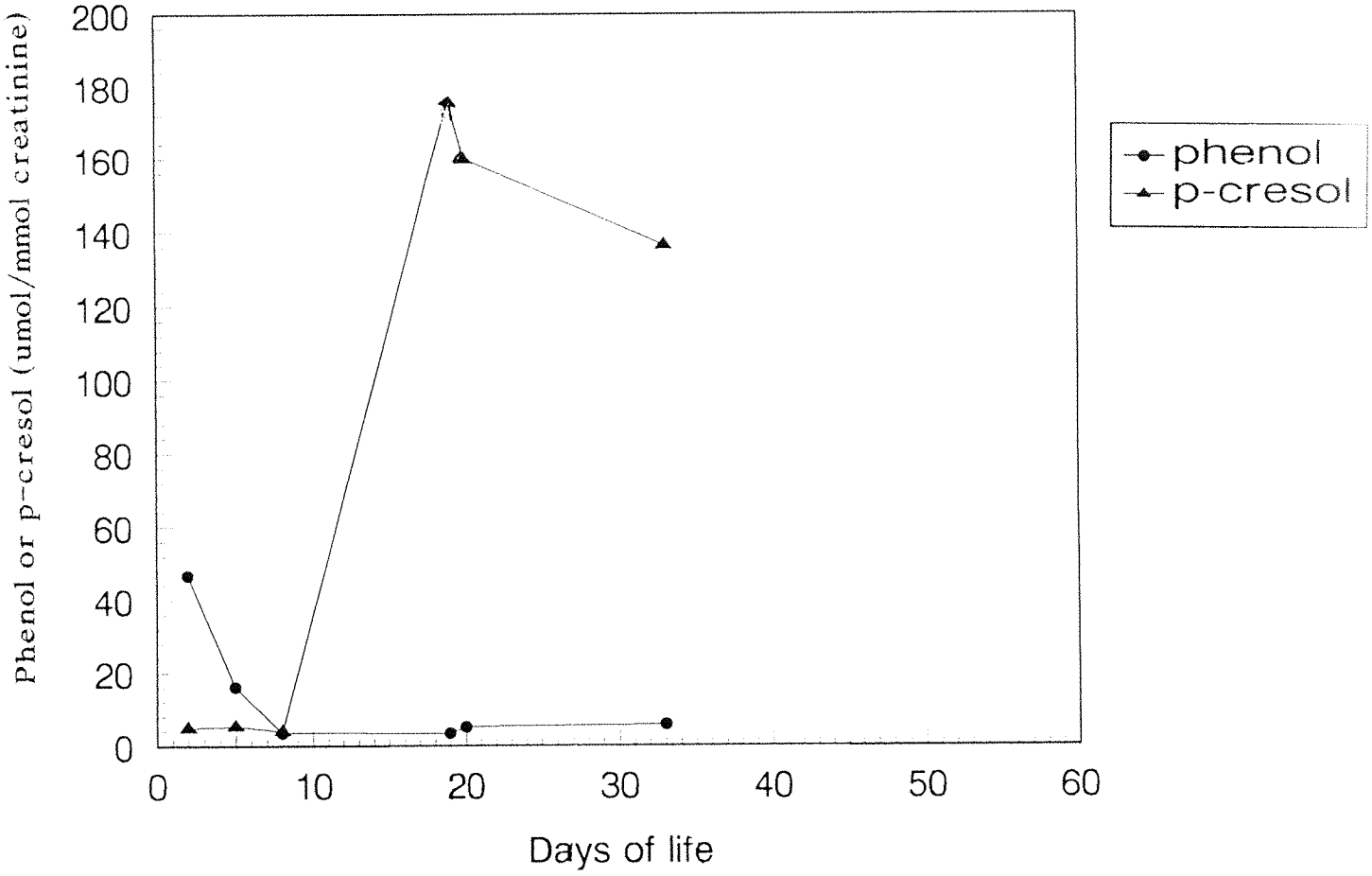
Age (days)	Phenol		ρ-Cresol		Ratio $\frac{\rho\text{-cresol}}{\text{phenol}}$
	No.*	Median (range)+	No.*	Median (range)+	Median (range)+
0-7	8/8	22.9 (11.1-61.4)	4/8	4.9 (4.1-6.7)	0.2 (0-4.5)
8-14	5/6	tr. (tr.-4.7)	5/6	tr. (tr.-8.7)	1.0 (0-1.4)
15-21	5/6	5.4 (tr.-22.8)	5/6	68.0 (tr.-145.0)	11.5 (0-26.4)
22-28	2/2	4.2, 9.2	2/2	4.5, 100.0	1.1, 10.8
29-35	3/3	5.0, 5.6, 64.0	3/3	6.2, 113.0, 245.0	0.1, 20.2, 49.0
>35	2/3	4.7, 8.1	2/3	4.2, 7.0	0.5, 1.5
Total	$\frac{25}{28}$	8.7 (tr-64.0)	$\frac{21}{28}$	6.6 (tr.-245.0)	

* Number of all samples that were positive

\+ Median (range) of positive samples only

Figure 3-1 Baby No. 20

Time course of excretion of urinary phenol and p-cresol



3.3.2.2 Effect of *Lactobacillus GG* colonisation on urinary phenol and ρ-cresol

Results were pooled for two babies who received no antibiotics (N 8, N 4) and four treated with cefotaxime only aged 1-5 days (N 6, N 7, N 9, N 10). They were all colonised with *Lactobacillus GG*. Their urinary phenol and ρ-cresol excretion is presented in Table 3-5. When compared with simiar data for Group B babies, it is evident that colonisation was not associated with significant changes in urinary phenol and ρ-cresol: there were no statistically significant differences between the *frequencies* of phenol or ρ-cresol excretion by Group A and Group B babies (X^2 test) or in the concentrations of phenol and ρ-cresol in positive samples of Group A and Group B (Mann-Whitney U test)

3.3.2.3 Urinary phenol and ρ-cresol of babies receiving antibiotics other than cefotaxime

Table 3-6 presents data for one Group B baby who was treated with erythromycin aged 16-28 days, and for 4 Group A babies who all had courses of flucloxacillin with netilmicin. One of the four group A babies did not colonise with *Lactobacillus GG*, one colonised initially, but lost the organism during antibiotic treatment and, in the fourth, *Lactobacillus GG* counts were decreased transiently. In three cases, antibiotic treatment was associated with decreased faecal anaerobes. Urinary phenol excretion of these babies was similar to that of Group A and Group B babies who received no antibiotics, or cefotaxime only (compare Table 3-5). In contrast, upto 35 days of life, excretion of ρ-cresol was significantly lower for the babies treated with antibiotics other than cefotaxime ($p<0.01$, Mann-Whitney U test). Data is presented separately (Table 3-7) for a sixth baby (from Group B). He was born at 24 weeks' gestation, developed staphylococcal septicaemia and chronic lung disease and received almost continuous antibiotic treatment up to 38 days of age, followed by a further course of netilmicin and flucloxicillin aged 48-53 days. By chance, monitoring was extended to 87 days of life. Up to 42 days of life, the only organisms grown from faecal samples were enterococci and coagulase negative staphylocci (all six samples) and enterobacteriaceae (one only). Strict anaerobes were not isolated. He was milk fed through out. Although phenol and ρ-cresol were detectable in all nine samples analysed, their concentrations were extremely low up to 56 days of age. (Figure 3-2 shows antibiotics and urinary phenol and ρ-cresol)

3.3.2.4 Bacterial sources of phenol and ρ-cresol

Phenol is produced by facultative anaerobes (*E. coli, Proteus* sp, *Enterococcus*); ρ-cresol is produced by strict anaerobes (*Bacteroides fragilis Fusobacterium* sp, *Bifidobacterium* sp, *Clostridium* sp). Appendix 4 shows when phenol-producing

Table 3-5 Urinary phenol (μmol/mmol creatinine) of six babies colonised with *Lactobacillus GG* (Group A) and eight controls (Group B) who received no antibiotics or cefotaxime only in the first 5 days of life

Age (days)	Group A		Group B	
	No.*	Median(range)+	No.*	Median(range)+
0-7	5/5	13.1 (4.9-31.1)	8/8	22.9(11.1-61.4)
8-14	4/5	4.1 (tr.-4.5)	5/6	tr. (tr.-4.7)
15-21	5/6	tr. (tr-7.8)	5/6	5.4 (tr.-22.8)
22-28	2/3	tr., 8.9,	2/2	4.2, 9.2
29-35	2/2	4.3, 9.4	3/3	5.0, 5.6, 64.0
>35	2/3	tr, 6.1	2/3	4.7, 8.1
Total	20/24	4.4 (tr.-31.1)	25/28	8.7 (tr.-64.0)

* Number of all samples that were positive

\+ Median (range) of positive samples only

Urinary ρ-cresol (μmol/mmol creatinine) of six babies colonised with *Lactobacillus GG* (Group A) and eight controls (Group B) who received no antibiotics or cefotaxime only in the first 5 days of life

Age (days)	Group A		Group B	
	No.*	Median(range)+	No.*	Median(range)+
0-7	4/5	5.1 (tr.-36.5)	4/8	4.9 (4.1-6.7)
8-14	4/5	4.3 (tr.-4.5)	5/6	tr. (tr-8.7)
15-21	5/6	7.7 (tr-120.5)	5/6	68.0(tr.-145.0)
22-28	2/3	4.5, 9.7	2/2	4.5, 100.0
29-35	2/2	40.1, 59.0	3/3	6.2, 113.0, 245.0
>35	3/3	65.2 (58.3-127.4)	2/3	4.2, 7.0
Total	20/24	6.4 (tr.-127.4)	21/28	6.6 (tr.-245.0)

* Number of all samples that were positive

\+ Median (range) of positive samples only

Table 3-6 Urinary phenol and ρ-cresol (μmol/mmol creatinine) of five babies (four Group A and one Group B) who received antibiotics other than cefotaxime

Age (days)	Phenol		ρ-Cresol	
	No.*	Median(range)+	No.*	Median(range)+
0-7	4/4	9.8 (9.0-54.0)	2/4	tr., tr.
8-14	5/6	5.0 (4.9-11.2)	3/6	tr. (tr.-4.6)
15-21	5/5	5.1 (4.7-11.6)	4/5	4.8 (tr.-6.1)
22-28	4/4	7.9 (tr.-22.8)	4/4	tr. (tr.-4.1)
29-35	2/3	tr., 8.1	1/3	tr.
>35	4/5	5.4 (4.0-6.4)	4/5	25.5 (4.1-82.5)
Total	24/27	5.4 (tr.-54.0)	18/27	tr. (tr.-82.5)

* Number of all samples that were positive

\+ Median (range) of positive samples only

Table 3-7 Urinary phenol and ρ-cresol (μmol/mmol creatinine) of baby No.12, who received antibiotics almost continuously to 38 days of age

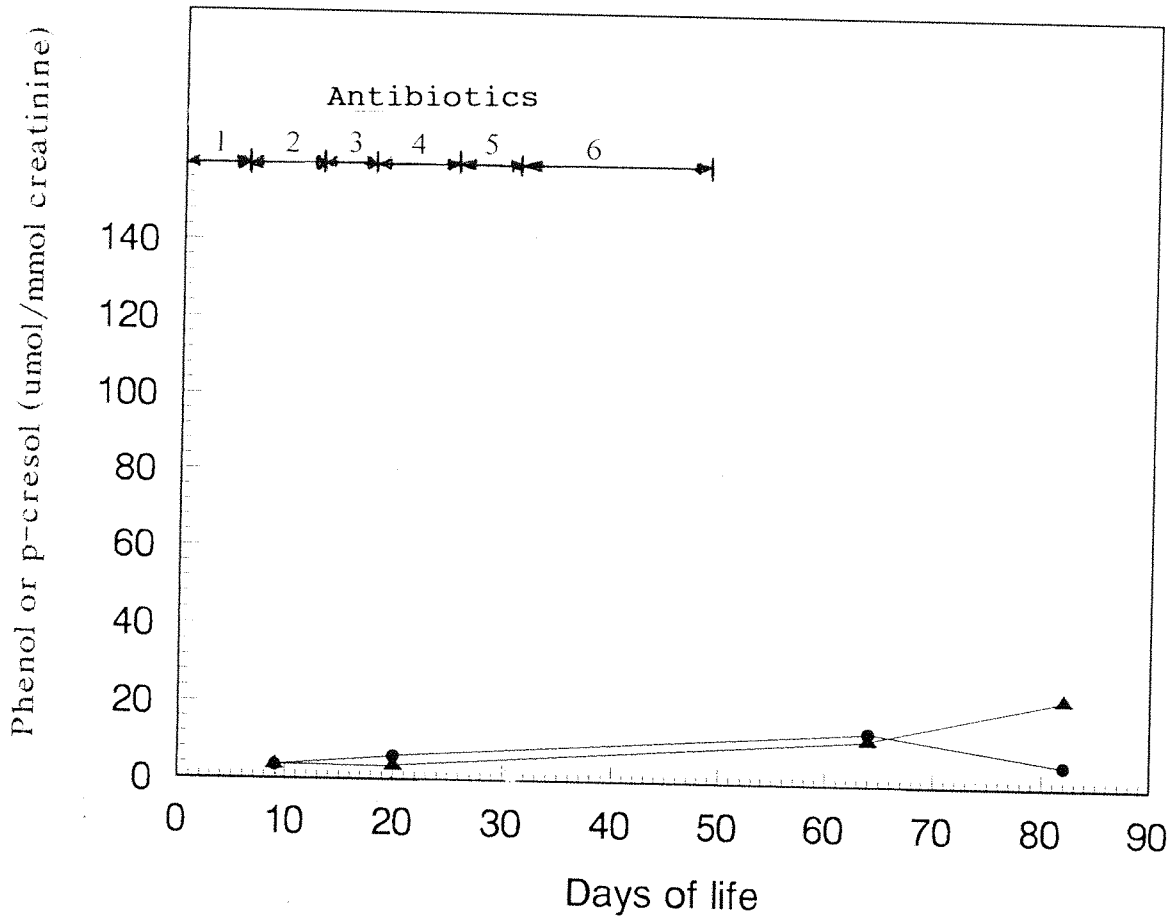
Age (days)	Phenol		ρ-Cresol	
	No.*	Concentation+	No.*	Concentation+
0-7	1/1	tr.	1/1	tr.
8-14	1/1	tr.	1/1	tr.
15-21	1/1	5.9	1/1	tr.
22-28	No samples			
29-56	2/2	tr., tr.	2/2	tr., 4.2
57-84	3/3	5.2, 5.7, 13.4	3/3	9.8, 18.9, 38.9
>84	1/1	5.2	1/1	21.1
Total	9/9	5.2 (tr.-13.4)	9/9	4.2 (tr.-38.9)

* Number of all samples that were positive

\+ Concentration of positive samples only

Figure 3-2 Baby No. 12

Antibiotics and urinary phenol and p-cresol



Antibiotic treatment
1. Cefotaxime
2. Fluconazole/Netilmicin/Fluconazole
3. Fluconazole
4. Fluconazole/Cefotaxime
5. Fluconazole
6. Day 31 Flucloxacillin/Netilmicin
   Day 35 Flucloxacillin/Amikacin
   Day 38 Flucloxacillin
   Day 49 Netilmicin/Flucloxacillin

organisms were isolated from the faeces of each baby, and Appendix 5 when ρ-cresol-producing organisms were cultured. On 50 occasions, urine samples were collected within +/- 48 hours of faecal samples examined by quantitative microbiology. The biochemical and microbiological findings for these paired samples could be related.

Phenol: Table 3-8 lists the major phenol producing gut bacteria and indicates the number of occasions when these were isolated from faeces and phenol was detected simultaneously in urine. Phenol-producing bacteria were isolated on 38 of 47 occasions (81%) when phenol was excreted. They were not cultured on 9 occasions (19%). The organism isolated most often was *Enterococcus*, found on all 38 occasions. In 26 faecal samples, this was the only phenol-producing organism identified, in 10, *E. coli* was also present and in two *E. coli* and *Proteus* sp. were found with *Enterococcus*. Thus it seems that *Enterococci* were likely to have been the major phenol producing bacteria. There were only three occasions when *Enterococci* and *E. coli* were cultured, but phenol was not detected in urine. The distribution of phenol producing organisms was similar for group A and group B babies, as were the urinary concentrations of phenol. Figure 3-3 shows the urinary phenol Excretion of baby N 7 and phenol-producing bacteria isolated from faeces.

ρ-Cresol: Table 3-9 lists the major ρ-cresol producing gut bacteria, and indicates the number of occasions when these were isolated from faeces and ρ-cresol was detected simultaneously in urine. ρ-Cresol-producing bacteria were isolated on 22 of 40 occasions (55%) when ρ-cresol was excreted. They were not cultured on 18 occasions (45%). The organism isolated most often was *Clostridia*, found on 21 occasions. In 19 faecal samples this was the only ρ-cresol-producing organism identified; in two, other ρ-cresol producers, *Bacteroides* and *Bifidobacteria* were found. There was only one occasion when *Clostridia* were cultured, but ρ-cresol was not detected in urine. Thus it seems likely that *Clostridia* may have often accounted for ρ-cresol production. However, on one occasion, *Bacteroides* only was identified as a possible source. The distribution of ρ-cresol producing organisms was similar for group A and group B babies, as were the urinary concentrations of ρ-cresol. Figure 3-4 shows the urinary ρ-cresol excretion of baby N 20 and ρ-cresol producing bacteria isolated from faeces.

3.3.2.5 Urinary 2,3-butanediol excreted by control babies (group B)

Individual results for all samples are presented in Appendix 6. From Table 3-10A it is evident that treatment with the antibiotic cefotaxime during the first five days of life had no demonstrable effect on urinary 2,3-butanediol excretion of babies in control Group B.

Table 3-8 Phenol producing bacteria isolated from faeces related to urinary phenol excretion

Faeces Phenol producing bacteria isolated	Urinary phenol (μmol/mmol creatinine)			
	Group A (n=26 sample pairs)		Group B (n=24 sample pairs)	
	Detected No.* Percentage+ (median range)**	**Not detected** No. Percentage++	**Detected** No.* Percentage+ (median range)**	**Not detected** No. Percentage++
Enterococci	13/13 100% (4.6, tr.-10.2)	0 0	13/13 100% (11.5, tr.-48.7)	0 0
E.coli	0 0	0 0	0 0	0 0
Proteus	0 0	0 0	0 0	0 0
Enterococci+ E.coli	6/7 86% (8.4, tr.-23.5)	1/7 14%	4/6 67% (4.8, tr.-23.5)	2/6 33%
Enterococci+ E.coli+Proteus	0 0	0 0	2/2 100%	0 0
None	6/6 100% (8.5, tr.-22.8)	0 0	3/3 100% (9.1, 5.9, 55.6)	0 0

* Number of urine samples containing phenol/number of faecal samples growing the listed organism(s).

\+ Positive urinary samples as a percentage of faecal samples growing organisms.

** Median (range) of positive urine samples only.

++ Number of urine samples with no detectable phenol as a percentage of faecal samples positive for the listed organism(s).

Table 3-9 ρ-Cresol producing bacteria isolated from faeces related to urinary ρ-cresol excretion

Faeces ρ-cresol producing bacteria isolated	Urinary ρ-cresol (μmol/mmol creatinine)			
	Group A (n=26 sample pairs)		Group B (n=24 sample pairs)	
	Detected No.* Percentage+ (median range)**	**Not detected** No. Percentage++	**Detected** No.* Percentage+ (median range)**	**Not detected** No. Percentage++
Clostridia	13/13 100% (9.3, tr.-145.0)	0 0	6/6 100% (63.8, tr.-176.0)	0 0
Bifidobacteria	0 0	0 0	0 0	0 0
Bacteroides	0 0	1/1 100%	1/2 50% (7.9)	1/2 50%
Clostridia+ *Bacteroides*	0 0	0 0	0 0	1/1 100%
Clostridia+ *Bacteroides*+ *Bifidobacteria*	0 0	0 0	2/2 100% (5.3, 4.1)	0 0
None	8/12 70% (4.8, tr.-44.2)	4/12 30%	10/13 77% (4.4, tr.-8.4)	3/13 23%

* Number of urine samples containing ρ-cresol/number of faecal samples growing the listed organism(s).

\+ Positive urinary samples as a percentage of faecal samples growing organisms.

** Median (range) of positive urine samples only.

++ Number of urine samples with no detectable ρ-cresol as a percentage of faecal samples positive for the listed organism(s).

Table 3-10 (A) Urinary 2,3-butanediol (μmol/mmol creatinine) of eight babies from Group B: three who received no antibiotics; five who received cefotaxime only in the first 5 days of life

Age (days)	No antibiotics		Cefotaxime only	
	No.*	Median(range)+	No.*	Median(range)+
0-7	1/3	23.3	0/2	
8-14	2/2	33.3, 203.0	3/4	4.6, 27.6, 314.0
15-21	2/3	63.5, 431.7	6/7	6.9 (3.4-23.6)
22-28	0/1		2/4	14.1, 53.0
29-35	2/2	89.3, 135.1	1/1	7.0
>35	No samples		3/4	3.6, 17.7, 38.5

* Number of all samples that were positive

\+ Median (range) of positive samples only

Table 3-10 (B) Combined urinary 2,3-butanediol (μmol/mmol creatinine) of three babies from Group B who received no antibiotics; and five who received cefotaxime only in the first 5 days of life

Age (days)	Urinary 2,3-butanediol	
	No.*	Median(range)+
0-7	1/5	23.3
8-14	5/6	33.3 (4.6-314.0)
15-21	8/10	10.5 (3.4-431.7)
22-28	2/5	14.1, 53.0
29-35	3/3	7.0, 89.3, 135.1
>35	3/4	3.6, 17.7,38.5

* Number of all samples that were positive

\+ Median (range) of positive samples only

Figure 3-3 **No. 7 baby (Group A)**

Urinary phenol and phenol-producing bacteria isolated from faeces

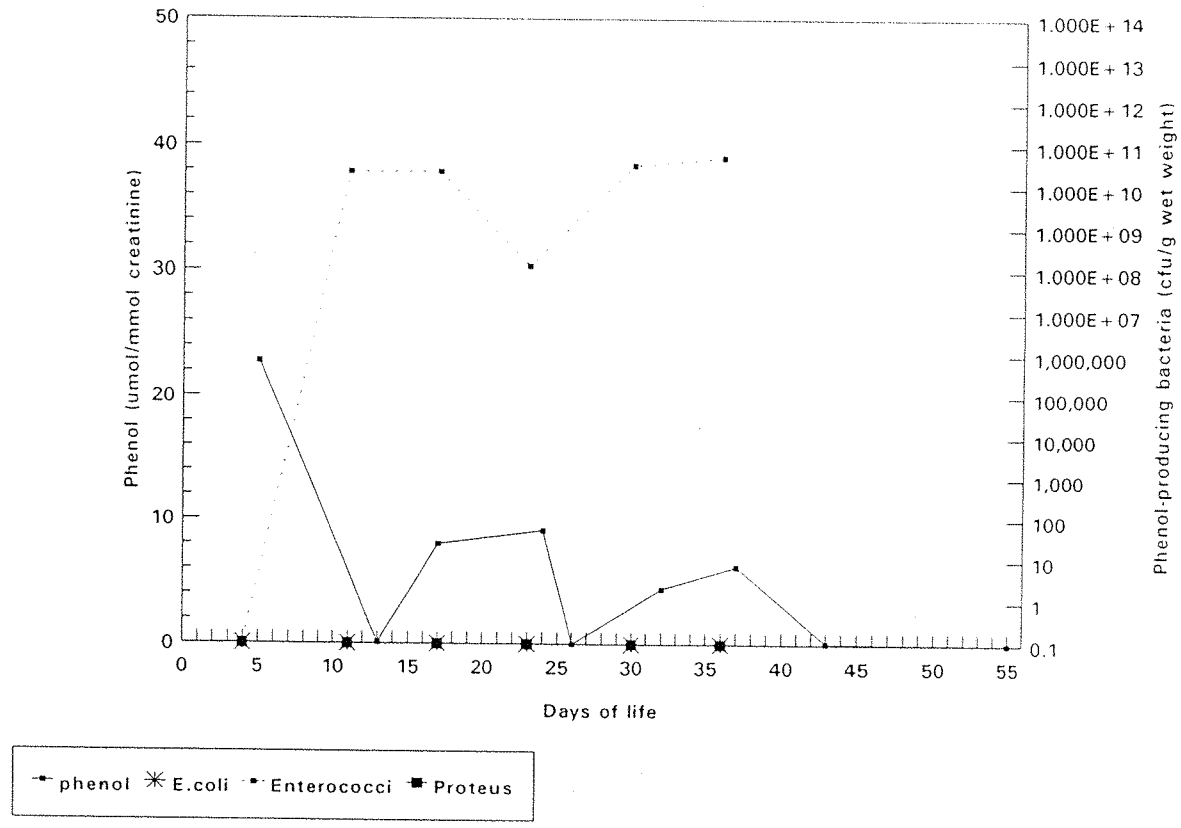
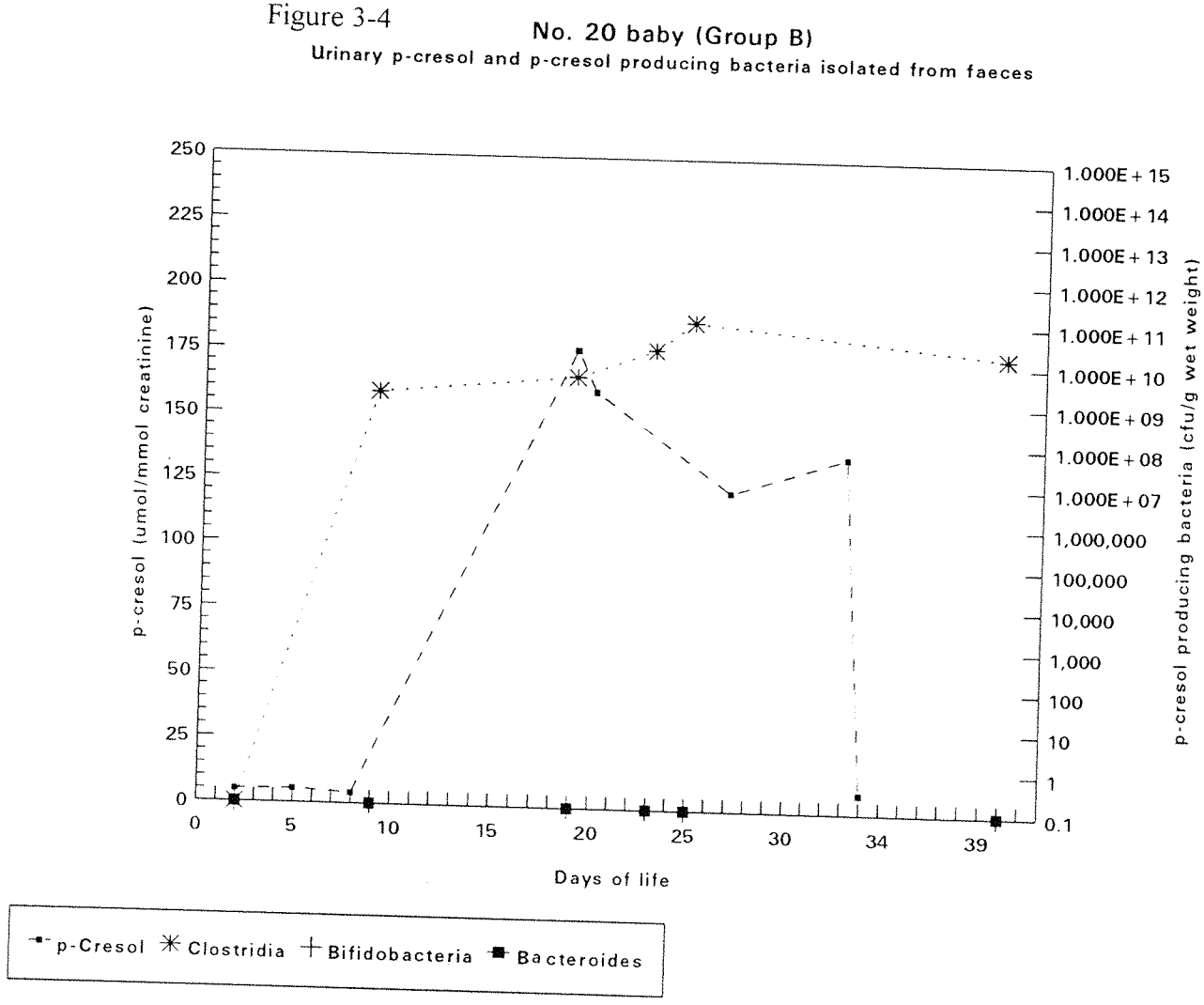


Figure 3-4


No. 20 baby (Group B)
Urinary p-cresol and p-cresol producing bacteria isolated from faeces
p-cresol (umol/mmol creatinine)
250
225
200
175
150
125
100
75
50
25
0
p-cresol producing bacteria (cfu/g wet weight)
1.000E+15
1.000E+14
1.000E+13
1.000E+12
1.000E+11
1.000E+10
1.000E+09
1.000E+08
1.000E+07
1,000,000
100,000
10,000
1,000
100
10
1
0.1
0
5
10
15
20
25
30
34
39
Days of life
p-Cresol
Clostridia
Bifidobacteria
Bacteroides

Data was, therefore, pooled for the three babies (N 13, N 19, N 20) who received no antibiotics and five (N 11, N 14, N 16, N 17, N 18) who had cefotaxime only (Table 3-10B). Results for the two other babies treated with erythromycin (baby N 15; days 16-28) and multiple antibiotics (baby N 12; days 1-28) have been excluded, as these antibiotic regimens were associated with decreased counts of faecal bacteria. During the first week, only one of 5 samples analysed contained 2,3-butanediol(23.3 μmol/mmol creatinine). Thereafter 2,3-butanediol was present in the majority of samples. The amount varied, with four samples having very high concentrations (> 100 μmol/mmol creatinine). Concentrations did not change significantly after the second week.

3.3.2.6 Effect of *Lactobacillus GG* colonisation on urinary 2,3-butanediol

Results were pooled for two babies who received no antibiotics (N 8, N 4) and four treated with cefotaxime only aged 1-5 days (N 6, N 7, N 9, N 10). They were all colonised with *Lactobacillus GG*. Their urinary 2,3-butanediol excretion is presented in Table 3-11. When compared with similar data for Group B babies, it is evident that colonisation was not associated with statistically significant changes in urinary 2,3-butanediol.

3.3.2.7 Urinary 2,3-butanediol of babies receiving antibiotics other than cefotaxime

Table 3-12 presents data for one Group B baby (N 15) who was treated with erythromycin aged 16-28 days, and for 4 Group A babies (N 1, N 2, N 3, N 5) who all had courses of flucloxacillin with netilmicin. One of the four Group A babies did not colonise with *Lactobacillus GG*, one colonised initially, but lost the organism during antibiotic treatment and, in the fourth, *Lactobacillus GG* counts were decreased transiently. In three cases, antibiotic treatment was associated with decreased faecal anaerobes. Urinary 2,3-butanediol excretion of these babies was similar to that of Group A and Group B babies who received no antibiotics, or cefotaxime only (compare Table 3-11). Data is presented separately for a sixth baby (N 12, from Group B) (Table 3-13), who received antibiotics almost continuously until 53 days of age. 2,3-Butanediol was detected in 6 of 9 samples from this baby (Figure 3-5 shows his urinary 2,3-butanediol results and antibiotic treatment.).

3.3.2.8 Bacterial sources of 2,3-butanediol

2,3-Butanediol is produced by *Klebsiella* sp, *Enterobacter* sp. and *Serratia* sp and yeasts. Appendix 7 lists the faecal samples that were positive for these organisms.On 47 occasions, urine samples were collected within +/- 48 hours of faecal samples

Table 3-11 Urinary 2,3-butanediol (μmol/mmol creatinine) of six Group A babies, who were colonised with *Lactobacillus GG* and eight Group B controls, who received no antibiotics or cefotaxime only in the first 5 days of life

Age (days)	Group A		Group B	
	No.*	Median (range)+	No.*	Median (range)+
0-7	0/6		1/5	23.3
8-14	8/8	21.2 (6.9-136.0)	5/6	33.3 (4.6-314.0)
15-21	6/6	69.7 (5.0-251.7)	8/10	10.5 (3.4-431.7)
22-28	6/7	22.9 (6.2-41.6)	2/5	14.1, 53.0
29-35	2/2	4.9, 52.8	3/3	7.0, 89.3, 135.1
>35	2/3	2.8, 24.7	3/4	3.6, 17.7, 38.5
Total	24/32	27.4 (2.8-251.7)	22/33	25.5 (3.4-431.7)

* Number of all samples that were positive

\+ Median (range) of positive samples only

Table 3-12 Urinary 2,3-butanediol (μmol/mmol creatinine) of five babies (four Group A and one Group B), who received antibiotics other than cefotaxime

Age (days)	Urinary 2,3-butanediol	
	No.*	Median(range)+
0-7	4/6	58.3, 156.7
8-14	3/5	2.8, 21.8, 69.7
15-21	1/4	29.6
22-28	2/4	86.3, 431.7
29-35	4/4	25.1 (3.1-71.3)
>35	2/4	11.5, 15.5

* Number of all samples that were positive

\+ Median (range) of positive samples only

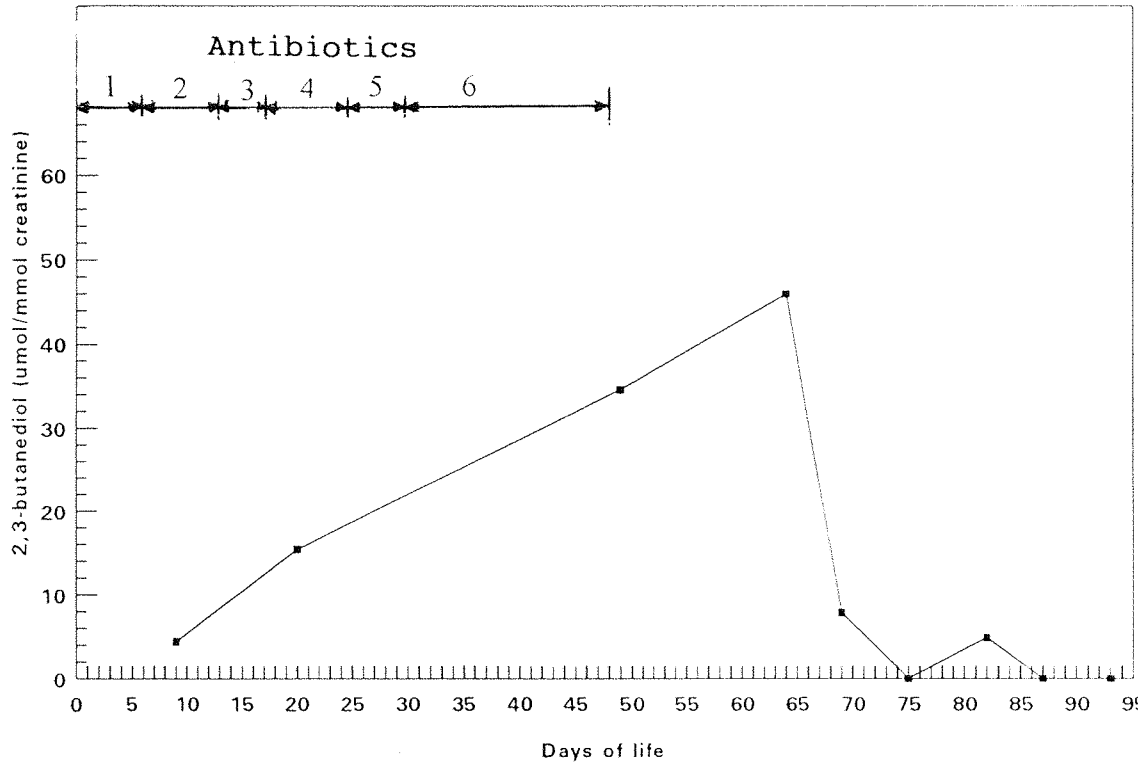
Table 3-13 Urinary 2,3-butanediol (μmol/mmol creatinine) of baby No. 12 (Group B) who received antibiotics almost continuously to 38 days of age

Age (days)	Urinary 2,3-butanediol	
	No.*	Median(range)+
0-7	0	0
8-14	1/1	4.4
15-21	1/1	15.4
22-28	0	0
29-56	1/1	34.6
56-84	3/5	4.9, 7.9, 46.0
>84	0/1	

* Number of all samples that were positive

\+ Median (range) of positive samples only

Figure 3-5 **No. 12 baby (Group B)**
Urinary 2,3-butanediol and antibiotics



Antibiotic treatment
1. Cefotaxime
2. Fluconazole/Netilmicin/Fluconazole
3. Fluconazole
4. Fluconazole/Cefotaxime
5. Fluconazole
6. Day 31 Flucloxacillin/Netilmicin
   Day 35 Flucloxacillin/Amikacin
   Day 38 Flucloxacillin
   Day 49 Netilmicin/Flucloxacillin

examined by quantitative microbiology. The biochemical and microbiological findings for these paired samples could be related. Table 3-14 lists the major 2,3-butanediol producing gut bacteria, and indicates the number of occasions when these were isolated from faeces and 2,3-butanediol was detected in urine. 2,3-Butanediol-producing bacteria were isolated on 17 of 32 occasions (53%) when 2,3-butanediol was excreted. They were not cultured on 15 occasions (47%). The diol-producing organisms isolated most often were *Klebsiella* sp. (11 occasions) and *Enterobacter* sp. (12 occasions), with *Serratia* sp. being isolated on only 3 occasions. In 9 faecal samples, two diol-producing orgnisms were found together: *Enterobacter* sp. with *Klebsiella* sp. (6) *Enterobacter* sp with *Serratia* sp. (2) and *Enterobacter* sp with yeasts (1). On 9 of the 15 occasions (60%) when 2,3-butanediol was not excreted in urine, no diol-producing organism was isolated from faeces. However, in the other 40% of cases, the faeces were positive for these microorganisms. The distribution of diol-producing organisms was similar for group A and group B babies, as were the urinary concentrations of 2,3-butanediol. Figure 3-4 shows the urinary 2,3-butandiol excretion of baby N 4 (Group A) and diol-producing bacteria .

Table 3-14 2,3-Butanediol producing bacteria from the faeces related to urinary 2,3-butanediol excretion

Faeces 2,3-butanediol producing bacteria isolated	Urinary 2,3-butanediol (μmol/mmol creatinine)			
	Group A (24 sample pairs)		Group B (23 sample pairs)	
	Detected No.* Percentage+ (median range)**	**Not detected** No. Percentage++	**Detected** No.* Percentage+ (median range)**	**Not detected** No. Percentage++
Klebsiella	1/2 50% (6.9)	1/2 50%	3/5 60% (3.3, 14.1, 17.7)	2/5 40%
Enterobacter	0 0	0 0	2/2 100% (4.0,71.0)	0 0
Serratia	0 0	0 0	1/1 100% (7.6)	0 0
Yeasts	0 0	0 0	0 0	3/3 100%
Klebsiella+ *Enterobacter*	6/6 100% 52.6 (6.2-251.7)	0 0	0 0	0 0
Serratia+ *Enterobacter*	0 0	0 0	2/2 100 6.1, 53.0	0 0
Yeasts+ *Enterobacter*	0 0	0 0	1/1 100% 364.8	0 0
Yeasts+ *Klebsiella*+ *Enterobacter*	1/1 100% 41.6	0 0	0 0	0 0
None	11/15 73% 22.9(4.7-65.7)	4/15 23%	4/9 44% 9.4(3.4-33.4)	5/9 66%

* Number of urine samples containing 2,3-butandiol/number of faecal samples growing the list organism(s).

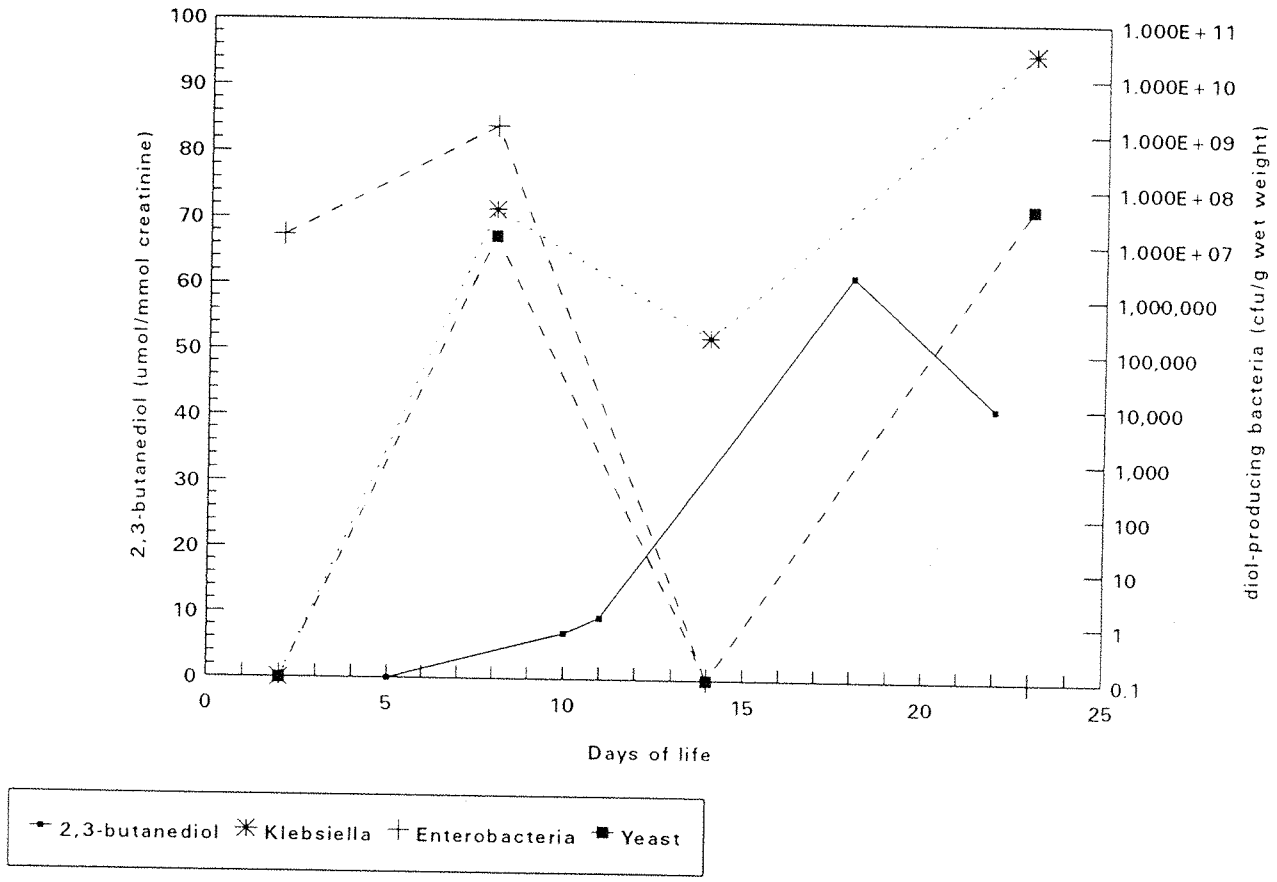
\+ Positive urinary samples as a percentage of faecal samples growing organisms.

** Median (range) of positive urine samples only.

++ Number of urine samples with no detectable pheol as a percentage of faecal samples positive for the listed organism(s).

Figure 3-6 **No. 4 baby (Group A)**

Urinary 2,3-butanediol and diol-producing bacteria isolated from faeces



CHAPTER FOUR

DISCUSSION

The intestinal microflora of preterm infants nursed on a neonatal intensive care unit is different from that of more mature individuals [13]. Because of treatment with broad spectrum antibiotics during their early life, such babies are at increased risk of intestinal bacterial colonisation with pathogens, which cause serious illness. Colonisation with a non-pathogenic organism might protect them from bacterial infections arising from the gut, and such protection might be achieved by feeding them with a probiotic. In this study, *Lactobacillus GG* was investigated for this purpose [84,97,98]. This was the first time it had been given to well premature babies. However, colonisation with such a probiotic could have widespread effects on the metabolic activity of the gut bacteria and this might not always be beneficial to the host. This project investigated the effects on bacterial phenylalanine and tyrosine metabolism, and on 2,3-butanediol produced by carbohydrate fermentation. Other researchers in the trial undertook clinical and microbiological studies and monitored faecal short chain fatty acids.

4.1 ANALYSIS OF URINARY PHENOL AND ρ-CRESOL

For this study, it was necessary to set up and validate a method to analyse phenol and ρ-cresol in urine of premature babies.

4.1.1 Validation of the urinary hydrolysis procedure

Methods of hydrolysing conjugated urinary phenol and ρ-cresol were investigated by Bakke and Scheline (1969) [101]. They compared acid (concentrated hydrochloric acid) and enzymic (β-glucuronidase) hydrolysis. More ρ-cresol was liberated using enzymic hydrolysis than with acid. The yield of phenol was similar with both methods. For enzymic hydrolysis, they incubated 5 ml of urine (pH 5.0) and 2ml of 0.2M acetate buffer (pH 5.0) with β-glucuronidase for 18 hours at 37°C. They used β-glucuronidase type H-1 (which also contained sulphatase) approximately 1,000-2,000 units per ml urine. The yields were considerably greater with a β-glucuronidase/sulphatase mixture than with β-glucuronidase alone. Other groups [76,77,106] have used acid (concentrated

sulphuric or hydrochloric acid), for urinary phenol hydrolysis in studies of patients with gastro-intestinal disorders or normal adults. For this study, we selected the enzymic method for hydrolysis and optimised the procedure for urine from premature babies. Maximal hydrolysis was achieved with an overnight incubation of 18 hours at 37°C. The amount of β-glucuronidase needed for urinary phenol and ρ-cresol hydrolysis was investigated. 0, 10μl, 20ul and 30μl of β-glucuronidase (Sigma, HP-type 2, 10,000 units of β-glucuronidase and 1140 units of sulphatase units per ml) were used to hydrolyse 500μl samples of urine. A plateau was gained with 20 μl of β-glucuronidase and this volume was selected. This corresponds to 4,280 units/ml of urine which was more than that used by Bakke and Scheline. The increase might be explained by differences in the enzyme preparations used.

4.1.2 Discussion of the analytical methods

Urinary phenols have been isolated and identified by paper and thin layer chromatography [107,108]. The quantitative determination of these compounds has been reported using spectrophotometric methods. However, these methods are nonspecific [107].

For this study, we aimed to measure volatile compounds, phenol and ρ-cresol, in babies' urine, which is very dilute compared with adult urine. We needed to process a large number of samples. We required a procedure that was relatively quick and simple, suitable for batch analysis, and which needed minimal sample preparation in order to avoid loss of the compounds during their isolation. HPLC has been used to analyse organic acids in biological samples. Rumsby et al (1987) published a comprehensive investigation of the use of a cation exchange column (HPX-87H) for urinary organic acid profiling [103] The method was modified by Chong et al, who reduced analysis time by using a short fermentation monitoring column [102]. This procedure is in current use to measure selected organic acids in clinical samples in the Department of Chemical Pathology, Southampton General Hospital. During Chong's original evaluation of the method, aqueous standards of phenol and ρ-cresol applied to the HPLC column were well resolved. Murray et al reported an HPLC procedure for determination of simple phenols [106]. They hydrolysed urine with acid and isolated phenols by steam distillation. However, the equipment for steam distillation was complicated. The procedure was laborious and not suitable for batch sample analysis.

The HPLC procedure of Chong et al was investigated first for analysis of total phenol and ρ-cresol in urine hydrolysed by β-glucuronidase. When diluted, hydrolysed, babies' urine was applied directly to the HPLC column, the chromatograms were grossly overloaded and phenol and ρ-cresol were poorly resolved. This showed that a

preliminary extraction procedure was essential. SAX columns (strong anion exchange columns), used by Chong et al to isolate organic acids, were investigated. Unlike the organic acids, more than 90% of phenol and ρ-cresol was found to elute from SAX columns in the preliminary water wash, with little more recovered in the subsequent sulphuric acid eluate. The water and sulphuric acid eluates were, therefore, both retained and combined for analysis. This extraction, and the HPLC analytical procedure, worked well with aqueous standards. The method gave good recoveries for phenol and ρ-cresol, and was sensitive (minimum detection limit 4 μmol/L for phenol and ρ-cresol). Recoveries for the full procedure were good (98.5% for phenol, 97.3% for ρ-cresol) as was the analytical precision:batch coefficient of variation for phenol 4.1% and for ρ-cresol 4.8%. However, when the procedures were applied to diluted hydrolysed urine samples (creatinine 0.1 mmol/L), the chromatograms showed only very small peaks of phenol and ρ-cresol, that could not be quantified accurately. This indicated that a larger urine volume was required for analysis. A five fold greater volume was investigated. The peaks for phenol and ρ-cresol were larger, but this overloaded the chromatograms, to a degree that was not acceptable. Contamination of the HPLC column was a considerable worry. Other extraction procedure, including SCX columns (strong cation exchange columns), Celute MX tubes (cellulose columns, Jones Company), and ethyl acetate and diethyl ether extraction were then investigated. The recoveries were very poor. The method of using HPLC for determination of urinary phenol and ρ-cresol had to be abandoned. Further work is required to find a more suitable urine extraction procedure.

Gas chromatography is used widely for organic acid profiling of biological samples and for the analysis of a few simple phenols in water [107]. Bakke and Scheline analysed simple phenols in standard aqueous solutions and urine, using ether to extract the compounds from acidified samples (pH 1.0) [101]. They used two kinds of GC columns and flame ionisation detection. Column C (1% w/w Carbowax 20M, temperature, 130°C-185°C) was used for quantitative measurements; Column S (15% w/w silicone rubber UC-W98, 120°C) for identification. Duran et al later reported using GC analysis for urinary volatile phenols of normal subjects and patients with gastro-intestinal disorders [76]. Urine was hydrolysed with 4M hydrochloric acid and extracted with ether. The extracts were dried by rotary evaporation and analysed using a gas chromatograph with dual hydrogen flame ionisation detectors and a multi-linear temperature programming unit. The columns used were stainless steel, filled with 5% neopentyl glycol adipate and 2% phosphoric acid on Chromosorb W,AW-DMCS 100-120 mesh [76]. Bone et al also used GC analysis of urinary phenols to study the possible role of gut bacterial products in causation of large bowel cancer [77]. Acid hydrolysed

urine (50ml of a 24hr urine collection) was adjusted to pH10 and extracted with ether. The ether extract was analysed on a 5 foot column packed with 2.5% trixylenylphosphate on diatomite at an operating temperature of 120°C.

Because of the volatility of phenol and ρ-cresol, losses are likely during complex sample preparation procedures, as used, for example, by Duran et al [76]. For this study, a simple ether extraction procedure was applied to acidified urine that had been hydrolysed by β-glucuronidase. For gas chromatography, the column selected was a megabore fused silica column (25m x 0.53mm I.D.) coated with polyethylene glycol, BP-20 (SGE Ltd, Milton, Keynes, UK). This type of column is used by our laboratory to analyse volatile fatty acids in faeces, and does not cause errors from 'ghosting'. In preliminary studies, 1ml aliquots of urine were spiked with standard phenol and ρ-cresol solutions and 30μl of 2-ethylbutyric acid as I.S, acidified to pH 1.0 and extracted twice with ether. The extracts were concentrated under nitrogen and injected onto the gas chromatograph. The GC conditions were those used for measuring volatile fatty acids, with modification of the temperature programme [104]. Standard phenol, ρ-cresol and internal standard were well resolved, and produced sharp peaks. Recoveries ranged from 89.3% to 106.0% for phenol and from 89.8% to 109.0% for ρ-cresol. The lower limit of sensitivity for phenol and ρ-cresol was 4 μmol/L and the method was linear to 1 mmol/L for both phenol and ρ-cresol. Within batch and between batch precision was good, CV% ranged from 0.23% to 9.70% for phenol and 0.25% to 12.0% for ρ-cresol. This procedure was then applied to analysis of urine from preterm babies, hydrolysed with β-glucuronidase. A volume equivalent to 0.1 μmol creatinine, was extracted with ether. The ether extracts were concentrated to 250 μl under nitrogen and 1.5 μl injected onto the gas chromatograph. Phenol and ρ-cresol were well resolved. This GC procedure for urinary phenol and ρ-cresol was simple and quick and suitable for analysis of batches of samples (about 12 per day). It was selected as the definitive procedure for the study.

4.1.3 Urinary phenol and ρ-cresol excretion of healthy adult controls

In order to confirm that the analytical procedure developed for this study was valid, urine was analysed from healthy adults. Others have published reference data which could be used for comparison. Phenol was present in all 27 samples analysed and ρ-cresol in 26 of 27 samples. Urinary phenol and ρ-cresol concentrations were similar for 19 adult men and 8 women. Generally, the concentrations of ρ-cresol were higher than phenol. For phenol: men, median 2.8, range: tr-8.7 μmol/mmol creatinine; women, median 2.8, range 2.4-4.5 μmol/mmol creatinine. For ρ-cresol: men, median 22.4, range 2.5-106.4 μmol/mmol creatinine; women, median 20.4, range tr.-75.9 μmol/mmol creatinine. Duran et al presented results for acid hydrolysed urine of four normal adults

subjects [76]. The concentrations for phenol were higher than in our study. Their mean value for phenol (range) was: 19.2 (3.4-36.2) μmol/mmol creatinine. Their mean value for ρ-cresol was also higher (56.5 μmol/mmol creatinine), but the range (18.1-73.5 umol/mmol creatinine) was similar to our study. These differences may be explained by differences in the methods used for urinary hydrolysis, phenol and ρ-cresol isolation procedures, and in the gas chromatographic analysis, and the small number of subjects in Duran's study. The ratio of ρ-cresol to phenol in our study was: median (range) 8.8 (4.2-25.3) for women and 6.9 (1.0-27.8) for men. These ratios are similar to those reported by Bone et al [77], who analysed 24 hour urine samples from normal adults: mean value for women 8.4, for men 4.0. These findings support the validity of the analyses. They show that phenol and ρ-cresol are almost always present in urine of healthy adults. They confirm other reports that the concentration of ρ-cresol is higher than phenol, indicating dominance of anaerobes over facultative anaerobes in the established gut microflora.

4.2 URINARY PHENOL, ρ-CRESOL AND 2,3-BUTANEDIOL OF PREMATURE BABIES AND THE EFFECTS OF FEEDING *LACTOBACILLUS GG*

4.2.1 Urinary phenol and ρ-cresol

Urinary phenol and ρ-cresol are gut bacterial degradation products of unabsorbed tyrosine and phenylalanine [76]. Phenol is produced by facultative anaerobes, ρ-cresol by strict anaerobes. They are absorbed via the portal blood. In the liver, most are conjugated with sulphuric and glucuronic acids, and then excreted in urine. Although conjugated phenols are probably not toxic, further study is needed to investigate the possible effects of large amounts of these compounds passing through the body constantly. Duran et al found that urinary excretion of phenol and ρ-cresol increased in patients with gastro-intestinal disorders [76].

4.2.1.1 Urinary phenol and ρ-cresol excretion of Group B (control) babies

In the first week, phenol was present in all samples (n=8) collected from 8 Group B babies, who received no antibiotic or cefotaxime only in the first 5 days of life. The median concentration of phenol 22.9 (range 11.1-61.4)μmol/mmol creatinine, was higher than for healthy adults in this study: median 2.8 (range tr.-8.7) μmol/mmol creatinine and similar to that reported by Duran et al for adults (mean 6.8 μmol/mmol creatinine , range 2.3-11.3); of 3.4 to 37.1 μmol/mmol creatinine for 5 babies under 1 year of age, and of

2.3 to 31.6 for older children [76]. There are no published reference values for urinary phenol and ρ-cresol excretion of premature and term newborn babies. In our study, ρ-cresol was present in only 50% of samples during the first week, and the concentrations (median 4.9, range 4.1-6.7 μmol/mmol creatinine) were lower than phenol. The ratio of ρ-cresol/phenol was, therefore, low ranging from 0 to 4.5, median 0.2. Thereafter, both phenol and ρ-cresol were present in the majority of samples. Generally, the concentrations of ρ-cresol were higher than phenol, with phenol ranging from trace to 64.0 μmol/mmol creatinine after the first 7 days, and ρ-cresol ranging from trace to 245.0 μmol/mmol creatinine. High concentrations of ρ-cresol were found in 8 samples (>100 μmol/mmol creatinine). The highest concentrations of ρ-cresol were observed during the third week of life with a median of 68.0 (range tr.-145.0) μmol/mmolcreatinine, and ρ-cresol/phenol ratio of 11.5 (range 0-26.4). These concentrations were similar to those of normal adults (page 67) and to values reported for five babies under 1yr of age (mean 56.5 μmol/mmol creatinine range 18.1-73.5) and older children (mean 68.2 μmol/mmol creatinine, range 15.8-109.6) [76]. The high ratios of ρ-cresol/phenol observed in the third week were greater than values reported for normal adults (mean 8.4 for women, 4.0 for men) [77].

The pattern of excretion observed in the premature babies, ie. high concentrations of phenol in the first week, followed by increasing excretion of ρ-cresol, reflects the process of gut colonisation in newborns. Aerobic and facultative bacteria are the first organisms to colonise the bowel and are predominant in the first week of life. Strict anaerobes start to colonise the intestine towards the end of the first week, and soon outnumber the aerobes. Part of the explanation for the relatively high concentrations of phenol and ρ-cresol in the preterm babies compared with older children and adults is probably that they were related to urinary creatinine. Creatinine is an end-product of muscle creatine. Because of their small muscle bulk, premature babies excrete much smaller amounts of creatinine than older individuals, and these low values increase the ratios of urinary metabolites to creatinine. Use of creatinine ratios, however, corrects for differences in the concentration of urine because of the state of hydration, and is preferable to expressing results as per litre.

4.2.1.2 Effect of *Lactobacillus GG* colonisation on urinary phenol and ρ-cresol excretion

Nine of the 10 babies from Group A, who received the probiotic *Lactobacillus GG* were colonised by the organism. Colonisation had little impact on urinary excretion of phenol and ρ-cresol: there were no significant differences between the frequencies or concentrations of phenol and ρ-cresol excreted by Group A babies colonised with

Lactobacillus GG and Group B control babies who received no antibiotics or cefotaxime only. These observations complement the microbiological findings that *Lactobacillus GG* did not affect colonisation with phenol and ρ-cresol producing bacteria (page 66).

4.2.1.3 Bacterial sources of urinary phenol and ρ-cresol

Phenol is produced by facultative anaerobic bacteria: *E.coli*, *Enterococcus* sp, *Proteus* sp and *Staphylococcus* sp. ρ-Cresol is produced by strict anaerobic bacteria: *Clostridium* sp, *Bifidobacterium* sp, *Bacteroides* sp, *Fusobacterium* sp and *Staphylococcus* sp [77].

In our study, when phenol was excreted in urine, *E.coli*, *Enterococcus* sp or *Proteus* sp were isolated from the faeces in 81% of cases. *Enterococcus* sp was isolated most often (100% of cases). These bacteria were also found by Bone et al when phenol was detected in adults' urine [77]. Thus these bacteria are likely to have been the sources of phenol in this study, with *Enterococcus* sp, the major phenol producer.

Clostridium sp, *Bacteroides sp* and *Bifidobacterium* sp were isolated from faeces in 55% of cases when ρ-cresol was excreted in urine. *Clostridia* were isolated most often (53% of cases). *Bacteroides* sp and *Bifidobacterium* sp were found in only two samples. Thus, in our study, *Clostridia* were probably the major source of ρ-cresol.

From these results, it is notable that only one phenol producing bacterial species, *Enterococcus*, and one ρ-cresol producing bacterial species, *Clostridium*, were isolated in most cases. This contrasts with the wide range of phenol and ρ-cresol producing bacteria in the adult bowel [76]. This difference might be explained by prematurity, milk feeding, treatment with broad spectrum antibiotics, and being nursed in an intensive care unit, where babies are significantly more likely to grow a single organism, than healthy babies at home or on a postnatal ward.

4.2.1.4 Effect of antibiotics on urinary phenol and ρ-cresol excretion

Generally, antibiotic administration substantially reduces the numbers of colonic bacteria [53]. Neomycin and erythromycin were reported to reduce the colonisation of the colon by both aerobic and anaerobic bacteria

In control babies from Group B, cefotaxime treatment during the first 5 days of life had no significant effects on the species or numbers of faecal bacteria isolated during the study. Urinary excretion of phenol and ρ-cresol was similar to that of babies who received no antibiotics - evidence that cefotaxime did not have a substantial effect on gut colonisation by phenol and ρ-cresol producing bacteria. Treatment with antibiotics other than cefotaxime (erythromycin, flucloxacillin, netilmicin), had no significant effects on urinary phenol excretion, or on phenol producing bacteria cultured from faeces. In

contrast, urinary ρ-cresol was significantly reduced compared with 8 Group B babies who received no antibiotic, or cefotaxime only ($P<0.01$). ρ-Cresol producing bacteria were isolated from only 6 of 29 faecal samples from the five antibiotic-treated babies, indicating that treatment suppressed growth of these anaerobes. The concentrations of phenol and ρ-cresol of baby No.12, who received antibiotics throughout, were strikingly low: only traces of phenol and ρ-cresol were detected upto 56 days of age. Up to 42 days, enterococci, coagulase negative staphylococci and enterobacteriaceae were the only organisms grown from faecal samples. Strict anaerobes were not isolated. In this baby prolonged treatment with broad spectrum antibiotics decreased both counts of facultative and anaerobic colonic bacteria, and reduced production of phenol and ρ-cresol.

In summary, the findings for the study of phenol and ρ-cresol excretion:

(1) support published observations that the first organisms to colonise the gut of newborns are facultative anaerobes, some of which are phenol producers, and that numbers of anaerobes, including ρ-cresol producing organisms, increase from the end of the first week.

(2) indicate that *Enterococcus* was probably the main phenol producing species in these babies and *Clostridium* sp, the main ρ-cresol producers.

(3) show that treatment with the antibiotic cefotaxime during the first five days of life did not affect bacterial colonisation after the first week, or excretion of phenol and ρ-cresol.

(4) show that other antibiotic regimens, which included erythromycin, flucloxacillin and netilmicin, were associated with significantly decreased excretion of ρ-cresol.

(5) show that colonisation with the probiotic *Lactobacillus GG* had no significant impact on colonisation with facultative anaerobes or anaerobes, or on urinary phenol and ρ-cresol excretion.

4.2.2 Urinary 2,3-butanediol excretion

2,3-Butanediol and its precursor acetoin are products of glucose fermentation. Endogenous production of these compounds probably only occurs rarely and under abnormal metabolic conditions: production of acetoin has been demonstrated in animal tissues in vitro in the absence of oxygen [73]; increased urinary excretion was reported in the inherited metabolic disorders, methylmalonic aciduria and propionic acidaemia. However, these compounds are also fermentation products of yeasts and certain species

of enterobacteriaceae which colonise the gut - *Klebsiella* sp, *Enterobacter* sp and *Serratia* sp. They are produced when the pH falls below 6.0 [73]. They are absorbed from the gut lumen by the host. Acetoin is readily reduced to 2,3-butanediol in the liver, and is excreted in urine. 2,3-Butanediol is not a normal constituent of urine. However, in a recent study of 20 babies (17 premature), who were nursed on the neonatal unit of Southampton General Hospital, 2,3-butanediol was detected in 45 of 272 urine samples analysed. Acetoin was also present in 20 of these samples. Butanediol producing organisms were isolated from the faeces on 10 occasions. In that study, urinary excretion of 2,3-butanediol was explained by: heavy gut colonisation with diol-producing bacteria, deficiency of intestinal lactase with impaired lactose absorption, and increased intestinal permeability in premature infants, so that more 2,3-butanediol was absorbed into the baby [73].

4.2.2.1 Urinary 2,3-butanediol excretion of Group B (control) babies

In the first week, 2,3-butanediol was detected in only one of 5 samples from 8 babies of Group B, who received no antibiotic or cefotaxime only in the first 5 days of life. Thereafter 2,3-butanediol was present in the majority of samples (21 of 28 samples). The amount varied (range: 3.4- 431.7 μmol/mmol creatinine) and very high concentrations (>100 μmol/mmol creatinine) were found in 4 of samples. Acetoin was detected in few samples and then only in trace amounts. These findings are similar to those of Mills and Walker [73], who investigated urinary 2,3-butanediol and acetoin excretion of term and preterm babies nursed on a neonatal unit. They reported that increased 2,3-butanediol excretion did not occur immediately after birth, but, in most babies, was delayed until the second week or later.

4.2.2.2 Effect of *Lactobacillus GG* colonisation on urinary 2,3-butanediol

Nine of the 10 babies from group A, who received the probiotic *Lactobacillus GG*, were colonised by the organism. Colonisation had no effect on urinary excretion of 2,3-butanediol: there were no significant differences between the frequencies or concentrations of 2,3-butanediol excreted by Group A babies colonised with *Lactobacillus GG* and Group B control babies who received no antibiotics or cefotaxime only. These observations complement the microbiological findings that *Lactobacillus GG* did not affect colonisation with 2,3-butanediol producing bacteria (page 66).

4.2.2.3 Bacterial sources for urinary 2,3-butanediol

In our study, when 2,3-butanediol was excreted in urine, diol-producing bacteria: *Klebsiella* sp, *Enterobacter* sp and *Serratia* sp, were identified in stool cultures on 53%

of occasions. *Klebsiella* sp (11 occasions) and *Enterobacter* sp (12 occasions) were isolated most often, followed by *Serratia* sp (3 times) and yeasts (twice). On 60% of occasions when 2,3-butanediol was not found in urine, no diol-producing organisms were grown from faeces. Conversely, on 40% of occasions when the urine was negative for 2,3-butanediol but the faeces grew diol-producing organisms. This might have been because the colonic pH exceeded 6.0, and 2,3-butanediol production was suppressed. It was surprising that on as many as 47% occasions when 2,3-butanediol was excreted in urine, no known diol-producing gut organism was isolated from the faeces. It is highly unlikely that the babies produced the compound endogenously - they were well, thriving and none had an inherited metabolic defect. The source must have been one of the small range of diol-producing organisms. Perhaps these failed to grow in stool culture, or the duration of gut colonisation with the responsible enterobacteriaceae was brief, so that the organisms had not appeared, or had been lost, at the time that the stool samples were passed.

4.2.2.4 Effect of antibiotic treatment on 2,3-butanediol production

In eight control babies from Group B, urinary concentrations of 2,3-butanediol of five babies who had no antibiotics were similar to those of three babies, who received cefotaxime only in the first 5 days of life. This shows that cefotaxime had no effect on urinary excretion of 2,3-butanediol. Concentrations of 2,3-butanediol of the six babies (two from Group B, four from Group A), who received antibiotics other than cefotaxime were not significantly different from these control babies. Thus, unlike the case for p-cresol, antibiotic treatment had no obvious effect on urinary excretion of 2,3-butanediol. 2,3-Butanediol-producing enterobacteriaceae were isolated during antibiotic administration.

In summary, the findings for the study of 2,3-butanediol excretion:

(1) show that this was common after the first week of life among premature babies nursed on the neonatal unit.

(2) that it was not influenced by colonisation with *Lactobacillus GG*.

(3) that on 53% of 32 occasions when 2,3-butanediol was present in urine, there was gut colonisation with known 2,3-butanediol organisms, enterobacteriaceae or yeasts. Of these, *Klebsiella sp* and *Enterobacter sp* were found most often. On 47% of occasions when a diol-producing organism was not isolated, it is possible that colonisation with these organisms was transient and did not coincide with the time that urine was collected.

(4) on 60% of occasions when 2,3-butanediol was not excreted in urine,

diol-producing microorganisms were also absent from the faeces. In 40% of cases, however, an organism was grown. It is speculated the pH of the gut lumen may have exceeded 6.0.

Thus, urinary excretion of 2,3-butanediol may indicate that the baby has an abnormal gut colonisation with enterobacteriaceae or yeasts. However, it cannot be inferred that if 2,3-butanediol is NOT found in urine, there is no colonisation with these organisms - they may be present, but not producing 2,3-butanediol, perhaps because the pH is too high.

The common occurrence of 2,3-butanediol excretion in this study can be explained by: firstly, the high frequency of abnormal colonisation with enterobacteriaceae observed. This is a known hazard of nursing on neonatal units. These organisms occasionally cause diseases and have been implicated in necrotising enterolitis. Secondly, the immaturity of the babies. Lactase production by premature babies and their capacity to digest lactose is reduced [39]. Undigested lactose entering the colon would provide substrate for bacterial fermentation. In addition, they would absorb 2,3-butanediol readily from the gut lumen, because their intestinal mucosa is more permeable than mature babies [73].

CHAPTER FIVE

CONCLUSIONS

In this study, HPLC and GC procedures were investigated for analysis of total urinary phenol and ρ-cresol from premature babies. An anion exchange extraction procedure and HPLC analysis worked well with aqueous standards. However, the method was not satisfactory for urinary analysis and was abandoned. Further work is needed to develop a suitable extraction procedure for urine. A gas chromatographic method was set up and investigated, using diethyl ether to extract urinary phenol and ρ-cresol. These compounds were well resolved. Recoveries from urine were good. The method was sensitive to 4 μmol/L. The procedure was simple and suitable for batch analysis. A β-glucuronidase/sulphatase enzyme preparation was used successfully for hydrolysis of conjugated urinary phenol and ρ-cresol.

As a part of a randomised double blind study, the effects of feeding premature babies with a probiotic, *Lactobacillus GG*, on urinary excretion of gut bacterial metabolites (phenol, ρ-cresol and 2,3-butanediol) was investigated. *Lactobacillus GG* colonised the gut of 9 of 10 premature babies. Colonisation had little effect on the species or numbers of other faecal bacteria, and did not have significant impact on urinary phenol, ρ-cresol and 2,3-butanediol. Generally, ρ-cresol was not detected in the first week of life. Thereafter, phenol and ρ-cresol were present in the majority samples and urinary excretion of ρ-cresol was higher than phenol. This pattern reflects the known sequence of bacterial colonisation of the intestine after birth. The major phenol producing organism was identified as *Enterococcus*. *Clostridium* sp were associated with ρ-cresol production.

2,3-Butanediol was detected in urine only once in the first week of life. Thereafter, it was present in around two thirds of urine samples. The association between urinary 2,3-butanediol excretion and faecal growth of diol-producing bacteria was investigated. On 53% of occasions when the diol was excreted, the faeces grew a diol-producing organism (most often *Klebsiella* sp and *Enterobacter* sp); on 60% of occasions when it was absent from urine diol-producing organisms were also absent from faeces. Detection of 2,3-butanediol in urine may imply that there is abnormal gut colonisation with diol-producing micro-organisms. This is important because they are

potential pathogens in premature babies. Absence of detectable 2,3-butanediol in urine does not definitely exclude abnormal colonisation,

Antibiotic regimens, including erythromycin, flucloxacillin and netilmicin, were associated with decreased faecal anaerobes and significantly decreased excretion of p-cresol. There was no effect on urinary phenol and 2,3-butanediol excretion.

Appendix 1

Urinary phenol and ρ-cresol of normal adults

Adult control patient	Sex (M/F)	Age (year)	Urinary Phenol (μmol/mmol creatinine)	Urinary ρ-cresol (μmol/mmol creatinine)
P1	M	49	1.9	21.7
P2	M	34	3.4	3.4
P3	F	44	2.9	15.1
P4	M	69	5.8	33.4
P5	M	56	2.9	4.0
P6	F	37	2.5	22.0
P7	M	27	2.3	24.5
P8	F	32	2.6	44.0
P9	F	42	2.4	7.8
P10	F	48	2.7	56.7
P11	M	70	trace	10.3
P12	M	40	2.0	13.8
P13	M	70	8.7	106.4
P14	F	68	3.5	trace
P15	M	67	1.2	33.4
P16	M	50	1.7	34.0
P17	F	46	3.0	75.9
P18	M	42	2.9	18.8
P19	M	23	2.8	12.1
P20	M	48	2.8	11.0
P21	F	20	4.5	18.8
P22	M	50	1.6	37.4
P23	M	20	2.5	34.8
P24	M	70	12.7	23.1
P25	M	44	2.8	34.8
P26	M	47	2.7	ND
P27	M	32	1.7	2.5

ND = not detected

Appendix 2

Lactobacillus GG isolated from faeces of Group A babies who received *Lactobacillus GG*

Babies	Days of life	*Lactobacillus GG* (cfu/g wet weight)
N.1	3	-
	8	-
	16	-
	24	-
	27	-
	36	-
N.2	4	-
	9	$3.8x10^{10}$
	15	$9.3x10^{7}$
	23	-
	30	-
	38	-
N.3	1	-
	7	$1.2x10^{9}$
	15	$5.5x10^{9}$
	23	$1x10^{5}$
	28	$4.3x10^{8}$
	37	$1x10^{10}$
N.4	2	$7.5x10^{8}$
	8	$6.5x10^{10}$
	14	$2.2x10^{10}$
	23	$5.7x10^{9}$
N.5	2	-
	9	-
	16	$1.7x10^{9}$
	24	-
	32	-
	34	-

-: not isolated

Appendix 2 (continued)

Lactobacillus GG isolated from faeces of Group A babies who received *Lactobacillus GG*

Babies	Days of life	*Lactobacillus GG* (cfu/g wet weight)
N.6	2	-
	9	$4.2x10^{10}$
	15	$5.1x10^{9}$
	23	$5.7x10^{8}$
	27	$8.7x10^{8}$
	33	$2.8x10^{8}$
N.7	4	-
	11	$7.6x10^{10}$
	17	$1.2x10^{10}$
	23	$1.1x10^{7}$
	30	$4.8x10^{8}$
	36	$6.4x10^{7}$
N.8	3	-
	10	$1x10^{11}$
	20	$7.5x10^{9}$
	26	$3.5x10^{9}$
	29	$2.9x10^{9}$
	42	$5.9x10^{9}$
N.9	2	-
	9	$2x10^{10}$
	16	$2.3x10^{10}$
	22	$3.3x10^{9}$
	29	$6.1x10^{8}$
N.10	4	-
	10	$1x10^{7}$
	17	$1.2x10^{7}$
	22	$1.3x10^{10}$

-: not isolated

Appendix 3

Urinary phenol and ρ-cresol of Group A babies, who received *Lactobacillus GG*

Number of babies	Days of life	Phenol (μmol/mmol creatinine)	ρ-Cresol (μmol/mmolcreatinine)
N.1	2	9.3	4.0
	11	5.1	ND
	18	7.7	4.4
	22	23.5	<4
	25	8.3	ND
	29	8.4	ND
	40	6.6	55.7
	50	5.1	99.9
N.2	3	10.2	ND
	16	4.9	7.4
	41	5.8	5.8
N.3	11	ND	ND
	13	<4	<4
	19	5.3	ND
	32	ND	ND
	48	4.0	ND
	59	ND	4.0
N.4			
	5	13.5	6.2
	10	ND	ND
	18	ND	ND
N.5			
	7	10.0	ND
	9	5.2	5.5
	13	<4	<4
	19	11.9	<4
	34	<4	<4

ND: not detected

Appendix 3(continued)

Urinary phenol and ρ-cresol of Group A babies, who received *Lactobacillus GG*

Number of babies	Days of life	Phenol (μmol/mmol creatinine)	ρ-Cresol (μmol/mmol creatinine)
N.6	5	9.0	4.4
	6	5.0	<4
	9	4.2	4.8
	12	5.0	5.2
	18	4.2	5.9
	26	<4	5.5
N.7	5	22.8	44.2
	13	<4	4.7
	17	8.0	9.3
	24	9.1	11.7
	26	ND	ND
	32	4.4	40.1
	37	6.2	78.9
	43	<4	71.3
	55	ND	154.2
N.8	8	4.6	5.4
	20	<4	145.0
	21	<4	117.0
	30	9.0	71.4
N.9	2	32.0	ND
	22	<4	4.1
N.10	No samples	No samples	No samples

ND: not detected

Appendix 3 (continued)

Urinary phenol and ρ-cresol of Group B control babies who did not receive *Lactobacillus GG*

Babies	Days of life	Phenol (μmol/mmol creatinine)	ρ-Cresol (μmol/mmol creatinine)
N.11	19	5.9	160.0
	30	5.1	296.0
N.12	9	<4	<4
	10	6.1	<4
	31	<4	<4
	49	<4	4.1
	64	13.8	11.8
	76	5.3	46.2
	82	5.8	22.9
	87	5.3	25.5
N.13	1	17.8	6.3
	5	11.4	ND
	11	5.2	5.5
	18	23.5	<4
N.14	1	63.2	ND
	2	48.7	ND
	3	29.3	ND
	16	17.7	4.8
	20	34.5	4.1
N.15	2	55.6	4.7
	8	11.5	ND
	15	<4	4.4
	26	<4	4.1

ND: not detected

Appendix 3 (continued)

Urinary phenol and ρ-cresol produced by Group B control babies who did not receive *Lactobacillus GG*

Babies	Days of life	Phenol (μmol/mmol creatinine)	ρ-Cresol (μmol/mmol creatinine)
N.16	23	4.3	5.3
	37	4.8	8.4
	40	8.3	5.1
	44	ND	ND
N.17	10	4.1	4.1
	13	ND	ND
	18	ND	ND
	22	ND	ND
	28	<4	<4
N.18	1	10.2	6.6
	12	<4	10.5
	14	4.6	7.9
N.19	13	4.8	4.1
N.20	2	46.8	4.9
	5	16.1	5.3
	8	<4	4.0
	19	<4	176.0
	20	5.1	160.0
	27	9.1	122.0
	33.4	5.8	136.0
	33.7	66.0	7.5

ND: not detected

Appendix 4

Phenol-producing faecal bacteria isolated from Group A babies who received *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)		
		E.coli	*Enterococci*	*Proteus*
N.1	3	-	-	-
	8	-	-	-
	16	-	-	-
	24	$3.7x10^{10}$	$1.5x10^{11}$	-
	27	$3.1x10^{9}$	$1.2x10^{11}$	-
	36	$1.1x10^{10}$	$4x10^{11}$	-
N.2	4	-	$1.2x10^{10}$	-
	9	-	$1.5x10^{11}$	-
	15	-	$4.8x10^{8}$	$9.3x10^{8}$
	23	-	$6.5x10^{5}$	$2.1x10^{10}$
	30	-	$1.7x10^{9}$	$8.3x10^{4}$
	38	-	$3.6x10^{10}$	$2.4x10^{10}$
N.3	1	-	-	-
	7	-	$5.7x10^{10}$	-
	15	-	$1.4x10^{10}$	-
	23	-	$4.2x10^{7}$	-
	28	-	$1.9x10^{10}$	$1x10^{10}$
	37	-	$5.2x10^{10}$	$1x10^{10}$
N.4	2	$3.2x10^{10}$	$6.2x10^{8}$	-
	8	$1.5x10^{10}$	$3x10^{10}$	-
	14	$1.8x10^{9}$	$2x10^{10}$	-
	23	-	$6.8x10^{9}$	-
N.5	2	$1.8x10^{10}$	$1.2x10^{10}$	-
	9	$8.3x10^{10}$	$1.5x10^{10}$	-
	16	$4.1x10^{8}$	$7.6x10^{9}$	-
	24	$2.8x10^{10}$	$2.9x10^{10}$	-
	32	$3x10^{11}$	$7.5x10^{10}$	-
	34	$1x10^{11}$	$1.6x10^{10}$	-

-: not isolated

Appendix 4 (continued)

Phenol-producing faecal bacteria isolated from Group A babies who received *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)		
		E.coli	*Enterococci*	*Proteus*
N.6	2	-	-	-
	9	-	$1x10^{10}$	-
	15	-	$3.6x10^{10}$	-
	23	-	$8.3x10^{10}$	-
	27	-	$7.9x10^{10}$	-
	33	-	$1.8x10^{10}$	-
N.7	4	-	-	-
	11	-	-	-
	17	-	$2.3x10^{10}$	-
	23	-	$1.3x10^{8}$	-
	30	-	$3.4x10^{10}$	-
	36	-	$5.3x10^{10}$	-
N.8	3	-	-	-
	10	-	$8.6x10^{10}$	-
	20	-	$2.1x10^{10}$	-
	29	-	$1.5x10^{10}$	-
	26	$7.5x10^{10}$	$5.7x10^{10}$	-
	42	$8.2x10^{10}$	$1.2x10^{11}$	-
N.9	2	-	-	-
	9	-	-	-
	16	-	$2.5x10^{11}$	-
	22	-	$1x1010^{11}$	-
	29	-	$5.1x10^{10}$	-
N.10	4	-	-	-
	10	-	$1x10^{7}$	-
	17	-	$1.2x10^{10}$	-
	22	-	$1.3x10^{10}$	-

-: not isolated

Appendix 4 (continued)

Phenol-producing faecal bacteria isolated from Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)		
		E.coli	*Enterococci*	*Proteus*
N.11	9	-	-	-
	14	-	-	-
	21	-	-	-
	27	$1.1x10^{11}$	-	-
N.12	4	-	$7.3x10^{9}$	-
	10	-	$7.9x10^{9}$	-
	17	-	$4.6x10^{9}$	-
	23	-	$5.6x10^{10}$	-
	27	-	$2.8x10^{10}$	-
	41	-	$2.5x10^{10}$	$3.7x10^{9}$
N.13	2	-	$8.7x10^{9}$	-
	9	-	$5.1x10^{10}$	-
	16	$1.5x10^{10}$	$1.7x10^{11}$	-
	23	$2x10^{11}$	$1.4x10^{11}$	-
	29	$7x10^{10}$	$8x10^{10}$	-
N.14	2	-	$1.9x10^{8}$	-
	8	-	$7.2x10^{10}$	-
	15	-	$9.3x10^{10}$	-
	20	-	$6.1x1010^{10}$	-
	27	-	$2x10^{11}$	-
	31	-	$5.7x10^{10}$	-

-: not isolated

Appendix 4 (continued)

Phenol-producing faecal bacteria isolated from Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)		
		E.coli	*Enterococci*	*Proteus*
N.15	3	-	-	-
	10	-	$1.6x10^{10}$	-
	18	-	$4.5x10^{10}$	-
	23	-	$3.8x10^{10}$	-
	29	-	$5.7x10^{10}$	-
N.16	5	-	-	-
	10	-	-	-
	19	-	$4.5x10^{8}$	-
	24	-	$2.3x10^{9}$	-
	32	-	$3.8x10^{9}$	-
	37	$4.1x10^{10}$	$1.1x10^{9}$	-
N.17	4	-	-	-
	9	-	$5.2x10^{10}$	-
	17	$5.9x10^{10}$	$1.5x10^{10}$	-
	22	$6.5x10^{10}$	$1.7x10^{9}$	-
	28	$1.6x10^{10}$	$1.4x10^{10}$	-
N.18	4	-	$8.5x10^{8}$	-
	8	-	$3.5x10^{10}$	-
	15	-	$7.6x10^{10}$	-
	18	$6.7x10^{8}$	$3.7x10^{10}$	-
N.19	1	-	-	-
	8	$8.9x10^{9}$	-	-
	15	$2.2x10^{10}$	$2.3x10^{9}$	-
	20	$5.8x10^{10}$	$1x10^{10}$	-
	32		$8.7x10^{9}$	-
	36	$2.2x10^{9}$	$8.9x10^{9}$	-
N.20	2	-	-	-
	9	-	$3.6x10^{10}$	-
	19	$1.7x10^{11}$	$1.1x10^{11}$	$4x10^{9}$
	23	$1.2x10^{11}$	$2x10^{11}$	$1.1x10^{10}$
	25	$2x10^{11}$	$2x10^{11}$	$2x10^{9}$
	40	$1.7x10^{10}$	$8.7x10^{10}$	$6.7x10^{9}$

-: not isolated

Appendix 5

ρ-Cresol producing bacteria isolated from Group A babies, who received *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)		
		Clostridia	*Bifidobacteria*	*Bacteroides*
N.1	3	-	-	-
	8	-	-	-
	16	-	-	-
	24	$1.5x10^{8}$	-	-
	27	$5.5x10^{8}$	-	-
	36	$1.6x10^{10}$	-	-
N.2	4	-	-	-
	9	$2.1x10^{8}$	-	-
	15	-	-	-
	23	-	-	-
	30	-	-	-
	38	-	-	-
N.3	1	-	-	-
	7	-	-	-
	15	-	-	-
	23	-	-	-
	28	-	-	-
	37	-	-	-
N.4	2	$1x10^{6}$	-	$1.9x10^{10}$
	8	-	-	$2.5x10^{8}$
	14	-	-	-
	23	$4.3x10^{10}$	-	$1.1x10^{11}$
N.5	2	-	-	-
	9	-	-	-
	16	-	-	-
	24	-	-	-
	32	$4.5x10^{9}$	-	-
	34	$1.6x10^{8}$	-	-

-: not isolated

Appendix 5 (continued)

ρ-Cresol producing bacteria isolated from Group A babies, who received *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)		
		Clostridia	*Bifidobacteria*	*Bacteroides*
N.6	2	-	-	-
	9	-	-	-
	15	-	-	-
	23	$7.x10^{8}$	-	-
	27	$8.7x10^{8}$	-	-
	33	$4x10^{6}$	-	-
N.7	4	-	-	-
	11	$1.3x10^{5}$	-	-
	17	$1x10^{9}$	-	-
	23	$6.7x10^{7}$	-	-
	30	$6.4x10^{8}$	-	-
	36	$6.4x10^{8}$	-	-
N.8	3	-	-	-
	10	$8.6x10^{9}$	-	-
	20	$2.8x10^{8}$	-	-
	29	$9x10^{8}$	-	-
	26	$1.1x10^{10}$	-	-
	42	$8.8x10^{9}$	-	$5.9x10^{9}$
N.9	2	-	-	-
	9	-	-	-
	16	$3.3x10^{10}$	-	-
	22	$2.5x10^{9}$	-	-
	29	$3x10^{9}$	-	-
N.10	4	-	-	-
	10	-	-	-
	17	$9.4x10^{5}$	-	-
	22	$2.2x10^{6}$	-	-

-: not isolated

Appendix 5 (continued)

ρ-Cresol producing faecal bacteria isolated from Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)		
		Clostridia	*Bifidobacteria*	*Bacteroides*
N.11	9	-	-	-
	14	$6.2x10^{8}$	-	-
	21	$1.1x10^{10}$	-	-
	27	$4.8x10^{9}$	-	-
N.12	4	-	-	-
	10	-	-	-
	17	-	-	-
	23	-	-	-
	27	-	-	-
	41	-	-	-
N.13	2	-	-	-
	9	$6.3x10^{6}$	-	-
	16	$8.3x10^{8}$	-	-
	23	$3.6x10^{10}$	-	-
	29	$1.8x10^{9}$	-	-
N.14	4	-	-	-
	11	-	$1x10^{10}$	-
	17	-	$3.6x10^{10}$	-
	23	-	$8.3x10^{10}$	-
	30	-	$7.9x10^{10}$	-
	36	-	$1.8x10^{10}$	-
N.15	3	-	-	-
	10	-	-	-
	18	-	-	-
	23	-	-	-
	29	-	-	-

-: not isolated

Appendix 5 (continued)

ρ-Cresol producing faecal bacteria isolated from Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	Faecal bacteria cfu/g wet weight)		
		Clostridia	*Bifidobacteria*	*Bacteroides*
N.16	5	-	-	$1.9x10^{7}$
	10	-	$8.9x10^{8}$	$5.5x10^{8}$
	19	$5.7x10^{5}$	$1.8x10^{10}$	$1.7x10^{10}$
	24	$6.1x10^{4}$	$1.8x10^{10}$	$9.2x10^{7}$
	32	$3.1x10^{4}$	$6.1x10^{10}$	$3.8x10^{7}$
	37	$1.4x10^{9}$	$2.7x10^{10}$	$2.4x10^{10}$
N.17	4	-	-	-
	9	-	-	-
	17	$1.2x10^{5}$	-	$6.6x10^{10}$
	22	-	-	$4x10^{10}$
	28	-	-	$7.5x10^{10}$
N.18	4	-	-	-
	8	$2.8x10^{4}$	-	-
	15	$6.9x10^{8}$	-	-
	18	$6.7x10^{8}$	-	-
N.19	1	-	-	-
	8	$4.4x10^{7}$	-	-
	15	-	-	-
	20	$2.5x10^{10}$	-	-
	32	$4.3x10^{3}$	-	-
	36	$3x10^{6}$	-	-
N.20	2	-	-	-
	9	$1.4x10^{9}$	-	-
	19	$4x10^{9}$	-	-
	23	$2x10^{10}$	-	-
	25	$1x10^{11}$	-	-
	40	$1.7x10^{10}$	-	-

-: not isolated

Appendix 6

Urinary 2,3-butanediol of Group A babies who received *Lactobacillus GG*

Babies	Days of life	2,3-Butanediol (μmol/mmol creatinine)
N.1	8	31.9
	20	251.7
	21	189.3
	35	52.8
N.2	16	33.9
	41	247.4
N.3	9	4.4
	20	15.4
	49	34.6
	64	46.0
	69	7.9
	73	ND
	75	ND
N.4	2	ND
	5	ND
	10	6.9
	11	9.3
	18	61.6
	22	41.6
N.5	7	156.7
	9	2.8
	13	69.7
	34	30.3

ND: not detected

Appendix 6 (continued)

Urinary 2,3-butanediol of Group A babies who received *Lactobacillus GG*

Babies	Days of life	2,3-Butanediol (μmol/mmol creatinine)
N.6	5	ND
	6	ND
	9	65.7
	2	136.8
	18	77.7
	26.2	22..7
	26.6	20.3
N.7	12	10.4
	13	7.7
	16	5.0
	24	ND
	26	ND
	32	4.9
	37	24.0
	43	ND
	55	2.8
N.8	8	31.9
	20	251.7
	21	189.3
	35	52.8
N.9	2	ND
	11	3.9
	22.6	15.9
	22.8	6.2
N.10	4	ND
	8	ND
	11	7.1

ND: not detected

Appendix 6 (continued)

Urinary 2,3-butanediol of Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	2,3-butanediol (μmol/mmol creatinine)
N.11	16	23.6
	19	4.0
N.12	9	4.4
	20	15.4
	49	34.6
	64	46.0
	69	7.9
	73	ND
	75	ND
	82	4.9
	87	ND
	93	ND
N.13	2	ND
	18	ND
N.14	2	ND
	3	ND
	16	7.6
	20	6.1
	25	53.0
N.15	2	ND
	3	ND
	15	ND
	26	ND

ND: not detected

Appendix 6 (continued)

Urinary 2,3-butanediol of Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	2,3-butanediol (μmol/mmol creatinine)
N.16	19	13.3
	23	14.1
	29	7.0
	37	17.7
	40	38.5
	44	3.6
	54	ND
N.17	10	364.8
	13	27.6
	18	ND
	22	ND
	28	ND
N.18	12	4.6
	14	ND
	15	3.4
N.19	13	33.3
N.20	2	ND
	5	23.3
	8	266.3
	19	431.7
	20	63.5
	26	ND
	35.4	89.3
	35.7	135.1

ND: not detected

Appendix 7

2,3-Butanediol producing faecal bacteria isolated from Group A babies who received *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)			
		Klebsiella	*Enterobacter*	*Serratia*	Yeasts
N.1	3	-	-	-	-
	8	-	-	-	$2x10^9$
	16	-	$9.8x10^{10}$	-	$3.9x10^5$
	24	-	$4.6x10^9$	-	-
	27	-	-	-	-
	36	-	-	-	-
N.2	4	-	-	-	-
	9	-	-	-	-
	15	-	-	-	-
	23	-	-	-	-
	30	-	$5.7x10^{10}$	-	-
	38	-	-	-	-
N.3	1	-	-	-	-
	7	-	-	-	$4.9x10^5$
	15	-	$3.9x10^9$	-	-
	23	$3.9x10^9$	-	-	-
	28	-	-	-	-
	37	-	-	-	-
N.4	2	-	$1.2x10^7$	-	-
	8	$3.7x10^7$	$1.2x10^9$	-	$1.2x10^7$
	14	$1.8x10^5$	-	-	-
	23	$2.8x10^{10}$	-	-	$4.5x10^7$
N.5	2	-	$1.3x10^{11}$	-	-
	9	$1.2x10^{11}$	-	-	-
	16	$2.6x10^{10}$	-	-	-
	24	-	$2.9x10^9$	-	-
	32	-	-	-	-
	34	-	-	-	-

-: not isolated

Appenix 7 (continued)

2,3-Butanediol producing faecal bacteria isolated from Group A babies who received *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)			
		Klebsiella	*Enterobacter*	*Serratia*	Yeasts
N.6	2	-	-	-	-
	9	-	-	-	-
	15	-	-	-	-
	23	-	-	-	-
	27	-	-	-	-
	33	$9.4x10^7$	-	-	-
N.7	4	-	-	-	-
	11	-	-	-	-
	17	-	-	-	-
	23	-	-	-	-
	30	$2.2x10^{10}$	-	-	-
	36	-	-	-	-
N.8	3	-	-	-	-
	10	-	$1.3x10^{11}$	-	-
	20	-	$3.7x10^9$	-	-
	26	-	-	-	-
	29	-	-	-	-
	42	-	-	-	-
N.9	2	$1.2x10^{10}$	-	-	-
	9	$3x10^9$	-	-	-
	16	$1.4x10^{10}$	-	-	-
	22	$4x10^{10}$	-	-	-
	29	$8.5x10^9$	-	-	-
N.10	4	-	-	-	-
	10	-	$1.6x10^6$	-	-
	17	$9.4x10^{10}$	-	-	-
	22	$6.9x10^{10}$	-	-	-

-: not isolated

Appendix 7 (continued)

2,3-Butanediol producing faecal bacteria isolated from Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)			
		Klebsiella	*Enterobacter*	*Serratia*	Yeasts
N.11	9	-	-	-	-
	14	-	-	-	-
	21	-	$7.5x10^9$ -	-	-
	27	-		-	-
			-		
N.12	4	-	-	-	$3.7x10^9$
	10	-	-	-	-
	17	$3.2x10^{10}$	-	$7x10^9$	-
	23	-	-	-	-
	27	-	$2.2x10^{10}$	-	-
	21	-		-	-
			-		
N.13	2	-	-	-	-
	9	-	$1.4x10^{10}$	-	-
	16	$3.9x10^9$	$1.5x10^{10}$	-	-
	23	-	-	-	-
	29	$2.3x10^8$		-	-
			-		
N.14	2	-	-	-	-
	8	-	-	$1.2x10^{11}$	-
	15	-	$1.5x10^{10}$	$3.6x10^{10}$	-
	20	-	$1.7x10^{10}$	$1.2x10^{11}$	-
	27	-	-	$1.7x10^{10}$	-
	31	-		-	-
			-		
N.15	3	-	$2.7x10^9$	-	$1.8x10^{10}$
	10	-	$2.2x10^{10}$	-	-
	18	-	$6.5x10^9$	-	-
	23	$7.1x1^9$	$1.8x10^9$	-	-
	29	$3.8x10^{10}$		-	-

-: not isolated

Appendix 7 (continued)

2,3-Butanediol producing faecal bacteria isolated from Group B babies who did not receive *Lactobacillus GG*

Babies	Days of life	Faecal bacteria (cfu/g wet weight)			
		Klebsiella	*Enterobacter*	*Serratia*	Yeasts
N.16	5	-	-	-	-
	10	-	-	-	-
	19	$6.5x10^{10}$	-	-	-
	24	$1.2x10^{10}$	-	-	-
	32	$6.1x10^{10}$	-	-	-
	37	$7x10^{8}$	-	-	-
N.17	4	-	-	-	-
	9	-	$1.8x10^{10}$	-	$1.1x10^{7}$
	17	-	-	-	-
	22	$6.1x10^{9}$	-	-	-
	28	$5.6x10^{9}$	-	-	-
N.18	4	-	-	-	-
	8	-	-	-	-
	15	-	-	-	-
	18	-	-	-	-
N.19	1	-	-	-	-
	8	-	-	-	-
	15	-	-	-	-
	20	-	-	-	-
	32	-	-	-	-
	36	-	-	-	-
N.20	2	-	-	-	-
	9	-	-	-	-
	19	-	$3.9x10^{9}$	-	-
	23	$3.9x10^{9}$	-	-	-
	25	-	-	-	-
	40	-	-	-	-

-: not isolated

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