Role of Gut Bacteria in Human Toxicology and Pharmacology

M.J. HILL

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Edited by
M.J.Hill
ECP (UK) Headquarters
Wexham Park Hospital
Slough, UK

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Introduction

All living bodies have on their surface a layer of dead and decaying exfoliated cells which provide an invaluable breeding ground for bacteria. The human body is, of course, no exception to this and, in consequence, has a rich bacterial flora on all surfaces. The local conditions differ and so the flora at a given site is characteristic of that site. For example, the nose is rich in salt and is a highly aerobic environment and so only salt-resistant, strictly aerobic organisms (such as staphylococci and micrococci) flourish there. The main nutrient sources are the dead exfoliating mucosal cells and nasal secretions such as nasal mucin. The skin is rich in long-chain fatty acids, which are bactericidal to most bacteria, and only resistant organisms such as the diphtheroids and coryneform bacilli flourish there. The major nutrient sources on the skin, in addition to the dead surface cells, are derived from sweat. Soaps, consisting of long-chain fatty acids, reinforce the natural host defence mechanism. The normal bacterial flora at various sites in the body was reviewed recently by Hill and Marsh (1990).

The most richly colonized site in the human body is the digestive tract and, particularly, the large intestine. The lumen of the digestive tract is external to the body and so is the site for exfoliation of dead gut mucosal cells; since intestinal turnover is rapid this alone is a rich source of nutrients. In addition there is a very large volume of intestinal secretions needed for digestion, and these provide a fluid environment also for the luminal bacterial flora. Finally, there is the human diet itself which is largely digested in, and absorbed from, the upper small intestine but which contains residues that are not digestible by the host enzymes in the small bowel but may be readily digested and utilized by the bacterial flora at any site in the gut.

The opportunities for bacteria in the gut are thus immense, but the host has a range of mechanisms for cleansing the upper intestine, thereby ensuring that, in the healthy person, digestion can take place to the maximum benefit of the host and unimpeded by the flora. These are discussed in more detail in Chapter 1. In the lower gut the bacteria survive (and flourish) on a diet consisting of exfoliated cells, secreted proteins and enzymes and the undigested or indigestible components of the food. A proportion of the food will be digestible but undigested because the digestive system is not 100% efficient. Part of the food will be in the form of indigestible components of plants (e.g. dietary fibre; some plant glucosides etc.), and part will be in the form of food additives which are not absorbed from the intestine. Despite the efficiency of the digestive system this apparently 'spartan' environment is sufficiently rich to support a profuse flora of approximately 10^{14} bacteria. Further, because of the type of scavenging conditions under which they live, this is perforce a highly metabolically-active flora capable of producing a very wide range of inducible enzymes and so capable of taking full nutritional advantage of anything that comes its way.

Consequently, the gut bacteria are in a highly favourable position to mediate between a person and their surrounding environment by interacting not only with the undigested food components but also with the full range of xenobiotics that reach the gut in the form of food additives, drugs, environmental contaminants and so on, either directly or after biliary secretion in the form of conjugates. Their importance in toxicology, although first noted and publicized many decades ago (e.g. Scheline, 1968; Williams, 1972; Drasar and Hill, 1974), is still only slowly being realized and fully recognized.

In this book the first Section deals with background data on the normal gut bacterial flora and its metabolic activity, and the use of gnotobiotic animals in toxicology. The second Section is concerned with various aspects of nitrogen metabolism. Section 3 covers carbohydrate metabolism and the toxicology of the end-products of fermentation. Section 4 deals with the metabolism of fats and fat substitutes; the chapter on fats includes data on bile acid metabolism since the bile acids are so crucial to efficient fat digestion. Section 5 is about the metabolism of sulphur compounds and about sulphatereducing bacteria. Section 6 is concerned with a miscellaneous group of compounds, and includes discussion of the metabolism of metal compounds and the production of vitamins by the gut flora. Section 7 covers the crucial area of the biliary excretion and enterohepatic circulation of xenobiotics. The final Section reviews the general area of probiotics; this is clearly an area that will grow rapidly over the next few years because probiotics offer a constructive route to combating the role of gut bacteria in toxicology, rather than the essentially destructive route of antibiotic use.

The book does not attempt to provide the last word on the subject of the role of gut bacteriology in toxicology; that would be impossible because the subject is as wide and as dynamically evolving as toxicology itself. However, an attempt has been made to offer an indication of the breadth and depth of the subject, and the current state of the art in the growth points of the field.

References

Drasar, B.S. and Hill, M.J., 1974, *Human Intestinal Flora,* London: Academic Press.

Hill, M.J. and Marsh, P.D., 1990, *Human Microbial Ecology,* Boca Raton: CRC Press.

Scheline, R.R., 1968, Drug metabolism by intestinal micro-organisms, *J. Pharm. Sci.,* **57,** 2021–37.

Williams, R.T., 1972, Toxicological implications of biotransformation by intestinal microflora, *Toxicol. Appl. Pharmacol.,* **23,** 769–81.

Contributors

J.K.Chipman
Department of Biochemistry
University of Birmingham
Edgbaston
Birmingham B15 2TT
United Kingdom

M.E.Coates
School of Biological Sciences
University of Surrey
Guildford
Surrey GU2 5XU
United Kingdom

R.Coleman
Department of Biochemistry
University of Birmingham
Edgbaston
Birmingham B15 2TT
United Kingdom

A.Csordas
Institut für Medizinische Chemie und Biochemie
Universität Innsbruck
Fritz-Pregl-Strasse 3
A-6020 Innsbruck
Austria

D.C.Ellwood
Industrial Research Laboratories
University of Durham
Durham DH1 3LE
United Kingdom

T.W.Federle
Corporate Professional and Regulatory Services Division
The Procter and Gamble Company
Ivorydale Technical Center
5299 Spring Grove Avenue
Cincinnati, Ohio 45217–1087, USA

M.J.Hill
ECP (UK) Headquarters
Lady Sobell Gastrointestinal Unit
Wexham Park Hospital
Slough, Berkshire SL2 4HL
United Kingdom

A.A.Jackson
Institute of Human Nutrition
University of Southampton
Biomedical Sciences Building
Bassett Crescent East
Southampton SO16 1BJ
United Kingdom

S.A.Leach
Pathology Division
PHLS-CAMR
Porton Down
Salisbury
Wiltshire SP4 0JG
United Kingdom

A.Lidbeck
Department of Microbiology
Huddinge University Hospital
F60
Novum
S-141 86 Huddinge
Sweden

J.C.Mathers
Department of Biological and Nutritional Sciences
University of Newcastle-upon-Tyne
Newcastle-upon-Tyne NE1 7RU
United Kingdom

B.J.Moran
Institute of Human Nutrition
University of Southampton
Biomedical Sciences Building

Bassett Crescent East
Southampton SO16 1BJ
United Kingdom

K.Orrhage
Department of Microbiology
Huddinge University Hospital
F60
Novum
S-141 86 Huddinge
Sweden

P.J.Packer
PHLS-CAMR
Pathology Division
Porton Down
Salisbury
Wiltshire SP4 0JG
United Kingdom

J.Rafter
Department of Medical Nutrition
Huddinge University Hospital
F60
Novum
S-141 86 Huddinge
Sweden

A.G.Renwick
Clinical Pharmacology Group
University of Southampton
Biomedical Sciences Building
Bassett Crescent East
Southampton SO9 3TU
United Kingdom

I.R.Rowland
BIBRA
Toxicology International
Woodmansterne Road
Carshalton
Surrey SM5 4DS
United Kingdom

T.G.Schlagheck
Regulatory and Clinical Development Division
The Procter and Gamble Company
Ivorydale Technical Center

5299 Spring Grove Avenue
Cincinnati, Ohio 45217–1087, USA

J.H.P.Watson
Department of Cryogenics
University of Southampton
University Road
Highfield
Southampton SO9 5NH
United Kingdom

Section 1

Gut bacteriology

Chapter 1
The normal gut bacterial flora

M.J.Hill

1.1
Introduction

It is now recognized that all external body surfaces have a normal resident bacterial flora, and this includes the digestive tract. Because of cell turnover, gut surfaces are coated with dead and desquamating cells, and these provide an excellent basal nutrient source, to which can be added nutrients passing through the lumen of the gut. During the last 25 years there has been a massive increase in our knowledge about the gut flora at different sites in the digestive tract (Drasar and Hill, 1974; Hill and Marsh, 1990). This has occurred as a result of improved anaerobic culture techniques, the recognition of the limitations on the data and attempts to moderate those limitations.

In this chapter I will first briefly review the factors that limit the quality of the data, then describe the flora at various sites in the digestive tract, and finally discuss the factors controlling the gut flora.

1.2
Limitations on the data

The problems with obtaining accurate data on the bacterial flora of specific sites in the digestive tract can be divided into two groups, namely sampling problems (including those associated with obtaining, transporting and storing of samples) and cultivation problems (including those associated with the quantitative isolation, enumeration and identification of the components of the flora).

1.2.1
Sampling problems

Good samples of saliva and of faeces can be obtained in a form suitable for quantitative bacteriology, with results expressed as number of organisms per gram; faeces can be assumed to be representative of the rectum and rectosigmoid region. Other sites are less readily sampled. Within the mouth there are distinct floras (in addition to saliva), on the tongue, tooth surface, gingival crevices, roof of mouth and cheek surface. In order to sample these quantitatively it is necessary to swab standard surface areas and then express the results as number of organisms per cm^2. This is

rarely practicable so that, in practice, nonstandard areas are swabbed and the results expressed as relative counts or proportions of the total count—a semi-quantitative technique.

Between the throat and rectum it is necessary to use other methods of sampling because of the inaccessibility of these sites, and no sampling method is satisfactory. Samples of luminal contents can be obtained at surgery from specific sites in the gut. However, surgery almost always involves pre-operative preparation to empty the bowel. Further, under anaesthesia there may be profound changes in bowel motility. This, of course, will compromise the validity of the specimen. Samples may also be obtained by intubation (upper gut) or endoscopically. Both intubation and upper gastro-intestinal tract endoscopy can be criticized for contamination of the sample with saliva and contamination of the tip of the tube during its descent to the sampling site. Various methods have been devised to overcome tip contamination. Samples of colonic contents can be obtained without bowel preparation using a rigid proctoscope (which permits sampling of the rectum and part of the rectosigmoid) or flexible sigmoidoscope (which samples up to the rectosigmoid; sampling from higher in the colon using colonoscopy requires bowel cleansing and so invalidates any luminal samples obtained). Mucosal biopsy samples can be obtained at endoscopy for study of the mucosa-associated flora. Analyses of these are usually expressed as counts per gram of biopsy; however, only the mucosal surface is colonized and estimates of the mucosal surface area in a biopsy are largely notional. In the future it is likely that results will be expressed using the amount of mucin as a measure of mucosal surface area.

Ideally the samples should be analyzed bacteriologically as soon as they are taken but this is rarely possible. The bacteriology laboratory is usually distant from the endoscopy unit, for example, and so the sample needs to be protected during transit. For transport within the hospital it is usually sufficient to transfer the sample to an anaerobic tube and to keep it cool (but not frozen). In most laboratories, samples are analyzed in batches and this entails samples being stored. Crowther (1971) studied storage of faeces and showed that the best results were obtained by freezing at −40°C in a cryoprotective medium containing 10% glycerol. This cryoprotective medium is also ideal for storage of salivary samples; gastric juice can also be stored in this way if it is first neutralized. Duodenal juice is rich in bactericidal bile components which are active even at −40°C and in the presence of glycerol; no good storage medium has been devised for such samples and this may explain why so many studies show duodenal contents to be sterile.

1.2.2
Cultivation problems

Despite the impressive progress made in cultivation methodology during the last 30 years there is still considerable scope for further improvement. *Helicobacter pylori*, now the most intensively studied gut organism and thought to be responsible for an array of peptic diseases, was not isolated until 1984 by Marshall and Warren, and it is unlikely that this is the last surprise that gut bacteriology has for us.

The problems can be divided into those related to the cultivation medium and those related to the cultivation environment. New media are constantly being devised for cultivation of organisms of medical importance but little has been done with respect to the cultivation of organisms with specific biochemical activities. The vast majority of bacteria in most digestive tract sites are strictly anaerobic and are killed by oxygen; in order to isolate quantitatively such organisms it is necessary to plate out samples in an anaerobic environment, using plates that had previously been

Table 1.1 Comparative effectiveness of methods of anaerobic culture.

Method	*Effectiveness*
1. Roll tube method of Hungate	Gold standard for culture of even very–oxygen–sensitive (VOS) anaerobes.
2. Anaerobe cabinet	Unable to cultivate the most OS organisms, but can be very effective.
3. Bench plating out, then immediate transfer to anaerobe jars for incubation	Sufficient for isolation of anaerobes from clinical infections, but not for the VOS normal flora.
4. Bench plating; delayed transfer to anaerobe jars	Will only cultivate the most robust anaerobes.

deoxygenated and then to incubate the plates in a suitable anaerobic environment of hydrogen, nitrogen and carbon dioxide (Table 1.1).

The problems of sampling and cultivation were discussed in detail by Borriello *et al.* (1978) and, more recently, by Borriello (1986).

1.3
Bacterial flora of the human digestive tract

1.3.1
Flora of the mouth

This has been reviewed in detail by Theilade (1990) and by Marsh and Martin (1984). It is the subject of books, because of the range of surfaces and hence of floras to be found within the oral cavity in health and disease. For the purposes of this book, the most important flora in the mouth is that of saliva. The reason for this is that contact time between oral surfaces and ingested compounds is relatively short and not of significance in toxicology and pharmacology. The exception to this is the salivary flora, because a number of ingested xenobiotics are secreted in saliva and so are in contact with the salivary flora between meals as well as during meals.

The salivary flora is, of course, derived from that on the oral surfaces. Table 1.2 lists the organisms commonly isolated from the resident oral microflora (Theilade, 1990) and gives the relative counts of the major bacterial genera. Note that the flora is dominated by Gram positive rods *(Lactobacilli andActinomyces)* and cocci (*streptococci* and *micrococci*).

The salivary flora is of importance in, for example, the pharmacology of nitrate (discussed later in Chapter 5) and of other xenobiotics secreted in saliva.

1.3.2
Flora of the stomach

All bacteria that are able to live as commensals in the human body are killed by incubation at pH values below 3. Drasar *et al.* (1969) analyzed a large number of gastric juice samples for bacterial flora and pH (and demonstrated that at acidic pH the samples were sterile whilst above pH 4–5, bacteria are able to survive and some can proliferate (Table 1.3). The normal resting gastric juice pH is below 3 and so the normal resting gastric juice is bacteria-free. Vanzant *et al.* (1932) reviewed

Table 1.2 The resident flora of the mouth.

Gram positive	*Gram negative*
Rods	
Actinomyces	*Bacteroides*
Arachnia	*Wallinella*
Bifidobacteria	*Selenomonas*
Lactobacilli	*Fusobacteria*
Bacterionema	*Leptotrichia*
Eubacteria	*Capnocytophaga*
Propionibacteria	*Haemophilus*
Cocci	
Streptococci	*Neisseria*
Micrococci	*Veillonella*
Enterococci	*Branhamella*

Table 1.3 The relation between pH and gastric juice bacteria.

pH	*Number of bacteria per ml*	*Composition of flora*
Less than 3	$<10^4$	No bacterial multiplication
3–5	10^5 to 10^8	Acid-tolerant organisms (micrococci, some streptococci and lactobacilli)
5–7	10^6–10^8	Mixed populations of acid-tolerant and acid-resistant organisms

Table 1.4 The relation between age and gastric hypochlorhydria (Vanzant *et al.*, 1932).

Age group	*Percentage with hypochlorhydria*	
	Males	*Females*
20–29	3.3	4.8
30–39	3.5	9.5
40–49	9.9	12.6
50–59	17.6	18.2
60–69	23.2	27.6

their data on pH of resting gastric juice in relation to age (Table 1.4) and noted that, even 60 years ago in the United States, the prevalence of anacidity was low in persons aged less than 50 years old; thus, in normal healthy young persons the resting gastric lumen is free of bacteria.

However, even in young normochlorhydric persons, the lumen is not bacteria-free for the whole day. During a meal the gastric acid is buffered, allowing swallowed salivary bacteria to survive or even to proliferate (Figure 1.1) as demonstrated by Milton-Thompson *et al.* (1981) and by Meyrick-Thomas *et al.* (1987). However, when the pH returns to less than 3 these swallowed organisms are killed. In consequence, a resident bacterial flora in the stomach can only occur when gastric acid secretion is impaired to the point that the pH does not fall below 3–4 even in the resting stomach. Under those conditions (i.e. when the resting gastric pH is between 4 and 5) only acid-tolerant

Table 1.5 The bacterial flora of gastric juice in pernicious anaemia.

Bacteria	*Log_{10} (number per ml) in gastric juice*	*Range*
Streptococcus spp.	7.7	(7–8)
Neisseria spp.	6.7	(6–8)
Veillonella spp.	6.7	(6–7)
Haemophilus spp.	7.0	(6–8)
Staphylococcus spp.	5.0	(4–6)
Bacteroides spp.	2.0	(0–6)
Lactobacillus spp.	7.0	(5–8)
Total anaerobes	8.0	(7–9)

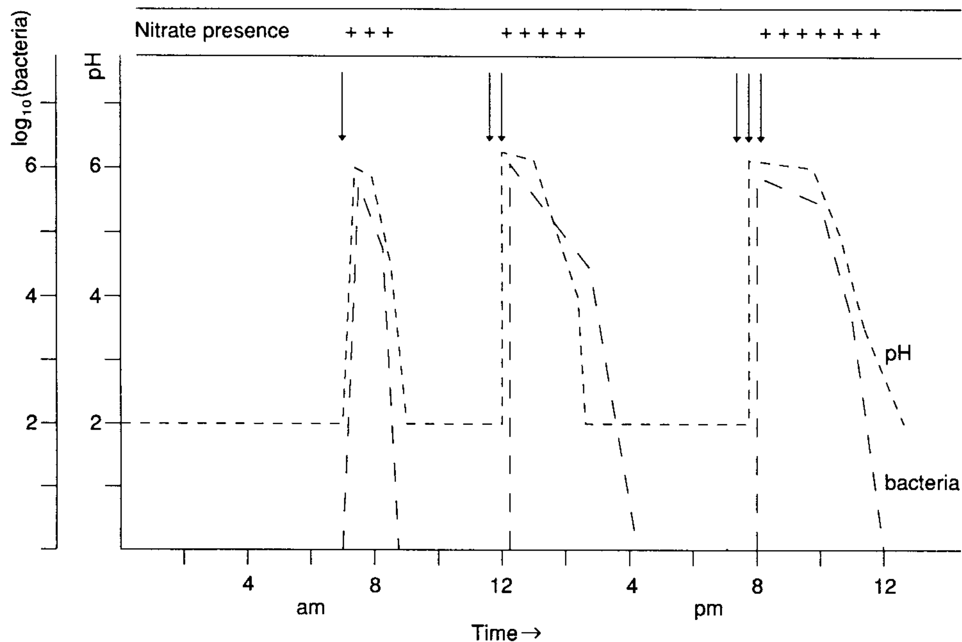


Figure 1.1 The variation in pH and bacterial counts (expressed as $\log_{10}$) and nitrite in the stomach during 24 hours. ↓ Breakfast; ↓↓ Lunch; ↓↓↓ Dinner

organisms proliferate and these include some *Streptococcus,* the *Lactobacillus* and *Micrococcus* spp. When hypoacidity is more severe and the pH does not fall below 5, almost all upper-gut organisms are able to proliferate, and the flora is determined by other criteria discussed later. Impaired gastric acid secretion can occur as a natural result of ageing (see the data of Vanzant *et al.,* 1932 in Table 1.4), or following any gastric surgery that includes vagotomy, or in certain disease states such as pernicious anaemia or hypogammaglobulinemia, or in the chronic atrophic gastritis that follows prolonged *H.pylori* infection. Table 1.5 gives data on the gastric bacterial flora in pernicious anaemia patients.

Table 1.6 Characteristics of *Helicobacter pylori*.

Morphology	Gram–negative short curved rods
Habitat	Surface of gastric mucosa
tolerance	Will not grow on intestinal mucosa or on intestinal metaplasia
Sensitivity to:	
acid	Sensitive
bile	Sensitive
antibiotics	Sensitive to combinations of antimicrobials, but not to any single agent

Table 1.7 Prevalence of *Helicobacter pylori* in various populations (data from Hill and Marsh 1990)

Age group	*UK*	*USA*	*China*	*Mexico*	*India*
20–30	32	21	60	33	46
31–40	43	–	–	–	–
41–50	59	–	–	–	–
51–60	69	–	–	–	–

An apparent anomaly to the above is infection with *Helicobacter pylori*. This organism was first isolated as part of the gastric mucosal flora by Marshall and Warren (1984) and has since become the most intensively studied organism in medical microbiology, because of its possible role in a range of peptic diseases such as peptic ulcer, gastritis and gastric cancer. Table 1.6 summarizes some of the important characteristics of *H. pylori,* and Table 1.7 summarizes its prevalence in various populations in relation to age. In most populations its prevalence increases with age and is low in persons less than 20 years old. However, in some populations it has a high prevalence at all ages (including those less than 10 years old). In most *H. pylori* carriers the gastric luminal pH is less than 3 and there are no symptoms such as dyspepsia or gastric pain. *H. pylori* is sensitive to strong acid and could not survive in the normal gastric lumen; infection must therefore occur during periods of transient anacidity probably associated with enterovirus infection. The organism then colonizes the mucosa below the mucin barrier and is able to inhibit local acid secretion; since the mucin will protect the organisms from luminal acid they are able to proliferate in a locally pH-controlled environment. They do not colonize intestinalized mucosa in intestinal metaplasia patients, and perhaps this is because of the failure of intestinal mucin to protect them from luminal acid. Interestingly, *H. pylori* is rarely found alone but is usually accompanied by a range of opportunistic organisms taking advantage of the local favourable conditions of bacterial growth.

The rich mixed bacterial flora found in the gastric lumen of persons with hypoacidity is ideally placed to have an important influence on the metabolism of xenobiotics, since in such patients the gastric emptying time of four to five hours offers ample time for bacterial metabolism.

Table 1.8 Numbers of bacteria per gram luminal contents at various subsites in the gut of humans and of laboratory animals (data expressed as $\log_{10}$ counts).

	Human	*Rat*	*Mouse*	*Rabbit*
Mouth	7–8	7–8	7–8	7–8
Stomach	<3	7	7	4
Jejunum	<3	7	7	4
Lower ileum	5	8	8	8
Colon	11	11	11	11

1.3.3 Flora of the proximal small intestine

When, during digestion, the gastric contents enter the small bowel they are mixed with large volumes of biliary and pancreatic secretions which include bicarbonate (to neutralize the gastric acid), bile (to emulsify fat to micelles small enough to be digested readily by lipases and phospholyases), and pancreatic digestive enzymes including proteinases, lipases and saccharidases. Many of these secretions are bactericidal and help to sterilize the material entering the small bowel. Further, there is extensive fluid secretion from the bowel mucosa which serves to flush the crypts and prevent colonization of the mucosal layer. Small bowel contents are very fluid but the bowel movements tend to slow transit thereby increasing the opportunity for digestion. Nevertheless, small bowel transit time is only two to four hours and this is a further barrier to small bowel colonization. For those reasons the normal small bowel contains a very sparse flora of transient organisms; a resident flora can only establish in areas of stasis (such as diverticulae or surgical blind loops) or as secondary infections in areas infected by higher microbes (e.g. *Giardiai*) or damaged in other ways (e.g. by immune reactions in coeliac disease) or in tropical sprue.

All of the foregoing applies to western persons, but studies of persons living in South America or in India indicate that in those populations colonization of the upper small intestine is common, or even normal. In such persons the flora is composed predominantly of Gram positive cocci (e.g. *streptococci, peptococci, peptostreptococci*) and rods *(lactobacilli, bifidobacteria)*. Bhat *et al.* (1972, 1980) have explored this further and have shown that small bowel colonization is associated with malnourishment; this could be an indirect relationship (in which both are associated with poor public health) or direct (and related, perhaps, to the role of diet in maintaining gastric acidity). Saltzman *et al.* (1994), Drasar *et al.* (1969) and many others have shown that the small bowel is colonized in gastric achlorhydria.

Bacterial colonization of the upper small intestine would be of major importance in toxicology and pharmacology. It is important to note that the normal lack of a small bowel flora distinguishes humans from the laboratory animals used in lexicological studies (where in animals fed *ad libitum* there is a profuse normal small bowel flora, as illustrated in Table 1.8).

1.3.4 Flora of the lower ileum

Whereas the upper small intestine is colonized from above, the lower small bowel is colonized as a result of the reflux of caecal contents through the ileocaecal junction. This is an area of the gut that

is extremely difficult to sample with confidence. It cannot be sampled from below since this would entail colonic cleansing before colonoscopy. It cannot now be sampled by incubation from above because of difficulties in earlier studies in recovering the tube. It cannot be sampled reliably at operation because more than any other site it is subject to gross contamination from below.

We can say from the few reliable reports (e.g. Drasar *et al.*, 1969; Drasar and Hill, 1974) that the flora of the terminal ileum is similar to that of the caecum and probably results at least in part from reflux through the ileocaecal junction. We do not know the extent of colonization, nor how this varies between persons, nor the temporal variations in extent of colonization in an individual.

1.3.5
Flora of the large intestine

Most information on the composition of the flora of the large bowel has come from analyses of faecal samples, reliable methods for which have been developed in the past 25 years. Table 1.9 summarizes data on the normal faecal flora of healthy adult western persons. The flora is dominated by the strictly anaerobic rod-shaped organisms, both Gram negative (*Bacteroidis* spp.; *Fusobacterium* spp.) and Gram negative (*Bifidobacterium* spp.; *Eubacterium* spp.; *Propionibacterium* spp.), which between them represent more than 99% of the total flora. Other genera that flourish in the gut are the strictly anaerobic *clostridia* and anaerobic *cocci;* the microaerophilic *lactobacilli* and *streptococci* are at an intermediate level and more numerous than the dominant facultative organisms (the coliform group). Whereas the counts of the dominant organisms are stable those of the minor organisms show considerable fluctuation, as is shown by the standard deviations in Table 1.9.

There is some limited information on the faecal flora in various populations around the world. The first study compared UK citizens and persons from Uganda who lived on a diet of matoke (which is prepared from bananas) and found considerable differences in the relative proportions of different genera (Aries *et al.*, 1969). Subsequent studies of other populations did not show similar differences and, in fact, tended to show little detectable difference in the profile of organisms (Table 1.10).

There have been a few attempts to study the flora of the proximal colon, all of which have been unsatisfactory. Berghouse *et al.* (1984) and Fernandez *et al.* (1985) all studied the effect of diet on the composition of ileostomy fluid, which is the nearest that we have to data on the terminal ileum or proximal colon. Their data are summarized in Tables 1.11 and 1.12. A feature is the simplicity of the flora and the absence of certain organisms that are well represented further down the gut.

The inoculum for the colonic flora is presumably from three sources, namely (a) the digesta entering the colon from above (which will be very sparse), (b) the residual colonic material and (c) the mucosal flora.

1.3.6
The colonic mucosal flora

There is a limited amount of information on the composition of the flora at the mucosal surface at various subsites within the large bowel. This comes from the analysis of biopsy material obtained at endoscopy and from specimens of surgically excised tissue. The human gut does not have a true mucosal flora analogous to that of the chicken gut (with its palisades of *lactobacilli*) or the rat (with

Table 1.9 The normal faecal flora of healthy adult humans.

Genus	*Log_{10} bacteria per gram faeces*
Non–sporing anaerobes	
Bacteroides spp.	10–11
Bifidobacterium spp.	10–11
Eubacterium spp.	9–11
Propionibacterium spp.	9–11
Veillonella spp.	5–8
Sporing anaerobes	
Clostridium spp.	5–9
Sporing aerobes	
Bacillus spp.	4–6
Microaerophiles	
Lactobacillus spp.	7–9
Streptococcus spp.	7–9
Enterococci	5–7
Facultative organisms	
Coliforms	7–9
other *Enterobacteria*	5–9

Table 1.10 The faecal flora of persons living in various countries (data from Drasar and Hill, 1974).

	UK	*USA*	*Uganda*	*India*	*Japan*
Non–sporing anaerobes					
Bacteroides spp.	9.7*	9.8	8.2	9.2	9.4
Bifidobacterium spp.	9.8	10.1	9.3	9.6	9.7
Sporing anaerobes					
Clostridia	5.7	5.4	5.1	5.7	5.6
Microaerophiles					
Lactobacilli	6.5	6.5	7.0	7.6	7.4
Enterococci	5.8	5.9	7.0	7.3	8.1
Facultative organisms					
Enterobacteria	7.9	7.4	8.0	7.9	9.4

*Counts are expressed as $\log_{10}$ counts per gram faeces.

its spiral flora). Nevertheless, it has a flora associated with the mucosa (the mucosa-associated flora, or MAF) which is distinct from that of the gut lumen and which is reproducible, stable, and responds to antibiotic treatment differently from that of the lumen. Table 1.13 summarizes the available information. Compared with the luminal flora, the MAF has a more equal representation of aerobic and anaerobic organisms and contains a relatively high proportion of cocci, particularly Gram positive cocci.

1.4
Factors controlling the gut flora composition

There has been a great deal of work both *in vivo* and *in vitro* on the factors which determine both the concentration of total bacteria and the relative proportions of different genera within that total.

Table 1.11 The effect of dietary fibre on the bacterial flora of ileostomy fluid (data from Berghouse *et al.*, 1984).

Organisms	*Log_{10} counts per gram*	
	Control	*High fibre*
Total facultative	5.3 ± 1.6*	6.8 ± 1.1
Escherichia coli	3.8 ± 1.6	4.7 ± 1.6
Streptococcus spp.	5.1 ± 1.7	6.3 ± 1.0
Microaerophiles		
Lactobacillus spp.	3.1 ± 1.2	3.6 ± 1.2
Total strict anaerobes	4.2 ± 1.9	5.1 ± 1.6
Bacteroides spp.	2.7 ± 0.9	3.2 ± 1.1
Clostridium spp.	3.7 ± 2.0	3.9 ± 1.5
Veillonella spp.	3.1 ± 0.8	4.2 ± 1.4
Total organisms	5.5 ± 1.7	6.8 ± 1.2

*± Standard deviation

Table 1.12 The effect of dietary change on the bacterial flora of ileostomy fluid (data from Fernandez *et al.*, 1985).

	Control	*High protein*	*High fat*
Total facultative	5.7*	6.7	6.2
Coliforms	4.5	6.0	5.3
Streptococci	5.6	6.3	5.6
Enterococci	2.9	4.2	3.9
Microaerophiles	ND†	3.7	1.9
Lactobacilli	ND†	3.7	1.9
Total strict anaerobes	4.5	4.9	5.6
Bacteroides	3.7	4.1	4.8
Bifidobacteria	1.4	1.8	1.8
Clostridia	3.8	3.6	4.7
Veillonella	2.0	2.6	1.9

*Log_{10} counts per gram
†Not detected

These are summarized in Table 1.14. They include physicochemical factors such as pH, oxidation-reduction potential and specific ion concentrations; host factors, including secretions such as saliva, bile, pancreatic juice, gastric juice; host-microbe interactions such as immune responses, and the lysozyme-complement-immunoglobulin complex; microbe-microbe interactions and nutrient composition. The most important of these will be considered in turn.

1.4.1 pH

Intestinal commensals are all acid-sensitive though to varying degrees. Thus, when saliva is swallowed and enters the normal acid stomach the acidity is eventually lethal to all of the organisms. In consequence the normal resting stomach between meals is essentially sterile. On occasions it is possible to cultivate organisms from gastric juice samples of pH less than 4 but these are of no significance since they represent the recovery of recently swallowed but dying salivary bacteria. In

Table 1.13 The bacterial flora of the human colonic mucosa.

Strict anaerobes	*Clostridia* *Eubacteria* *Peptococci* *Bacteroides* *Fusobacteria* *Bifidobacteria* *Veillonella*
Microaerophilic	*Lactobacilli* *Streptococci* *Enterococci*
Facultative and aerobes	*Enterobacteria* *Micrococci* *Staphylococci* *Bacilli*
Ratio anaerobes: aerobes	1 to 10

Table 1.14 The factors controlling the composition of the gut bacterial flora.

Physicochemical factors	pH Oxidation–reduction potential Oxygen tension Nutrient supply
Host–bacteria interactions	Saliva Bile Gastric secretions Pancreatic secretion Immune systems
Microbe–microbe interactions	Bacteriophages Bacteriocines Toxic metabolites

samples above pH 4, high concentrations of bacteria can be recovered; these include organisms that are relatively acid-tolerant and multiplying (e.g. *lactobacilli, streptococci, micrococci*) together with salivary organisms that are not multiplying but are not dying either. Above pH 5.5 most organisms are able to proliferate and neither the composition nor the density of the flora is determined by pH.

The pH is an important determinant of the composition of the flora in the hypochlorhydric stomach, in the caecum of, for example, alactasia patients or persons eating diets readily fermented in the caecum, and in the vagina (which is normally at pH below 5.5 but is neutral in infected patients).

1.4.2 Nutrient supply

This, together with pH, is the main determinant of the density of the flora but is also important in determining its composition. In considering nutrient supply consideration needs to be given to (a) nutrients that are available to all components of the flora and so contribute to the magnitude of the

overall flora; (b) nutrients that are available only to specific components of the flora (because those components produce the enzymes necessary to utilize the nutrients). Different aspects of the nutrient supply are important at different sites in the gut. In the mouth the major sources of nutrients are saliva and other secretions and, of course, the diet. The latter is sufficiently rich in glucose to inhibit the inducible enzymes, particularly disaccharides such as those hydrolysing lactose, lactulose and the nonsucrose sweetening sugars such as lactitol, xylitol etc. In contrast, in the colon there is no luminal glucose and all inducible enzymes, when presented with their substrate, will be fully induced. In consequence, in the colon all potential substrates (i.e. those for which bacteria produce degradative enzymes) will be substrates, whereas this is far from true in the mouth and it is not a simple matter to determine the nutritional value to the oral flora of the food.

1.4.3
Redox potential (E_h) and oxygen tension

Bacteria may be classified into strict aerobes, facultative anaerobes, microaerophiles, strict anaerobes and very-oxygen-sensitive (VOS) anaerobes, based on their requirements with respect to oxygen tension and redox potential.

1.4.4
Strict aerobes

Strict aerobes or *obligate aerobes* have an absolute requirement for oxygen for energy production from carbon sources. Classically, they use glycolysis to convert glucose to pyruvate and then the Krebs cycle for removal of pyruvate as CO_2 and water. This is energetically the most efficient pathway available and does not generate potentially toxic byproducts. As the oxygen tension decreases the pathway slows so that at low oxygen tensions the rate of energy production and new cell mass production is insufficient to compensate for cell death. Rapid oxidative metabolism results in the formation of a range of highly toxic oxygen radicals and these are detoxified by a range of enzymes such as catalase, peroxidase, and superoxide dismutase (SOD). Obligate aerobes use oxygen as the terminal hydrogen acceptor and so need high redox potentials, but these are normally present whenever the oxygen tension is suitable.

1.4.5
Facultative anaerobes

Facultative organisms such as the coliform bacteria utilize the Krebs cycle in the presence of an adequate oxygen tension but, in its absence, have an array of anaerobic pathways to dispose of pyruvate. The end-products of such fermentation include lactate, acetate, formate, CO_2, ethanol and hydrogen. Because the facultative organisms retain the enzymic capacity for both aerobic and anaerobic growth they are unable to compete with strict aerobes in the presence of suitable oxygen tensions; in compensation they are able to survive and flourish during periods of oxygen deprivation. Their pathways for anaerobic fermentation are not oxygen-sensitive and they retain the capacity for oxygen radical detoxification.

1.4.6
Microaerophilic bacteria

Microaerophiles such as *streptococci* are similar to the facultative bacteria in their enzymic armoury but have lost the ability to produce catalase, SOD, etc. In consequence, in the presence of oxygen *streptococci* will undergo a rapid burst of oxidative growth followed by cell death as a result of oxygen radical toxicity. They grow optimally in the presence of an atmosphere of CO_2 and nitrogen from which, however, it is unnecessary to completely exclude oxygen (since the organisms are not sensitive to oxygen *per se* but to oxidative growth). *Streptococci* will grow well in à normal atmosphere on blood agar because they are protected by the oxygen radical detoxification systems of the blood. *Lactobacilli* are more sensitive than *streptococci* to oxygen radical damage and so most species cannot be grown in air even on blood agar.

1.4.7
Strict anaerobes

Obligately anaerobic bacteria are not only unable to utilize oxygen but are killed by it. In general, they are deficient in the oxygen radical detoxification systems, and contain essential enzyme systems that are oxygen-sensitive, presumably due to the presence of sulphydryl groups. Anaerobic fermentation of pyruvate yields a wide range of short chain fatty acids including formate, acetate propionate, butyrate and higher aliphatic acids together with lactate, fumarate and succinate (Table 1.15). The obligately anaerobic bacteria show different levels of oxygen sensitivity, with the very-oxygen-sensitive (VOS) anaerobes needing special techniques for quantitative culture that are much more exacting than those required for the main group of human commensal anaerobes.

1.4.8
Relevance to the human gut

Ecological sites in the gut vary greatly in their redox and oxygen tension status and this is reflected in the composition of the flora at those sites. In saliva there is ample opportunity for oxygen to dissolve and it might thus be expected to have an aerobic flora. In fact, the rate of oxygen solution is slower than the rate of oxygen utilization by facultative organisms, and in consequence the conditions are favourable for the growth of some more robust strict anaerobes. In the lower gut the conditions are highly reducing and the oxygen tension is so low that even VOS anaerobes can flourish; under these conditions facultative organisms compete poorly for nutrients, and anaerobes outnumber facultative organisms by a factor of 1,000. At the colon mucosal surface, in contrast, oxygen diffusion from the mucosa is sufficient to inhibit VOS anaerobes and facultative organisms are as numerous as the anaerobes. Table 1.16 summarizes the situation at various ecological sites in the gut. Only on the skin and in the nose are conditions suitable to support growth of strict aerobes (such as *staphylococci*).

Table 1.15 The acid end products of fermentation produced by various bacterial genera.

Non–sporing anaerobes	
Bifidobacteria	Acetate, lactate
Bacteroides spp.	Acetate, propionate, succinate
Fusobacteria	Acetate, butyrate, lactate
Propionibacteria	Acetate, propionate, lactate
Sporing anaerobes	
Clostridium spp.	Acetate, propionate, butyrate, pentanoate, hexanoate, etc.
Microaerophiles	
Lactobacilli } *Streptococci* }	Lactate
Facultatives	
E. coli	Acetate, formate, lactate

Table 1.16 The relative proportions of anaerobes and aerobes at various subsites in the human gut.

Site	*Counts of anaerobic/aerobic bacteria*
Mouth	10–100
Stomach	<1
Jejunum	1–10
Terminal ileum	1–100
Ileostomy fluid	<1
Caecum	100–10,000
Colon	100–1,000
Colon mucosa	1–10

1.5 Interbacterial interactions

Whereas pH and E_h control the composition of the flora at the gross level, determining which genera are present, interbacterial interactions subtly control the flora composition at the species or strain level. The major interactions are those controlled by (a) bacteriophage, (b) bacteriocines and (c) bacterial metabolites.

1.5.1 Bacteriophage

A wide range of bacterial species produce bacteriophage—virus particles that enter and lyse certain strains of the same species. Sensitivity to phage is determined by the presence of phage receptor particles in the bacterial cell wall, and consequently strains tend to be sensitive to a small number of phages rather than the full range. A given ecological niche is likely to be equally favourable to all members of a species; phage production can protect an ecological niche from colonization by strains of the same species.

1.5.2
Bacteriocines

Most bacterial species produce bacteriocines, which are antibiotic substances that are lethal to a proportion of strains of the same or related species. They are named from the producer species; thus *Escherichia coli* produce colicines, *Pseudomonas pyocyanaeus* produce pyocines etc. The mode of action varies greatly between bacteriocines, but their function is very similar to that of bacteriophage in helping a colonizing strain to protect its ecological niche from strains of the same or related species.

1.5.3
Bacterial metabolites

Bacteria produce a range of metabolites that have antibacterial action, usually against unrelated species. Thus, bifidobacteria produce large amounts of acetic and lactic acid from glucose fermentation and this influences the rest of the flora through its effect on pH. In addition, it has been suggested that acetic and lactic acids have an inhibitory effect on coliform organisms. Most of these effects of bacterial interferences and the related phenomenon of colonization resistance are little understood but can have dramatic effects. *Clostridium difficile* is unable to colonize the human gut unless the anaerobic flora is suppressed with antibiotics such as lincomycin and clindamycin, under which circumstances the *Cl. difficile* is able to proliferate and cause the severe gut disease, pseudo-membranous colitis (PMC). In earlier decades neomycin had been reported to permit the growth of *Staphylococcus aureus* in the colon, resulting in *staphylococcal enterocolitis* (which has a 50% mortality rate).

In vitro, clostridia would appear to be the most likely organisms to flourish in the gut, vagina, etc and to out-compete the rest of the anaerobic flora. In fact, in such situations clostridia tend to be minor components of the gut flora because they are suppressed by the rest of the flora.

1.6
Host bacteria interactions

The host is not a passive supporter of the commensal flora but produces a range of products, enzymes and secretions that have antibacterial action and which have a profound effect on the composition of the flora at a specific site.

1.6.1
Lysozyme

Lysozyme is an antibacterial enzyme which hydrolyzes the peptidoglycan component of the cell wall. Since this gives the bacterium its shape and its ability to resist the 10–20 atmospheres pressure within the cell, its hydrolysis results in bacterial lysis and death. The peptidoglycan is protected in Gram negatives but is exposed in many Gram-positive organisms. Consequently, *in vitro,* it is only active against certain Gram-positive organisms; most Gram-positive human commensal bacteria are resistant (since the lysozyme sensitive bonds are readily protected, e.g. by N-acetylation). *In vivo* secretions containing lysozyme also contain secretory antibody (IgA) and complement; the antibody-

complement-lysozyme system is an extremely potent lytic system for Gram-negative species and this may explain the dominance of Gram-positive bacteria in, for example, saliva (a rich source of lysozyme).

1.6.2 Bile and pancreatic secretions

Bile is a rich mixture of bile acids, cholesterol, fatty acids, phospholipid, bile pigment and an array of detoxified xenobiotics. Pancreatic secretion contains an array of digestive enzymes, the lipolytic ones being activated by the surface-active properties of bile. The combination is highly bactericidal to all human commensals except those resident in the colon. Thus, qualitatively the oral and colonic flora are similar in containing the same major genera *(bacteroides, streptococci, lactobacilli, bifidobacteria)* but whilst the mouth contains bile-sensitive species the colon has bile-resistant species, including some (e.g. *Bacteroides fragilis*) which are stimulated to faster growth in the presence of bile.

1.6.3 Immune systems

The antibody-complement-lysozyme system has already been mentioned but it has been proposed that immune systems per se play a role in determining the composition of the gut flora. This has been studied most closely in persons who are deficient in IgG, IgM or IgA (Brown *et al.* 1972; Hersh *et al.* 1970); no detectable difference was seen in the small bowel flora between normal persons and groups with IgA or IgM deficiency but those with IgG deficiency have a more profuse flora.

1.7 Conclusions

The normal human gut bacterial flora is extremely complex both in itself and in the web of factors influencing it and controlling its actions. Studies of its role in toxicology have given us some insights and enable us to make some simple predictions. However, at this stage, when wishing to study the role of the gut flora in the toxicology of any new compound, there is no alternative to actually doing the experiment.

References

Aries, V.C., Crowther, J.S., Drasar, B.S., *et al.,* 1969, Bacteria and the etiology of cancer of the large bowel, *Gut,* **10,** 334–35.

Berghouse, L., Hori, S., Hill, M.J., *et al.,* 1984, Comparison between the bacterial and oligosaccharide content of ileostomy effluent in subjects taking diets rich in refined and unrefined carbohydrate, *Gut,* **25,** 1071–77.

Bhat, P., Shantakumari, S., Rajan, D., *et al.,* 1972, Bacterial flora of the gastrointestinal tract in Southern India control subjects and patients with tropical sprue, *Gastroenterol.,* **62,** 11–15.

Bhat, P., Albert, M., Rajan, D., *et al.,* 1980, Bacterial flora of the jejunum: a comparison of luminal aspirate and mucosal biopsy, *J. Med. Microbiol.,* **13,** 247–56.

Borriello, S.P., 1986, Microbial flora of the gastrointestinal tract, in Hill, M.J. (Ed.) *Microbial Metabolism in the Digestive Tract,* pp. 1–20, Boca Raton: CRC Press.

Borriello, S.P., Hudson, M.J. and Hill, M.J., 1978, Investigations of the gastrointestinal bacterial flora, *Clinics Gastro.,* **4,** 329–49.

Brown, W.R., Savage, D.C., Dubois, R.S., *et al.,* 1972, Intestinal flora of immunoglobulin-deficient and normal human beings, *Gastroenterol.,* **62,** 1143–52.

Crowther, J.S., 1971, Transport and storage of faecal specimens for quantitative bacteriology, *J. Appl. Bact.,* **34,** 477–83.

Drasar, B.S. and Hill, M.J., 1974, *Human Intestinal Flora,* London: Academic Press.

Drasar, B.S., Shiner, M. and McLeod, G.M., 1969, The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons, *Gastroenterol.,* **56,** 71–77.

Fernandez, F., Kennedy, H., Hill, M.J. and Truelove, S., 1985, The effect of diet on the bacterial flora of ileostomy fluid, *Microbiol. Aliments Nutr.,* **3,** 47–52.

Hersh, T., Floch, M.H., Binder, H.J., *et al.,* 1970, Disturbance of the jejunal and colonic bacterial flora in immunoglobulin deficiencies, *Am. J. Clin. Nutr.,* **23,** 1595–1601.

Hill, M.J. and Marsh, P.D., 1990, *Human Microbial Ecology.* Boca Raton: CRC Press.

Marsh, P.D. and Martin, M., 1984, *Oral Microbiology,* Washington DC: Am. Soc. Microbiol.

Marshall, B.J. and Warren, J.R., 1984, Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration, *Lancet,* **1,** 1311–15.

Meyrick-Thomas, J., Misiewicz, J.J., Cook, A.R., *et al.,* 1987, Effects of one year's treatment with ranitidine and of truncal vagotomy on gastric contents, *Gut,* **28,** 726–38.

Milton-Thompson, G.J., Ahmet, Z., Lightfoot, N.F., *et al.,* 1981, Intragastric acidity, bacteria, nitrate and N-nitroso compounds before, during and after cimetidine treatment, *Lancet,* **1,** 1091–95.

Saltzman, J.R., Kowdley, R.V., Pedrosa, M.C., *et al.,* 1994, Bacterial overgrowth in elderly hypochlorhydric patients, *Gastroenterol.,* **106,** 615–23.

Theilade, E., 1990, Factors controlling the microflora of the healthy mouth, in Hill, M.J. and Marsh, P.D. (Eds.) *Human Microbial Ecology,* pp. 1–56, Boca Raton: CRC Press.

Vanzant, F.R., Alvarez, W.C., Eusterman, G.B., *et al.,* 1932, The normal range of gastric acidity from youth to old age, *Arch. Int. Med.,* **49,** 345–59.

Chapter 2
The metabolic activity of gut bacteria

M.J.Hill

2.1
Introduction

In relation to pharmacology and toxicology it is clearly more important to know what the bacteria do rather than what names we choose to give them. Despite this the number of papers on bacterial taxonomy greatly outnumber those on bacterial metabolism, and a significant proportion of work on bacterial metabolism has been aimed at improving the ways to monitor changes in the flora rather than at learning more about the role of the flora in toxicology or pharmacology.

In this chapter the methods currently available for determining the metabolic activities of gut bacteria and the methods of expressing the results will be reviewed, and the interpretations that can and cannot be made of the data will be discussed. In addition there will be a discussion of the factors affecting the enzymic activity of the gut bacterial flora.

2.2
Bacterial enzyme activity

There is no single way to quantify the enzymic activity of the gut bacterial flora. In general, a role for the gut bacteria in toxicology can be demonstrated qualitatively by comparing the metabolism of the test compound in germ-free and in conventional animals and this is discussed in more detail in Chapter 3. Direct assays can be made using homogenates of faeces or caecal contents and more detailed data can be obtained using pure strains of bacteria or isolated cell-free enzymes.

2.2.1
Methods of assay—pure strains versus caecal contents

The simplest type of assay uses homogenates of faeces or caecal contents. In order to simulate the gut conditions most exactly, the homogenate should be made up using deoxygenated distilled water in an oxygen-free atmosphere (usually in an anaerobic cabinet). The substrate is added, also as a solution in oxygen-free water or solvent, and the metabolism monitored. If this method is followed, the conditions regarding oxygen tension, pH, nutrient supply, etc., will closely mimic those in the gut. Some form of gentle shaking to mimic the effects of bowel muscular activity would also be needed.

In general, such a simple approach would not be followed by those with a lexicological or biochemical background because the conditions lack reproducibility. In order to standardize the pH the sample of gut contents is usually suspended in buffer. To assist the bacteria to grow faster (and to give a faster result) some form of nutrient medium is used. To assist the assay the pH is often adjusted to the pH-optimum of the enzyme being tested. These are all changes which will improve the speed and reproducibility of the assay but do not simulate the true conditions in the gut. Further, if the medium is one which supports active division of the bacteria this will allow enzyme induction to occur. Such an assay will therefore measure the maximum potential induced enzyme activity but will give no information on the actual enzyme activity in the animal or individual being studied.

It is important to recognize that the conditions in the colon are extremely 'spartan' and that the normal gut bacterial flora is superbly adapted to such an environment. Provision of, for example, broth media as the suspension fluid, will provide the mixed flora with a range of nutrients that they would rarely experience *in vivo* except in trace quantities. Thus by incubating the assay mixture in broth media, not only are enzymes being induced that might never be produced under normal conditions, but their total activity is also influenced by the outgrowth of minor components of the flora that would not normally be able to flourish. Such methods are widely used in the assay of 'sentinel enzymes' used to monitor changes in the flora (discussed later in this chapter).

Because of the success of studying pure strains of bacteria in the determination of the causation and mechanism of disease, the same techniques have been applied by microbiologists in toxicological investigations. Assay of the enzyme activity in pure strains of gut bacteria can be made using methods similar to those described above for faecal enzymes. Pure culture is highly artificial and the activity of the enzyme will depend on the cultural as well as the assay conditions; culture is rarely under habitat-simulated conditions and so interpretation of the results is difficult. Nevertheless, such studies have provided useful results. In early studies of the enterohepatic circulation (EHC) of glucuronides it was shown that *Escherichia coli* produces a highly active β-glucuronidase, and much of the early discussion of EHC centred on the role of the enzyme produced by *E. coli*. Later studies by Hawksworth *et al.* (1971) showed that, whilst *E. coli* produces the most highly active enzyme in terms of activity per 10^8 cells, the numerical dominance of the non-sporing anaerobic bacteria ensure that they contribute more than 90% of the small bowel enzyme and more than 99% of the large bowel enzyme in rats. Further, in toxicological studies the differences in enzyme activity and consequently the rate of EHC between species of test animal can be rationalized in terms of the differences in composition of the flora in the small bowel of those animals.

2.2.2
Methods of expressing the data

Enzyme activity can be expressed in various ways (Table 2.1), and the method of choice depends on circumstances. For pure cultures activity is usually expressed per unit cell mass; this unit may be measured gravimetrically, or in terms of numbers of bacteria (e.g. per 10^8 cells), or from the optical density of the culture.

Faecal enzyme activity is usually expressed per gram wet weight. The water content of faeces is variable and some groups prefer to correct for the water content and express the activity per gram dry weight (the water content being assayed on a separate sample of faeces).

Table 2.1 Methods of expressing faecal enzyme activity

Sample	*Activity measure*
Pure cultures	per gram dry weight per gram wet weight per optical density unit per unit DNA per number of cells per mg protein
Faeces	per gram wet weight per gram dry weight per day stool weight per mg protein
Caecum (animal studies)	per caecum

Table 2.2 Ways in which diet may affect faecal enzyme activity.

Modify flora profile	Increase in proportion of a highly active or a low activity species relative to the total flora.
Modify enzyme induction	Induction or inhibition of enzyme activity.
Affect stool bulk	Dietary fibre has stool bulking properties. Laxatives increase water content of stools.

Daily faecal output is related to diet and is increased, for example, by increased dietary fibre intake, and decreased by residue-free diets. It has been suggested that enzyme activity should be measured on a per day stool weight basis. In animal studies where the whole gut can be excised, caecal enzyme activity is often expressed on a per whole caecum basis.

2.2.3 Interpretation of faecal enzyme assays

The interpretation of the results of enzyme assays should depend on the assay method used and the way in which the results are expressed. Amongst the matters to be considered are the assay conditions (e.g. pH, oxygen tension, nutrient supply), the enzyme induction (or otherwise) and the way in which the results are expressed.

2.3 Effect of diet on faecal enzyme activity

Diet may affect the faecal enzyme activity in various ways (summarized in Table 2.2). The diet may contain components which induce the enzyme, or stimulate or inhibit its activity. It may modify the relative proportions of the flora, thereby changing the net enzyme activity. It may affect stool bulk, by laxation, by increasing the water content of the stools or by increasing the rate of bacterial synthesis in the colon and so increasing the dry as well as the wet weight of the daily stool output.

Table 2.3 summarizes the effect of various changes in diet on the activity of various faecal enzymes. How these results might be rationalized is now discussed.

Table 2.3 The effect of diet on the faecal activity of various enzymes.

	β–glucosidase activity	*β–glucuronidase activity*	*azo reductase activity*	*nitro reductase activity*	*nitrate reductase activity*
Pectin supplements	Decreased	Decreased		No effect	No effect
Bran supplements	Decreased	Decreased		No effect	No effect
Increased fat	No effect	No effect		No effect	No effect
High meat *vs* low meat	Decreased	Increased	Increased	Increased	
Increased protein	Increased	No effect		Increased	Decreased
Diet restriction	No effect	No effect	Decreased	Decreased	Decreased

2.3.1 Balance of the flora

Studies on the effect of diet manipulation on the faecal bacterial flora have been disappointingly unconvincing. For example, in a survey of studies of the effects on the faecal bacterial flora of dietary supplementation with various dietary fibre sources or fractions Hill and Fernandez (1990) were able to find no evidence of any effect, and the same would be true for other diet supplements (Table 2.4).

They concluded that these negative results were misleading and were due to the effect being sought at the wrong site. Because digestion is so efficient, variations in the composition of food entering the gut will be greatly decreased by the time the digesta reaches the ileocaecal junction, but some residual effect should still be detectable because digestion is not 100% efficient. In particular, most of the dietary fibre but only a small fraction of supplements of meat, protein or fat, reaches the large bowel.

However, these small effects are decreased even more during colonic transit and large changes in diet composition result in only small changes in faecal composition. Thus, studies by Cummings *et al.* (1978) of the effect of different levels of fat intake (60 gm compared with 140 gm) showed no effect on the faecal bacterial flora, whilst Fernandez *et al.* (1985), studying similar fat intakes, were able to demonstrate large effects on the terminal ileal flora of ileostomists. The effect of changes in various components of the diet on the ileostomy flora are summarized in Table 2.5. They confirm that dietary changes modify the composition of the gut flora; perhaps the changes in enzyme activity achieved in faeces by Goldin and Gorbach (1976) following changes in meat intake are a measure of the greater sensitivity of enzyme assays compared with quantitative bacteriology and give a truer measure of changes in the composition of the flora.

2.3.2 Specific substrate supply

In 1979 Cummings and colleagues published a report of the effect of changes in meat protein intake on the urinary excretion of the urinary volatile phenols (UVP) produced by gut bacterial metabolism of phenolic amino acids. The increased meat intake, which presumably increased the supply of phenolic amino acid to the colonic flora, resulted in a 46% increased excretion of UVP.

The disaccharide lactulose is resistant to intestinal *β*-galactosidase and so passes unmetabolized into the lower bowel, where it is readily hydrolyzed by the bacterial galactosidase to release its

Table 2.4 The effect of diet change on the composition of the faecal bacterial flora as measured by classical bacteriological techniques (data from various sources).

Diet change	*Effect on the flora*
Increase protein	No effect
Increase fat	No effect
Increase bran	No effect
Increase bagasse	No effect
Increase pectin	No effect
Increase guar	No effect
Increase cellulose	No effect

Table 2.5 The effect of diet change on the composition of ileostomy effluent (Data from Fernandez *et al.*, 1985; Berghouse *et al.*, 1984).

Diet change	*Effect*
Fibre increased 4–fold	Fibre increased 4–fold
Protein increased 155%	Protein increased 36%
Fat increased 150%	Fat increased 40%

component monosaccharides galactose and fructose, which are then rapidly metabolized to short chain fatty acids (SCFA). Terado *et al.* (1992) showed that feeding lactulose causes an increase in faecal *bifidobacteria* and a decrease in lecithinase-positive *clostridia* (as would be expected if there was caecal acidification). Bown *et al.* (1974) demonstrated, using a radiotelemetry device that was swallowed and allowed to reach the colon, that after lactulose ingestion the colonic pH decreased to less than 5 (Table 2.6); a similar effect on caecal pH was observed by Pye *et al.* (1987) when they fed dietary fibre supplements to volunteers. Florent *et al.* (1985) showed that, with chronic ingestion of lactulose, the rate of acidification of the caecum was increased showing direct enzyme induction by the substrate. In all of these studies the pH increased along the colon, presumably as a result of the absorption or utilization of the SCFA.

Starch malabsorption also delivers a readily fermentable substrate to the gut bacterial flora and results in caecal acidification, favouring the outgrowth of the more acidtolerant bacteria such as the *streptococci, lactobacilli* and *bifidobacteria* at the expense of the *bacteroides* and *clostridia*. This has a profound effect on the metabolism of bile acids (Bartram, 1991) which may have implications in colorectal carcinogenesis.

2.3.3 Induction of specific enzymes

The best examples of induction of specific enzymes by dietary change are from the effects of dietary fat on bile salt metabolism and of dietary fibre on bile pigment metabolism.

The metabolism of bile salts is discussed in detail in Chapter 10. The major enzymes, and in particular the 7-dehydroxylase, are inducible and it was shown by Hill (1971) that the rate of dehydroxylation increased with faecal bile acid concentration; the latter was increased with increased fat intake, and so the inducible 7-dehydroxylase also increased with dietary fat intake.

Table 2.6 The effect of lactulose supplementation on the pH of the lower ileum and large bowel (Data adapted from Bown *et al.,* 1974)

	Lower ileum	*Colon*		*Rectum*
		Right	*Left*	
Control diet	7.5	6.0	7.0	6.8
Lactulose	7.1	4.9	6.7	6.6
Sodium sulphate	7.5	6.5	6.0	6.1
Lactulose + sodium sulphate	7.6	4.5	6.3	6.4

In persons on a residue-free diet the stools are green because of the failure of the gut bacterial flora to metabolize biliverdin to the usual range of urobilins and urobilinogens (Drasar and Hill, 1974). Any 'cheating' on the diet (e.g. grapes, chocolate, crisps) is immediately evident from the induced biliverdin metabolism resulting in the usual brown stool colour. The same residue-free diet results in the excretion of faecal bile acids rich in unhydrolyzed bile acid conjugates, due to the failure to induce the bile acid metabolizing enzymes. It is important to note that the above observations cannot be explained by decreased bacterial numbers; although the daily stool mass during consumption of a residue-free diet is greatly decreased the numbers of bacteria per gram of faeces remains similar to that in people consuming their normal diet.

2.3.4 Selection of metabolizing strains

It is possible that increased metabolism of a substrate with chronic feeding may be due to selection of metabolizing strains, and it is difficult to distinguish between such a mechanism and enzyme induction or a change in the composition of the flora. An example where the mechanism is relatively clear was in the study of cyclamate metabolism. Under normal circumstances healthy persons do not metabolize cyclamate; however, on chronic feeding it was observed by Renwick and Williams (1969) that some animals and some humans acquired the ability to hydrolyze the N-sulphate linkage to release cyclohexylamine and sulphate. The very wide variation between animals indicated that this was not an example of simple enzyme induction, but was due to the acquisition of a metabolizing strain which then was able to outcompete and outgrow the non-metabolizing organisms of the same or different species. The subject of cyclamate metabolism has been reviewed by Renwick (1988).

2.4 Relevance to toxicological studies

The importance of the gut bacterial flora in the metabolism of some xenobiotics is well established, and it is widely recognized that it is the metabolic activity of the gut microflora and not its taxonomic qualities that is of most interest. It is important, therefore, that the immediate experiments on the metabolism of a xenobiotic should involve assays in the whole animal and then using caecal contents or faeces. Nevertheless, there is a wealth of basic data on the bacterial flora of man and laboratory animals that is wasted unless we are able to translate bacterial numbers into an

approximation of enzymic activity. That is why, in order to be able to utilize these indirect data, it is essential to have information on the metabolic activity of pure strains of gut bacteria.

References

Bartram, A., 1991, Effect of starch malabsorption on faecal bile acids and neutral steroids in humans: possible implications for colonic carcinogenesis, *Cancer Res.,* **51,** 4238–42.

Berghouse, L., Hori, S., Hill, M.J., Hudson, M.J., Lennard Jones, J. and Rogers, E., 1984, Comparison between the bacterial and oligosaccharide content of ileostomy fluid in subjects taking diets rich in refined and unrefined carbohydrate, *Gut,* **25,** 1071–77.

Bown, R.L., Gibson, J.A., Sladen, G.E. *et al.,* 1974, Effects of lactulose and other laxatives on ideal and colonic pH as measured by a radiotelemetry device, *Gut,* **15,** 999–1004

Cummings, J.H., Wiggins, H., Jenkins, D. *et al.,* 1978, The influence of different levels of fat intake on faecal composition, microflora and gastrointestinal transit time, *J. Clin. Invest.,* **61,** 953–61.

Cummings, J.H., Hill, M.J., Bone, E.S. *et al.,* 1979, The effect of meat protein with and without dietary fibre on colonic function and metabolism, *Am. J. Clin. Nutr.,* **32,** 2094–2101.

Drasar, B.S. and Hill, M.J., 1974, *Human Intestinal Flora,* London: Academic Press.

Fernandez, F., Kennedy, H., Hill, M.J. and Truelove, S., 1985, The effect of diet on the bacterial flora of ileostomy fluid, *Microbiol. Aliments Nutr.,* **3,** 47–52.

Florent, C., Flourie, B., Leblond, A. *et al.,* 1985, Influence of chronic lactulose ingestion on the colonic metabolism of lactulose in man an *in vivo* study, *J. Clin. Invest.,* **75,** 608–13.

Goldin, B. and Gorbach, S.L., 1976, The relationship between diet and rat faecal bacterial enzymes implicated in colon cancer, *J. Nat. Cancer Inst.,* **57,** 371–75.

Hawksworth, G.M., Drasar, B.S. and Hill, M.J., 1971, Intestinal bacteria and hydrolysis of glycosidic bonds, *J. Med. Microbiol.,* **4,** 451–58.

Hill, M.J., 1971, The effect of some factors on the faecal concentration of acid steroids, neutral steroids and urobilins, *J. Path.,* **104,** 239–45.

Hill, M.J. and Fernandez, F., 1990, Bacterial metabolism and colorectal cancer, in Kritchevsky, D., Bonfield, C. and Anderson, J.W. (Eds) *Dietary Fiber,* pp. 417–29, New York: Plenum Press.

Pye, G., Crompton, J., Evans, D.F. *et al.,* 1987, The effect of dietary fibre supplementation on colonic pH in healthy volunteers *Gut,* **28,** A1366.

Renwick, A.G., 1988, Intense sweeteners and the gut flora, in Rowland, I.R. (Ed.) *Role of the Gut Flora in Toxicity and Cancer ,* pp. 175–206, London: Academic Press.

Renwick, A.G. and Williams, R.T., 1969, Gut bacteria and the metabolism of cyclamate in the rat, *Biochem. J.*, **114,** 78.

Terado, A., Hara, H., Kataoka, M. and Mitsuoka, T., 1992, Effect of lactulose on the composition and metabolic activity of the human faecal flora, *Micro. Ecol. Hlth. Dis.,* **5,** 43–50.

Chapter 3
The use of gnotobiotic animals in studies of toxicology

M.E.Coates

3.1
Introduction

The human gastrointestinal tract is home to myriad microorganisms actively metabolizing food residues, physiological secretions and cell debris in the luminal contents. Their activities may be beneficial, detrimental or of no account to the host. In order to unravel the different mechanisms that maintain the balanced ecosystem within the gut, and to determine their effects on the host, it is necessary to resort to an animal model in which the gastrointestinal flora can be controlled.

The standard laboratory animal, like its human counterpart, carries a burden of microorganisms that cannot reliably be totally eliminated, even by administration of heroic doses of antibacterials. The gnotobiotic animal is a much more useful tool. The term 'gnotobiote' (from the Greek *gnosos,* knowledge and *bios,* life) describes an animal in which only known life forms are present. It may be germ-free (GF), i.e. totally devoid of any detectable microbial associates such as bacteria, fungi or parasites, or it may be harbouring one or more species of microorganism, the identity of which is known. If any biological reaction in a germ-free animal differs from that in its conventional counterpart it must be concluded that microbial activity is influencing that reaction. Inoculation of GF animals with known strains of organism(s) may uncover the identity of those responsible. The terms mono-, di- or poly-associated describe animals harbouring one, two or many *known* microbial associates.

Animals kept in the open laboratory and harbouring their indigenous microflora are usually referred to as conventional (CV). Specified-pathogen-free (SPF) animals are maintained in carefully protected areas where exposure to pathogens is minimized. They are tested regularly to demonstrate the absence of stipulated pathogens but, because of the strict hygienic control of their environment, they may not carry the full range of organisms characteristic of the species. They do not therefore constitute an ideal control in experiments with gnotobiotes.

3.2
The germ-free animal

3.2.1
Derivation of germ-free animals

Detailed accounts of the equipment and techniques used for the production and management of GF animals are given elsewhere (Coates and Gustafsson, 1984). Briefly, their derivation depends upon the fact that mammals developing in the uterus of a healthy dam, or embryos in eggs from a disease-free hen, are microbiologically sterile. If delivered aseptically just before birth into a sterile environment they remain free from microbial contamination, unlike conventional creatures that acquire a heavy burden of microorganisms at birth or hatching from their dams and from the environment. So long as they are provided with sterile food, water and air they will remain 'germ-free' indefinitely. Rigorous sterility tests must be performed at intervals to check the continued absence of all detectable microbial contaminants. Most laboratory species and some farm animals have been produced germ-free. Rats and mice, most commonly chosen for toxicological studies, are obtainable from commercial sources. Other species must be derived by the user.

3.2.2
Characteristics of germ-free animals

There are important differences in the physiological and biochemical parameters of GF animals and their counterparts carrying their indigenous microflora (Table 3.1). These must be taken into account when comparing the responses of GF and CV animals to any experimental treatment. The characteristics of a CV animal reflect the combined effects of the animal host and its resident microflora. Those of the GF animal represent the intrinsic characteristics of the animal *per se,* devoid of any microbial influences. For instance, the immune system remains virtually unstimulated and, although competent, is slow in response to challenge. The liver of CV animals is heavier than that of their GF counterparts, possibly because of a greater 'workload' imposed by the need to metabolize microbial products. Differences in concentration of some metabolic enzymes and increased requirements by CV animals for some vitamin coenzymes may be similarly explained.

Not surprisingly the greatest differences are seen in the gut, where the heavy burden of organisms induces a mild inflammatory state. There is a faster rate of turnover of epicytes, an increase in thickness of the lamina propria and alterations in transit time of digesta. Microbial metabolites such as ammonia and short chain fatty acids modify the pH of the luminal contents in the conventional animal. An important anomaly particularly pronounced in germ-free rodents and lagomorphs is the development of a grossly enlarged caecum. The enlargement appears to result from the combined effects of accumulated substances that in the conventional animal would be degraded by microbial action. Mucins, for instance, which have strong water-retaining properties cause an increase in volume of the lower bowel contents. Bioactive substances such as kallikreins and vasodepressant peptides reduce muscle tone and intestinal mobility. The resulting fluid retention in the caecum may account for up to 30% of the animal's body weight, which renders invalid comparisons of growth rate between GF and CV rodents. The administration of drugs according to body weight is also open to question.

3.3 Experimental uses of gnotobiotic animals in toxicology

3.3.1 Metabolism of xenobiotics

Foreign substances entering the body in the food, or as orally administered medicaments, are directly exposed to the action of the gut microflora. So, also, are drugs given parenterally if they pass across the gut wall or are excreted in the bile. There is further opportunity for microbial action if the substance or its metabolites re-enter the gut via the enterohepatic circulation. In spite of the limitations described above, experiments with gnotobiotic animals have made significant contributions to our understanding of the role of the microflora in toxicology. If comparison between a group of GF animals and their CV counterparts reveals a difference in response to a xenobiotic, the difference may be due to a direct metabolic effect of microorganisms on the xenobiotic, or it may indicate a more subtle microbial involvement in the overall metabolism of the foreign substance.

A classic example of a direct microbial effect was clearly demonstrated in studies of the hepatotoxic action of cycasin, a glycoside that is lethal when given orally to CV rats but harmless in corresponding GF animals. Most of the administered cycasin was found unchanged in excreta from the GF rats, but only a quarter of the dose could be recovered from CV rats (Spatz *et al.,* 1967). Since many components of the gut flora possess *β*-glycosidase activity it seemed likely that the cycasin aglycone, methylazoxymethanol, might be the effective toxin. The suggestion was upheld when this compound was shown to induce liver tumours in GF rats. The role of the gut flora was finally established when rats monoassociated with a *lactobacillus* capable of hydrolysing cycasin developed the typical hepatotoxic lesions (Laqueur and Spatz, 1975).

A similar experimental protocol was used to demonstrate that the toxicity of amygdalin is largely due to the release of cyanide by microbial glycosidases. An oral dose of amygdalin induced high concentrations of cyanide in the blood of CV rats but not of their GF counterparts. More amygdalin was recovered in the excreta of the GF rats. Benzaldehyde was formed on aerobic incubation of amygdalin *in vitro* with intestinal contents from CV rats. The most likely explanation of these findings is that microbial enzymes cleave the *β*-glycosidic bond to give the unstable aglycone, mandelonitrile, which is spontaneously converted to benzaldehyde and HCN (Carter *et al.,* 1980).

Elucidation of a more complicated involvement of microorganisms is exemplified by studies of the metabolism of the herbicide, propachlor (2-chloro-N-isopropylacetanilide) in GF and CV rats. In the CV rat propachlor was converted to methylsulphonylcontaining compounds, and these accounted for six out of eleven metabolites detected in the urine (Bakke and Price, 1979). In CV rats with cannulated bile ducts the only metabolites found in the bile were the glutathione and cysteine conjugates, the mercapturate and its sulphoxide. GF rats given propachlor excreted two-thirds of the dose in the urine as the mercapturate, one-fifth in the faeces as the cysteine conjugate plus a small amount of the mercapturate sulphoxide in both faeces and urine. Since all the propachlor was metabolized by the mercapturic pathway in the GF rat it was concluded that the transformations necessary to form methylsulphonyl-containing metabolites must be performed by the intestinal flora. A scheme for the metabolic fate of propachlor in the conventional animal was proposed in which the 'first pass' metabolites excreted in the bile are subjected to microbial activity, the products of which are reabsorbed. Experiments in which some of the intermediate metabolites were administered to

Table 3.1. Characteristics of germ-free (GF) animals in comparison with their conventional (CV) counterparts

Characteristic	*GF vs CV*	*Species*	*Reference*
General physiology			
Cardiac output	lower	rat	Gordon *et al.*, 1963
O_2 consumption	lower	rat, mouse	Wostmann *et al.*, 1968
Liver weight	less	rat, mouse, chick, pig	Gordon, 1959 Gordon and Cherian, 1979
Liver blood flow	lower	rat	Gordon, 1968
Bile flow	decreased	rat	Wilson *et al.*, 1985
Defence mechanisms			
Immune response	delayed, then prolonged	rat mouse	Many authors, see review by
Lymphatic organs	development retarded	chick pig	Bealmear *et al.*, 1984
Haematopoetic activity	reduced		
Water balance			
Water intake	higher	rat	Kellogg and Wostmann, 1969
Urine output	lower		
Faecal water	higher		
Total water excretion	higher		
Liver enzyme activity			
Glucose 6–P dehydrogenase	lower	rat	Reddy *et al.*, 1973
6–P gluconate dehydrogenase	lower		
Succinate dehydrogenase	lower		
ATP–citrate lyase	higher		
Fatty acid synthetase	higher		
α–glycerylphosphate dehydrogenase	lower	rat	Sewell and Wostmann, 1975
Glucuronyl transferase	lower	rat	Ward *et al.*, 1990a
Gastrointestinal tract			
Small intestine	lighter	rat chick	Gordon *et al.*, 1966 Coates and Jayne-Williams, 1966
Lamina propria	reduced	mouse rat, chick	Abrams, 1969 Gordon and Bruckner-Kardoss, 1961a
Villus shape	longer and more slender	mouse pig	Abrams *et al.*, 1963 Kenworthy, 1967
Villus: crypt cell	higher	mouse, rat, dog, chick, pig	Heneghan, 1979
Reticuloendothelial elements	less	chick mouse, rat, guinea pig rat	Gordon and Bruckner-Kardoss, 1961b Miyakawa, 1966 Meslin and Sacquet, 1979
Epithelial cell turnover	slower	mouse chick pig	Abrams *et al.*, 1963 Rolls *et al.*, 1978 Kenworthy, 1967

Transit time	slower	mouse	Abrams and Bishop, 1967
		rat	Gustafsson and Norman, 1969
Caecum	enlarged	guinea pig	Nuttall and Thierfelder 1896–7
		rat	Wostmann and Bruckner-Kardoss, 1959
		mouse, rabbit	Pleasants, 1968
Intestinal contents			
pH			
small intestine	higher }	rat	Ward and Coates, 1987b
large intestine	lower }	chick	Ford, 1974
Redox potential	more +ve	rat	Wostmann and Bruckner-Kardoss, 1966

bile-cannulated CV rats showed that at least two further cycles of the enterohepatic circulation, with microbial metabolism of the resulting biliary components, were necessary to account for the range of metabolites of propachlor in the conventional rat (Bakke *et al.,* 1980).

3.3.2
Secondary effects of the presence of the flora

Toxic effects may be the primary results of microbial action, as in the examples quoted above, or they may be secondary to more general effects of the flora on the host. For instance, nitrosamines are formed to a lesser extent in GF rats than in their CV counterparts (Ward *et al.,* 1986). A direct microbial effect could be inferred, since organisms from the rat intestine have been shown *in vitro* to catalyse the nitrosation reaction (Klubes *et al.,* 1972). However, nitrosation can occur nonenzymatically at low pH, so the more acid nature of the gut contents in conventional rats would be expected to enhance nitrosamine formation. It remains unclear which of these mechanisms is most important.

The presence of the flora may indirectly affect the disposition of a xenobiotic, whether or not it is subject to microbial metabolism. This possibility was explored in a study of the biliary excretion of amaranth, indocyanine green and nitrazepam by GF and CV rats (Wilson *et al.,* 1985). The first two substances are eliminated unchanged in the bile whereas nitrazepam is excreted in the bile almost entirely as its metabolites. Bile flow measured in cannulated rats was consistently lower in the GF groups and, with the exception of the female given amaranth, this was reflected in a lower rate of excretion of the xenobiotics. Liver weights were about 20% lower in the GF animals, which may have accounted for the reduced bile flow. There was no microbial involvement in the metabolism of nitrazepam, since the metabolites were similar in germ-free and conventional rats. So also was the rate of urinary excretion of a gavaged dose of ^{14}C-nitrazepam, but faecal excretion was much slower in GF animals. This must have been partly due to the reduced rate of biliary excretion but would also result from the longer transit time in GF animals. Thus although these comparisons revealed differences between the findings in GF and CV rats, none could be attributed to direct microbial action.

3.3.3 Protective effects of the gastrointestinal flora

Although the majority of studies of the effects of the gastrointestinal flora on xenobiotics have exposed actions that increase toxicity, the reverse possibility should not be ignored. Microorganisms are responsible for detoxifying certain endogenous compounds, e.g. the peptides and kallikreins involved in the caecal enlargement in germ-free rodents (see Section 3.2.2). The elimination of bilirubin from the body is facilitated by microorganisms. Bilirubin, a catabolite of haem, undergoes hepatic conjugation with a glucuronyl moiety and the water-soluble conjugates are excreted in the bile. Comparisons between GF and CV rats show that microbial enzymes deconjugate and reduce the bilirubin conjugates to a complex mixture of urobilins collectively termed urobilinogen (Gustafsson and Swenander-Lanke, 1960). Many intestinal organisms produce the deconjugating enzyme *β*-glucuronidase but so far only one, *Clostridium ramosum,* has been found capable of forming urobilinogen (Midtvedt and Gustafsson, 1981). GF rats associated with *Cl. ramosum* alone or in combination with a *peptostreptococcus* species produced less urobilinogen than corresponding CV rats. Thus optimal production apparently depends on the presence of yet other organisms, which may be required to contribute more enzymes or to establish a more favourable milieu for the process. It has been pointed out that bilirubin is an interesting endogenous substance that could provide a model for investigation of the uptake, intercellular transport and excretion of lipophilic xenobiotics, and that studies on microbial deconjugation of bilirubin might facilitate understanding of the intestinal metabolism of xenobiotics that are eliminated from the body by glucuronide formation (Saxerholt, 1989).

The protective effect of the flora may be indirect, as in the case of some food mutagens such as the heterocyclic amines that are formed in meat cooked at high temperatures. The compounds IQ (2-amino-3-methylimidazo (4, 5-f) quinoline) and its methyl derivative (MeIQ) require activation for their conversion to the substance directly responsible for their mutagenicity, and liver enzymes of the cytochrome P_{450} series constitute an activation system. When liver fractions from GF and CV rats were examined for their ability to activate MeIQ or PhIP (2-amino-1-methyl-6-phenylimidazo (4, 5-b) pyridine), significantly greater activation occurred with the GF liver fractions, implying that the presence of a flora exerts a depressing effect on the hepatic activation system. The depression could not be explained in terms of cytochrome P_{450} content, which was similar (per mg protein) to that of the CV fractions. Although glucuronyl transferase activity was significantly higher in the livers from CV rats, there were no differences in microsomal enzyme activities between liver fractions from the two sources. As the livers of CV rats are generally heavier than those of their GF counterparts it is likely that the total hepatic activity, and hence the capacity for mutagen activation, was greater in the CV animals. This seeming paradox has still to be resolved (Ward *et al.,* 1990b).

3.4 The human-flora-associated (HFA) rat

The relevance to man of findings in experimental animals is frequently called into question. The question is particularly germane to problems involving the gut flora, which is generally characteristic of each species. Marked differences have been recorded in the types and numbers of organisms inhabiting the gastrointestinal tract of the rat and man (Drasar, 1988). In order to obviate this species difference in flora, mice (Ghnassia *et al.,* 1975) and rats (Mallett *et al.,* 1987) born

GF and maintained in isolators have been associated with organisms from the human gastrointestinal tract. Though not strictly gnotobiotic they provide a model more comparable to man than their conventional counterparts.

Rats orally inoculated with a suspension of human faecal organisms were used to examine changes in the flora in response to diet. The activities of four bacterial transformation enzymes were measured in the caecal contents of the HFA and CV rats. The activities (calculated per 10^{10} cells) of β-glucosidase, β-glucuronidase, nitrate reductase and nitroreductase in the caecal contents of HFA rats were similar to those in the human faecal preparation, but nitrate reductase activity in the contents from CV rats was negligible. When the diet was supplemented with pectin the enzyme profile of the HFA rats and of human subjects remained unchanged but nitrate reductase activity was significantly increased in caecal contents from CV rats. This difference in enzyme response suggests that HFA rats would be a more satisfactory model than CV rats for lexicological studies relevant to man (Mallett *et al.,* 1987).

In studies of nitrosamine formation a reaction observed in conventional rats differed from that in human subjects. Inclusion of high amounts of fat in the diet depressed the amount of nitrosamines excreted (and, by implication, formed) by CV but not by GF rats (Ward and Coates, 1987a). Inhibition of microbial nitrate reductase appeared to be the most likely explanation. The inhibitory action was shared by all the fats tested, but butterfat had a far greater effect than any of several polyunsaturated fats. In a small trial with human subjects the depressing effect of high fat diets on nitrosamine excretion was again observed, but butterfat was not more effective than maize oil (Ward *et al.,* 1988). A comparison was then made between the effects of butterfat and maize oil on nitrosamine excretion by CV and HFA rats. As previously observed, butterfat caused the greater depression of nitrosamine excretion in the CV rats but in the HFA rats, as in the human subjects, there was no difference between the effects of the two fats (Ward *et al.,* 1990). The mechanism of the inhibition by dietary fats, and butterfat in particular, has not been resolved but these studies strengthen the suggestion that HFA rats may prove more reliable models for the human condition than the ordinary CV rat.

Although the major site of activation of IQ is the liver, it has been shown to be converted *in vitro* by organisms from the human gut flora to its 7-hydroxy-derivative (7-OHIQ), which is a direct-acting mutagen (Bashir *et al.,* 1987). Caecal contents of rats and mice were found to convert IQ to 7-OHIQ at a much faster rate than did human faecal samples. When rats associated with a human faecal flora were used to study the effects of different dietary components on the metabolism of IQ, the rates of metabolism of IQ were generally lower than those of rats with a CV flora, supporting the authors' claim that the use of HFA rats made the results more applicable to the human condition (Rumney *et al.,* 1993).

3.5
Comments and conclusions

Although the involvement of the gastrointestinal flora in the metabolism of xenobiotics has long been recognized (see, for example, review by Scheline, 1973) comparatively little attention has been directed at elucidating the mechanisms of that involvement. Anaerobic incubations *in vitro* can reveal the capabilities of intestinal organisms to metabolize foreign compounds, but they do not necessarily reflect the behaviour of the organism *in situ*. In the complicated milieu of the gastrointestinal tract the reactions observed *in vitro* may be seriously modified by the activities of

other components of the flora as well as by the host's secretions and digestive processes. The ultimate fate of a xenobiotic can be observed in the whole conventional animal but the steps leading to the final end-product, and the distinction between host and microbial influences, are difficult to disentangle.

The gnotobiote offers a model which helps to resolve these difficulties. Experiments in GF animals clearly show the part played by the host. Comparisons in their CV counterparts reveal the modifications brought about by the microflora. It must be remembered that these modifications may not result from direct microbial metabolism but can be brought about through more general effects of the flora on the host's physiology, for instance a faster rate of transit through the gut, increased bile flow, lower pH of the gut contents or alterations in activities of metabolic enzymes.

Where a direct metabolic effect of the gut microbes is indicated factors such as the site of action and absorbability of the product become important. If a metabolite is formed in the small intestine and enters the enterohepatic cycle the complicated succession of host and microbial activities can be investigated in experiments with bile-cannulated animals, as in the case of propachlor.

Most toxicological investigations are undertaken to find out the likely effects of drugs, food additives or other types of xenobiotic on human subjects. If gut microorganisms play a part in their metabolism or disposition misleading conclusions may be reached if ordinary laboratory animals are used. The gut microflora tends to be characteristic for each species, and the floras of laboratory rodents differ in types, numbers and sites of activity from that of man. The difficulty can be at least partly resolved by the use of rats or mice born GF and then associated with a flora of human origin. Differences in the major sites of microbial action are more difficult to overcome, and it may be necessary to prevent coprophagy in laboratory rodents to eliminate recycling of a test material or its metabolites. Experiments with gnotobiotes such as the examples quoted above have emphasized the important role of microorganisms in the metabolism of xenobiotics. Elucidation of the mechanisms involved is much more than an intriguing academic exercise. If the part played by microorganisms is understood, ways can be sought to control either the organisms or their metabolism to the advantage of the host.

Diet can be an important regulator of microbial populations in the gastrointestinal tract, and hence of the toxicity of foreign compounds (Mallett and Rowland, 1988). For instance a high fat diet inhibited production of nitrosamines (Ward and Coates, 1987a) but increased conversion of IQ to 7-OHIQ (Rumney *et al.,* 1993). Gnotobiotes and HFA animals are essential tools for studies of the interrelationships between diet, gut flora and toxicity.

Disturbances of the normal ecology of the gut, such as occur with prolonged antibiotic treatment or in certain diseases of the gastrointestinal tract, could conceivably influence the toxicity of xenobiotics or the efficacy of therapeutics. Better understanding of the metabolic role of components of the gut flora would enable the likelihood of such consequences to be assessed. Gnotobiotic technique demands patience and meticulous attention to detail, but it can uncover manifestations of microbial activities not readily recognized by other means.

References

Abrams, G.D., 1969, Effects of normal flora on host defences against microbial invasion, *Advances Exper. Med. Biol.,* **3,** 197–206.

Abrams, G.D. and Bishop, J.E., 1967, Effect of the normal microbial flora on gastrointestinal motility, *Proceedings of the Society for Experimental Biology and Medicine,* **126,** 301–4.

Abrams, G.D., Bauer, H. and Sprinz, H., 1963, Influence of the normal microbial flora on mucosal morphology and cellular renewal in the ileum, *Lab. Invest.,* **12,** 355–64.

Bakke, J.E. and Price, C.E., 1979, Metabolism of 2-chloro-N-propylacetanilide (propachlor) in the rat, *J. Environ. Sci. Health,* **14,** 427–41.

Bakke, J.E., Gustafsson, J.Å. and Gustafsson, B.E., 1980, Metabolism of propachlor by the germfree rat, *Science,* **210,** 433–5.

Bashir, M., Kingston, D.G.I., Carman, R.J., Van Tassell, R.L. and Williams, T.D., 1987, Anaerobic metabolism of 2-amino-3-methyl-3H-imidazo[4, 5-f]quinoline (IQ) by human fecal flora, *Mut. Res.,* **190,** 187–90.

Bealmear, P.M., Holtermann, O.Q. and Mirand, E.A., 1984, Influence of the microflora on the immune response, in Coates, M.E. and Gustafsson, B.E. (Eds) *The Germ-free Animal in Biomedical Research,* pp. 335–84, London: Laboratory Animals Ltd.

Carter, J.H., McLafferty, M.A. and Goldman, P., 1980, Role of the gastrointestinal microflora in amygdalin (laetrile)-induced cyanide toxicity, *Biochem. Pharmacol.,* **29,** 301–4.

Coates, M.E. and Gustafsson, B.E., 1984, *The Germ-free Animal in Biomedical Research,* London: Laboratory Animals Ltd.

Coates, M.E. and Jayne-Williams, D.J., 1966, Current views on the role of the gut flora in nutrition of the chicken, in Horton-Smith, C. and Amoroso, E.C. (Eds) *Physiology of the Domestic Fowl,* pp. 181–8, Edinburgh: Oliver and Boyd.

Drasar, B.S., 1988, The bacterial flora of the intestine, in Rowland, I.R. (Ed.) *Role of the Gut Flora in Toxicity and Cancer,* pp. 23–38, London: Academic Press.

Ford, D.J., 1974, The effect of the microflora on gastrointestinal pH in the chick, *Brit. Poultry Sci.,* **15,** 131–40.

Ghnassia, J.C., Veron, M., Ducluzeau, R., Muller, M.C. and Raibaud, P., 1975, Cinétique d'établissement d'une flore fécale humaine chez des souris axènique et essai de décontamination par antibiothérapie, *Annales de Microbiologie (Institute Pasteur),* **126B,** 367–79.

Gordon, H.A., 1959, Morphological and physiological characterization of germ-free life, *Annals New York Acad. Sci.,* **78,** 208–20.

Gordon, H.A., 1968, Is the germ-free animal normal? A review of its anomalies in young and old age, in Coates, M.E. (Ed.) *The Germ-free Animal in Research,* pp. 127–50, London: Academic Press Ltd.

Gordon, H.A. and Bruckner-Kardoss, E., 1961a, Effect of normal microbial flora on intestinal surface *area, Am. J. Physiol.,* **201,** 175–8.

Gordon, H.A. and Bruckner-Kardoss, E., 1961b, Effects of the normal flora on various tissue elements of the small intestine, *Acta Anatomica,* **44,** 210–25.

Gordon, H.A. and Cherian, S., 1979, Does the intestinal flora influence the growth of the liver lobule? *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene,* 7 (Suppl.), 151–2.

Gordon, H.A., Wostmann, B.S. and Bruckner-Kardoss, E., 1963, Effects of microbial flora on cardiac output and other elements of blood circulation, *Proceedings of the Society for Experimental Biology and Medicine,* **114,** 301–4.

Gordon, H.A., Bruckner-Kardoss, E., Staley, T.E., Wagner, M. and Wostmann, B.S., 1966, Characteristics of the germ-free rat, *Acta Anatomica,* **64,** 367–89.

Gustafsson, B.E. and Swenander-Lanke, L., 1960, Bilirubin and urobilins in germ-free, ex-germfree and conventional rats, *J. Exper. Med.,* **112,** 975–81.

Gustafsson, B.E. and Norman, A., 1969, Influence of the diet on the turnover of bile acids in germ-free and conventional rats, *British Journal of Nutrition,* **23,** 429–442.

Heneghan, J.B., 1979, Enterocyte kinetics, mucosal surface area and mucus in gnotobiotes, in Fliedner, T., Heit, H., Neithammer, D. and Pflieger, H. (Eds) *Clinical and Experimental Gnotobiotics,* pp. 19–27, Stuttgart: Gustav Fischer Verlag.

Kellogg, T.F. and Wostmann, B.S., 1969, Stock diet for colony production of germ-free rats and mice, *Lab. Animal Care,* **19,** 812–14.

Kenworthy, R., 1967, Influence of bacteria on absorption from the small intestine, *Proceedings of the Nutrition Society,* **26,** 18–23.

Klubes, P., Cerna, I., Rabinowitz, A.D. and Jondorf, W.R., 1972, Factors affecting dimethylnitrosamine formation from simple precursors by rat intestinal bacteria, *Food Cosmet. Toxicol.,* **10,** 757–67.

Laqueur, G.L. and Spatz, M., 1975, Oncogenicity of cycasin and methylazoxymethanol, *GANN,* **17,** 189–204.

Mallett, A.K. and Rowland, I.R., 1988, Factors affecting the gut microflora, in Rowland, I.R. (Ed.) *Role of the Flora in Toxicity and Cancer,* pp. 347–82, London: Academic Press.

Mallett, A.K., Bearne, C.A., Rowland, I.R., Farthing, M.T.G., Cole, C.B. and Fuller, R., 1987, The use of rats associated with a human faecal flora as a model for studying the effects of diet on the human gut microflora, *J. Appl. Bacteriol.,* **63,** 39–45.

Meslin, J.C. and Sacquet, E., 1979, Effets de la flore microbienne sur la morphologie et le rénouvellement de l'epithelium intestinal. Comparison entre le rat axénique et le rat holoxénique, *Med. Nutr.,* **15,** 49–53.

Midtvedt, T. and Gustafsson, B.E., 1981, Microbial conversion of bilirubin to urobilins *in vitro* and *in vivo, Acta Path. Microb. Scandinavica, Section B,* **89,** 57–60.

Miyakawa, M., 1966, The morphological characteristics of the germ-free mammals, including the results of recent studies on the adrenal in the germ-free rats at the Department of Pathology, Nagoya University, *Proceedings of the IXth International Congress of Microbiology.,* pp. 291–8, Moscow.

Nuttall, G.H.F. and Thierfelder, H., 1896–7, Thierisches Leben ohne Bakterien im Lebenskanal, *Hoppe Seyler's Zeitschrift für Physiologische Chemie,* **33,** 62–73.

Pleasants, J.R., 1968, Characteristics of the germ-free animal, in Coates, M.E. (Ed.) *The Germfree Animal in Research,* pp. 13–25, London: Academic Press

Reddy, B.S., Pleasants, J.R. and Wostmann, B.S., 1973, Metabolic enzymes in liver and kidney of the germ-free rat, *Biochim. Biophysica Acta,* **320,** 1–8.

Rolls, B.A., Turvey, A. and Coates, M.E., 1978, The influence of the gut microflora and of dietary fibre on epithelial cell migration in the chick intestine, *Brit. J. Nutr.,* **39,** 91–9.

Rumney, C.J., Rowland, I.R. and O'Neill, I.K., 1993, Conversion of IQ to 7-OHIQ by gut microflora, *Nutr. Cancer,* **19,** 67–76.

Saxerholt, H., 1989, The normal microflora and bilirubin, in Grubb, R., Midtvedt, T. and Norin, E. (Eds) *The Regulatory and Protective Role of the Normal Microflora,* pp. 169–99, Basingstoke and London: The Macmillan Press.

Scheline, R.R., 1973, Metabolism of foreign compounds by gastrointestinal microorganisms, *Pharmacol. Rev.,* **25,** 451–532.

Sewell, D.L. and Wostmann, B.S., 1975, Thyroid function and related hepatic enzymes in the germfree rat, *Metab.,* **24,** 695–701.

Spatz, M., Smith, D.W.E., McDaniel, E.G. and Laqueur, G.L., 1967, Role of intestinal microorganisms in determining cycasin toxicity, *Proceedings of the Society for Experimental Biology and Medicine,* **124,** 691–97.

Ward, F.W. and Coates, M.E., 1987a, Dietary fat and N-nitrosation in the rat, *Brit. J. Nutr.,* **58,** 221–31.

Ward, F.W. and Coates, M.E., 1987b, Gastrointestinal pH measurement in rats: influence of the microbial flora, diet and fasting, *Lab. Animals,* **21,** 216–22.

Ward, F.W., Coates, M.E. and Walker, R., 1986, Nitrate reduction, gastrointestinal pH and N-nitrosation in gnotobiotic and conventional rats, *Food Chem. Toxicol,* **24,** 17–22.

Ward, F.W., Coates, M.E., MacDonald, I. and Sims, A., 1988, The effect of dietary fats on N-nitrosamine formation in man, *Proceedings of the Nutrition Society,* **47,** 21A.

Ward, F.W., Coates, M.E., Cole, C.B. and Fuller, R., 1990a, Effect of dietary fats on endogenous formation of N-nitrosamines from nitrate in germ-free and conventional rats and rats harbouring a human flora, *Food Additives Contamin.,* **77,** 597–604.

Ward, F.W., Mallett, A.K., Rowland, I.R. and Coates, M.E., 1990b, Mutagen activation and foreign compound metabolism by hepatic fractions from germ-free and conventional rats, *Biochem. Pharmacol. (Life Science Advances)*, **9,** 135–43.

Wilson, S.A., Tavendale, R. and Hewick, D.A., 1985, The biliary elimination of amaranth, indocyanine green and nitrazepam in germ-free rats, *Biochem. Pharmacol.,* **34,** 857–63.

Wostmann, B.A. and Bruckner-Kardoss, E., 1959, Development of cecal distension in germ-free baby rats, *Am. J. Physiol.,* **197,** 1345–6.

Wostmann, B.S. and Bruckner-Kardoss, E., 1966, Oxidation-reduction potentials in cecal contents of germ-free and conventional rats, *Proceedings of the Society for Experimental Biology and Medicine.,* **121,** 1111–14.

Wostmann, B.S., Bruckner-Kardoss, E. and Knight, P.L., 1968, Cecal enlargement, cardiac output and O_2 consumption in germ-free rats, *Proceedings of the Society for Experimental Biology and Medicine,* **128,** 137–41.

Section 2

Nitrogen metabolism

Chapter 4
Intestinal flora in the conservation of body nitrogen

A.A.Jackson and B.J.Moran

4.1
Introduction

All life is characterized by turnover and change. Nutrition is an integral part of this process and as a matter of course all living organisms take energy and nutrients from the environment. In humans this represents the normal dietary intake. The chemical reactions through which the dietary constituents exchange with constituents of the body are controlled to ensure that the metabolic function is maintained. As a general principle, the body adapts to conserve those components of limited availability in the environment. By and large, important mechanisms exist to ensure an adequate supply of protein, amino acids and nitrogen (Schoenheimer, 1942; Waterlow, 1968; Jackson, 1993). The chemical interchange is continuous, and ultimately the end-products pass out of the body as gases, solutes or solid material. The characterization of nutritional state is a formal expression of the extent to which diet has enabled normal function to be maintained. There are a number of different ways in which the nutritional state of an individual can be assessed and characterized. As our understanding develops the subtlety of the characterization increases in sophistication.

The shape, size and relative proportions of the body are expressions of body composition. A model comprising levels of body composition of increasing complexity has been proposed as an index of nutritional state, from the atomic, through the molecular to the cellular, the cells themselves being organized into tissue systems which are functionally integrated within the whole body (Wang *et al.*, 1992). Each level of interaction represents a statement about nutritional state and function and these underlie all attempts at the non-invasive assessment of nutritional state. At the atomic level nitrogen has no known function within mammals, but at the molecular level it is especially associated with amino acids and protein. Protein is the major constituent of lean tissue which, as the body cell mass, represents the major functionally active compartment of the body (Moore *et al.*, 1963).

The flow of material through the body is assessed by determining the dietary intake, or by using this information to determine the overall balance of energy or nutrients within the body. Stable isotopes, which can be used to probe metabolism non-invasively, have been available as reagents for over 50 years (Schoenheimer, 1942). The more recent technical and analytical advances have made these methods more accessible on a wider basis. With these approaches it is possible to determine the relative contribution of the dietary intake to the total extent of the body's metabolic activity, and

to follow the specific fate of individual items of the diet (Young and Marchini, 1990). Because the probes are atomic by nature, it is also possible to obtain information on interactions which take place at the molecular and cellular levels as well as to trace the flow of material between tissues and organs.

4.1.1
Nitrogen as an element essential for life

Nitrogen is the most abundant element in the atmosphere and the processes through which nitrogen is fixed in an organic form which is of metabolic use are of fundamental interest. The only known reactions through which fixation can take place are bacterial or algal in origin. At one time it was considered that the fixation of gaseous nitrogen might represent one important mechanism through which individuals could cope with a diet low in protein (Oomen, 1970). No evidence was found to support the suggestion of nitrogen fixation by the colonic bacteria in humans, but the problem is outstanding and its importance underlies the general issue under consideration, about which we are still not sufficiently clear.

The chemical nature of the organic nitrogenous materials in the body was first investigated by Mulder (1802–1880), who gave the name 'proteins' to these compounds (Munro and Allison, 1964). Chemical analysis of the body demonstrates that by weight about 85% is comprised of lean tissue, of which around 20% is protein. The proteins are a series of macromolecules which fulfil a range of functions. However, it was not until 1900, when new methods of chemical analysis became available, that amino acids were identified as the structural components of proteins, and the characteristic feature of individual proteins is the nature and pattern of the constituent amino acids which have nitrogen as an integral part of their structure. This discovery paved the way to modern concepts of protein metabolism. Proteins in the diet and in the body itself are of complex and varied composition and, moreover, represent the very nature of life (Cathcart, 1921). In quantitative terms they are clearly the most abundant form in which amino acids are found, comprising the constantly turning over structural elements of the body as well as forming the machinery that carries out this inexorable process of construction and destruction (Schoenheimer, 1942). However, there are also a range of other compounds derived in smaller amounts from amino acids, which perform a range of vital functions, e.g. nucleic acids, creatine, carnitine, vitamins, neurotransmitters.

The absolute requirement for nitrogen as a constituent of the diet was recognized more than a century ago and has been reviewed by Munro and Allison (1964). Magendie (1783–1855) conducted the first long-term feeding experiments in which dogs fed nitrogen-free diets eventually died, whereas those fed nitrogenous foods survived indefinitely. These observations led to the widely accepted conclusion that the nitrogenous constituents of the body are derived from the proteins and amino acids in food and are essential components of the dietary intake (Cathcart, 1921). It is now well established that some protein is an essential constituent of the human diet, although the extent to which individual amino acids or other nitrogenous compounds can effectively replace some or all of the dietary protein (Kies, 1972) is less clear.

There are about twenty amino acids which are commonly found as the constituents of proteins and it has long been recognized that eight of these need to be taken preformed in the diet by all animals for health to be maintained (Munro and Allison, 1964). These have been classed as 'essential' or 'indispensable' (Rose, 1957), and there has been the tendency to assume that these amino acids cannot be formed at all in the body. This assumption is not strictly correct. Those

amino acids which can be formed in the body, and therefore do not have to be provided preformed in the diet have been classified as 'non-essential' or 'dispensable' (Rose, 1957). The tendency has been to consider these as being of less importance, nutritionally (Jackson, 1993). It is now increasingly clear that the capacity to form these amino acids may not be infinite, and under appropriate circumstances the metabolic demand for the amino acids is not satisfied by endogenous formation. In this situation there is the need to provide the amino acid in the diet, making it 'conditionally essential' (Jackson, 1983; Laidlaw and Kopple, 1987).

At the present time there is an active debate about the minimal dietary requirements for the indispensable amino acids (Millward, 1992; Millward *et al.,* 1989; Young and Pellet, 1990; Young, 1992), and the resolution of. this debate hinges upon our appreciation of the metabolic fate of nitrogen within the body and the ability to utilize nitrogen and amino acids usefully within the system (Millward and Rivers, 1988). The fundamental concept which we would challenge, is the idea that the most important *metabolic* (as opposed to dietary) element is the indispensable amino acids. If the indispensable amino acids are essential for life, and if the pathways for their formation in the body have been lost, then in Darwinian terms, it must be expected that under most normal circumstances they are found in sufficient or even abundant amounts in the diet. On the other hand, the preservation of the pathways for the formation of the dispensable amino acids might be taken to imply, either that these amino acids perform a critical function which has to be protected, or that the amounts required usually exceed the amounts normally found in the diet, or both (Carpenter, 1992; Jackson, 1992a). Diets made up solely of the indispensable amino acids are not efficient in supporting health and growth. The addition of nitrogen in the form of ammonium salts, urea or as dispensable amino acids enhances the quality of the diet substantially (Kies, 1972; Millward and Rivers, 1988). The evidence is that there is an essential requirement for 'dispensable' nitrogen in the diet. Here we are interested to explore some of the ways in which this nitrogen might be made available to the system in a functionally useful form.

4.2
Nitrogen balance, amino acid flux and protein turnover

The nutritional assessment of protein and nitrogen status of an individual has been determined classically by the assessment of nitrogen balance (Munro and Allison, 1964; FAO/WHO/UNU, 1985) and the relative proportion of the body which comprises protein containing lean tissue through the assessment of body composition (Moore *et al.*, 1963). Both of these criteria are important and necessary, but they fail to take adequate note of the dynamic aspects of the internal exchange of nitrogen, amino acids and protein. When protein- or nitrogen-containing compounds are omitted from the diet there are continuing losses of nitrogen in urine, stool, sloughed skin, and hair, identified as the obligatory nitrogen loss. To maintain the *status quo* a daily intake is required to at least offset these losses, and the minimum intake upon which balance can be achieved represents the minimum physiological requirement. In practice the minimum intake required to achieve nitrogen balance exceeds the obligatory nitrogen losses on a protein-free diet. In a steady state, body composition is constant and intake must be equivalent to losses. Positive balance is required for laying down of body tissues as in normal growth, pregnancy or recovery from injury or illness: intake must exceed losses if health is to be maintained or restored. For many years the assessment of protein adequacy in diets and the assessment of protein quality has been based on nitrogen balance techniques (FAO/WHO/UNU, 1985). The intake of protein, amino acids or nitrogen can be

measured by determining the composition of foods and the measurement of food intake. Seventy-five to ninety-five per cent of nitrogen losses are in the urine and stools, with stool nitrogen normally accounting for 9–12% of the total (McGilverry, 1970). Thus the majority of nitrogen lost from the body is urinary nitrogen, and of the urinary nitrogen approximately 80% is in the form of urea (Allison and Bird, 1964). Urea is the major excretory end-product of nitrogen from protein metabolism and is produced almost exclusively in the liver (Bollman *et al.,* 1926). The ability of the body to accommodate a range of dietary intakes without apparent adverse effects implies complex response mechanisms which appear to be associated with the formation and excretion of urea. This response has been characterized as an adaptive response (Waterlow, 1968). Thus the nitrogen derived from an intake of protein in excess of the requirements is excreted as urinary urea (Allison and Bird, 1964).

The system itself is dynamic, and therefore a model which identifies and characterizes external balance makes presumptions about a process of internal exchange which enables the external balance to be achieved. Proteins taken in the diet are presented to the system as amino acids, through the process of digestion and absorption. All metabolic exchange is considered to take place through a metabolic pool of amino acids (Figure 4.1) (Schoenheimer, 1942; Jackson, 1993). The amino acids have one of two potential major fates: either they can be used as the building-blocks for the formation of more complex molecules which eventually function as metabolically active compounds (in quantitative terms, primarily proteins), or they can be catabolized with the carbon skeleton contributing to the energy needs of the body and the amino group giving rise to an end-product of metabolism (in quantitative terms, primarily urea). Both options are followed on a continuous basis and the factors which exert influence and control over these fundamental processes are of intrinsic interest (Waterlow, 1984).

In the adult, under conditions of normal health, overall balance is maintained, which means that the intake of nitrogen in protein must be equivalent to the losses of nitrogen in all forms, and hence equivalent amounts of amino acids are oxidized as are taken in the diet. The overall process is led by the demand for protein synthesis, whose magnitude at any point in time is determined by a range of factors including the genetic profile of an individual, his or her nutritional state and recent nutritional intake, and hormonal influences. There are additional factors which come into effect with the normal physiological changes associated with growth in pregnancy or childhood or during the response to pathological insults such as trauma and infection.

The intensity of protein turnover in adults is at least 5 times as great as the protein intake, and in infancy might be 10–15 times the protein intake (Waterlow, 1984; Jackson and Forrester, 1994). The processes associated with protein turnover are energetically expensive and may consume as much as 30–50% of resting energy expenditure (Jackson, 1986). In the normal course of events amino acids contribute to satisfying the requirements of the body for energy. In adults the oxidation of amino acids accounts for about 15–20% of the energy required. In the fetal lamb, by comparison, at least 40% of energy is derived from the oxidation of amino acids, and in situations of maternal nutrient deprivation this may rise to 60% of the total energy consumption of the fetus. It is not clear why there should be such a considerable dependency in early life upon amino acid oxidation; apparently an inefficient use of amino acids (Jackson, 1994).

There is a stoichiometric match between amino acid oxidation and urea formation. In the unstressed fetal lamb, urea formation is around 12–15 mgN/kg/h, increasing to 40 mgN/kg/h with maternal nutrient deprivation (Owens *et al.,* 1989). In the human neonate protein oxidation, based on the measurement of leucine kinetics, is about 9.3 mgN/kg/h (Denne and Kalhan, 1991), similar to

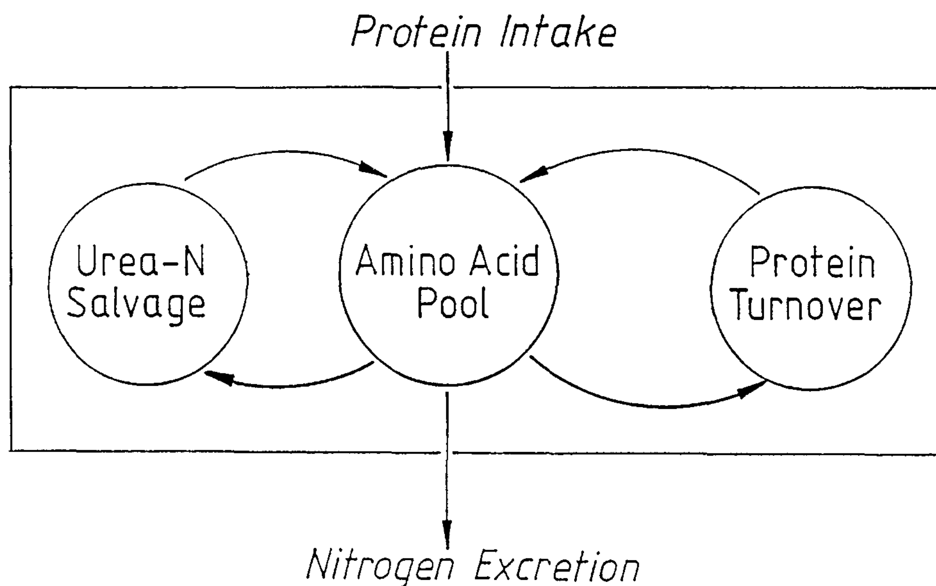


Figure 4.1. A model for assessing the nitrogen-amino acid-protein requirements of the diet has to take account of external balance, the relationship between protein intake and nitrogen excretion, and also has to embrace a consideration of the two major internal cycles: the movement of amino acids into and from proteins, and the salvage of urea-nitrogen through the metabolic activity of the colonic microflora (Jackson, 1993)

direct measures of urea formation at 10 mgN/kg/h on an established dietary intake (Wheeler *et al.*, 1991). Both are similar to the unstressed fetal lamb, which implies a similar rate of utilization of amino acids as a source of energy in the newborn as in the fetus. This suggests that the oxidation of amino acid as a source of energy is a major drive for urea formation. Whereas in the fetus the large amounts of urea produced can be handled metabolically, simply by the product moving across the placenta to the maternal circulation, in the newborn the renal excretion of such large quantities of urea would require a free water clearance of considerable magnitude. Therefore, if the renal capacity is not to be overwhelmed, there is the need for a substantial portion of the urea nitrogen to be handled safely. Under usual circumstances, colonic salvage is important for the handling of urea-nitrogen, with the nitrogen being returned to the metabolic pool (Jackson, 1994). The system requires the establishment of a normal colonic flora. In fasted, young neonates without an established dietary intake urea appearance, 5.3 mgN/kg/h (Kalhan, 1994), was similar to that in neonates on total parenteral nutrition who had never had a full dietary intake, 3.5 mgN/kg/h (Wheeler *et al.*, 1993a).

The rate at which urea nitrogen is salvaged is determined by the dietary intake of protein in relation to the metabolic demand for protein synthesis and net protein accretion. For a fixed demand, salvage increases as intake falls. For a fixed intake, salvage increases as the demand increases. Therefore salvage is enhanced when the metabolic demand increases for either physiological (e.g. in pregnancy or during childhood) or pathological reasons (e.g. following surgery, in hypersplenism in sickle cell disease or during catch-up growth) (Jackson and Wootton, 1990; Jackson, 1993).

There has been considerable confusion in the literature, primarily because there has been an assumption, which is readily made, that the rate at which urea is excreted in the urine is similar to the rate at which urea is produced in the body. We consider that the evidence does not support this position, and indeed demonstrates that there might be a considerable difference in the rates of production and excretion depending upon the overall metabolic state of the host. Indeed, the ability to produce urea which is salvaged rather than excreted represents the other major internal cycle of nitrogen exchange within the body. In humans adaptation to different levels of protein intake can be achieved over a wide, but defined range of intakes. In animal studies the diets used have often been more extreme, beyond the range of normal adaptation. The response to extreme diets, therefore, is to an excess or deficiency, rather than an indication of adaptation within the more limited range of protein intakes characteristic of adequate diets which are taken habitually in good health.

Animal experiments and biochemical measurements have shown short- and long-term control, both related to the availability of substrate. From animal experiments it has been concluded that the activity of the urea cycle enzymes is determined by the dietary intake (Meijer *et al.,* 1990). In biochemical studies it can be shown that the rate at which urea is synthesized in the liver can be influenced by the availability of nitrogenous substrate, as amino acids or as ammonia. The activity of the enzymes of the urea cycle is determined by the preceding dietary intake. In rats, on moving from a higher to a lower protein intake the rate of urea synthesis is reduced with a fall in both the activity and the amount of the urea cycle enzymes (Das and Waterlow, 1974). The demonstration of similar enzyme changes in liver biopsy specimens taken from children who had died of severe malnutrition have given rise to the presumption that the response is general, and that there is a dose-response relationship across a range of intakes (Stephen and Waterlow, 1968). These authors' findings, along with physiological studies, have given rise to the idea that adaptation to a low protein intake is primarily determined by the rate of synthesis and the pattern of excretion of urea (Waterlow, 1968). Implicit to this perception is the idea that the requirement for protein is a direct reflection of the fate of individual amino acids, either to synthetic pathways or to degradation by oxidation and urea synthesis. Recent data from human studies have caused us to review this position critically and have led us to consider that there are two major loci of control, at the level of production and at the level of salvage of urea nitrogen, and that salvage may be of considerable significance at levels of dietary protein which are habitually consumed (Jackson, 1992b).

4.3 *Urea hydrolysis*

For nearly 50 years there has been evidence that urea produced in the body is not excreted quantitatively in the urine. Despite the assertion by Bloch in 1946 that urea was effectively an end-product of metabolism, evidence has been forthcoming from studies in the mouse (Leifer *et al.,* 1948; Dintzis and Hastings, 1953), cat (Kornberg and Davies, 1952), rat (Chao and Tarver, 1953), pig (Liu *et al.,* 1955), pony (Prior *et al.,* 1974) and human (Walser and Bodenlos, 1959) that significant amounts of urea might be salvaged within the body. Evidence from germ-free rats and animals treated with antibiotics demonstrates unequivocally that the process of urea hydrolysis and salvage is exclusively a function of the gastrointestinal microflora (Dintzis and Hastings, 1953; Levenson *et al.,* 1959). The ability of *Helicobacter pylori* to hydrolyze urea has been used as the basis of a diagnostic test for upper gastrointestinal infection with this organism, but this represents a special case of a more general phenomenon (Graham *et al.,* 1987). The hydrolysis of urea in normal health

is essentially a function of the colonic microflora, although it is difficult to exclude absolutely any contribution from lower ileal organisms. Although it has been accepted for most of the century that a symbiotic relationship exists between the gastrointestinal flora and the health and well-being of the host in ruminants, the tendency has been to consider that for non-ruminants, the interaction with the colonic microflora is of little or no metabolic significance. The basis of the unwillingness to consider the interaction of the host and their microflora of relevance to nitrogen or amino acid metabolism has centred upon three objections:

1. that the colon is relatively impermeable to urea and other materials (amino acids);
2. that the fate of urea nitrogen cannot be determined with isotopic probes, and the results of experiments of this kind might simply reflect isotopic exchange reactions of no metabolic significance rather than being in any way representative of substrate flow;
3. that it is unlikely or impossible for amino acids, either indispensable or dispensable, to be made in functionally significant amounts by the colonic microflora and made metabolically available to the host.

The demonstration of the responsiveness of urinary urea to the dietary protein intake, on the basis of limited animal data, justified the position for many years that urea was simply an end-product of protein metabolism in man. It was concluded that a reduction in urea excretion, on a reduced protein intake, merely represented decreased urea production. However, Walser and Bodenlos (1959), using a stable isotope technique, demonstrated that 15–30% of urea produced was not excreted in the urine. They were able to show that the urea not excreted was hydrolyzed as a bacterial function, because urea hydrolysis could be virtually abolished by antibiotics (oral neomycin). The method used for measuring urea kinetics was to follow the pattern of excretion of label following a single dose by mathematically stripping the decay curve. This method is technically difficult and a tedious approach to arrive at a value for production which makes assumptions which cannot always be justified. Because of the insensitivity of the method and difficulties in obtaining reliable results only a few studies have been carried out (Long *et al.,* 1978). Nevertheless, in general the data have been confirmed and similar quantitative findings have been obtained by several investigators (Jones *et al.,* 1969; Long *et al.,* 1978; Jackson *et al.,* 1984; Hibbert and Jackson, 1991). In 1972, Picou and Phillips introduced a non-invasive method, in which a stochastic model was used. By adopting a model based upon a steady isotopic state, it was easier in principle to carry out the study and to analyze the results. Picou and Phillips carried out a series of studies in children with severe malnutrition on two different levels of protein intake before and after recovery. At the time of the study all the children were on very generous intakes of energy: a point not made in the paper but of great importance in the interpretation of the data (Jackson and Wootton, 1990). Picou and Phillips (1972) found little difference in urea kinetics between the malnourished and recovered children. Children on low protein diets had very low rates of urea production with a high proportion of the urea produced being salvaged (about 66%); hence in situations where the nitrogen intake was insufficient to meet the metabolic demand (in malnourished children) a greater proportion of the urea production was hydrolyzed and a large proportion salvaged and retained. The detailed nature of the conditions under which urea nitrogen is effectively salvaged may be critically dependent upon the energy intake, the protein intake, the rate of weight gain and the pattern of tissue being deposited at the time of the study (Jackson, 1986; Jackson *et al.,* 1990; Jackson and Wootton, 1990). A modification of the method of Picou and Phillips (1972) has been

developed and applied in a large number of individual studies (Jackson, 1993). More recently we have validated a simpler non-invasive method, based upon the cumulative excretion of isotope in urine following a single oral dose of label (Jackson *et al.*, 1993). This method holds promise for application under field conditions to free-living human populations.

The data from all the studies confirm that anything from 10% to 90% of the urea produced might be hydrolyzed and salvaged under appropriate conditions (Jackson, 1993). Our interest has been to identify the mechanisms which exert control over the process which, as a bacterial function, is beyond standard ideas about physiological control, and to determine the fate and functional significance of the nitrogen salvaged.

4.4
Role of microflora

The controversy over the extent to which hydrolyzed urea is metabolically available to the host hinges on our perceptions of the potential ways in which the microflora might handle available nitrogen. Clearly the microflora require a source of nitrogen for their own metabolic function, and the source of the nitrogen has never been formally identified. It is generally considered that the microflora are likely to reduce nitrogen presented in any form down to ammonia, which is then used as the building block for the bacteria's own metabolic synthetic processes. Although, in principle, urea should form as effective a source of nitrogen as any other potential nitrogen donor, the prejudice has been that the colonic flora might hydrolyze urea to ammonia, but the ammonia so formed is absorbed and passes to the liver. In the liver it has a number of possible fates, to be fixed, either by resynthesis to urea or as amino acids (in practice either glutamate or glycine/serine). Experience with *Helicobacter pylori* has served to reinforce this impression as oral urea is rapidly hydrolyzed and the ammonia appears to return preferentially to urea formation (Hibbert *et al.*, 1992). Studies *in vitro* indicate that the ammonia-nitrogen derived from urea hydrolysis by the *Helicobacter pylori* is unlikely to be recovered from the organism in mixed protein (Hawtin, personal communication). However, these observations fit well with the general perception that organisms which hydrolyze urea are not likely to fix the ammonia generated as amino acids.

One other contentious issue has been the conflicting findings regarding the site of urea hydrolysis. In early studies, there were suggestions of a mucosal urease, unassociated with any bacterial activity (Kornberg and Davies, 1955; Aoyagi *et al.*, 1966), but it is difficult to rule out the presence of deep mucosal organisms, or preparations which were poor for other reasons. It is almost certain that in the human the hydrolysis of urea is exclusively by bacterial urease. In health, with the exception of the skin and vaginal flora, substantial numbers of bacteria are associated with the gastrointestinal tract, and 99% of the bacteria are confined to the colon (Gorbach, 1971; Gustafsson, 1982). The identification of organisms which are specific for urea hydrolysis, and which appear to increase in numbers in a host with uremia due to renal disease (Brown *et al.*, 1971) argue in favour of an important functional role for the host's microflora. We had the opportunity to follow the establishment of the system in one child who was born without a colon (Wheeler *et al.*, 1993b). Early in life the child required the creation of an ileostomy. Sequential studies of urea kinetics were carried out as the ileostomy gradually came to take on the role of a functional colon. Whereas initially, at 15 days of age virtually all (95%) of the urea produced was excreted, by 23 days of age 79% of the urea produced was salvaged. It may be coincidental but the infant only went into positive nitrogen balance and started to gain weight as the salvage system was established. Infants in

whom urea kinetics are measured during the first week of life, before a colonic flora is well established, or who have been on total parenteral nutrition all their life, show evidence of a limited ability to salvage urea effectively (Kalhan, 1994; Wheeler *et al.,* 1993a). In adults, from whom the colon has been removed, the lower ileum appears to function as an effective colon as it becomes colonized, and may salvage significant amounts of urea nitrogen (Gibson *et al.,* 1976a).

Silen *et al.,* (1955) demonstrated that blood draining the colon has a much higher concentration of ammonia than blood from other segments of the intestine. They found that the ammonia concentration in blood draining the colon was 10 times that in the inferior vena cava and that colonic venous concentration could be reduced by 65% by oral antibiotics. This suggests that the colonic microflora is involved in the production of ammonia, and as a result antibiotics and colonic purgation have been key areas in the management of hepatic encephalopathy right up to the present day.

4.5
Colonic nitrogen metabolism

The idea that substantial amounts of urea might be hydrolyzed by the colonic contents sits uneasily with the idea that the colon is impermeable to urea. Hence, controversy continues as to the source of this substantial amount of colonic nitrogen. Indeed, isotopic data suggest that the movement of all nitrogen-containing compounds through the colon might be very much greater than can be identified from measurements of faecal losses and external balance (Jackson *et al.,* 1984; Wrong *et al.,* 1985). The suggestion is of a pool through which the flux is considerable and for which faecal losses represent only a small proportion. No doubt contributions to the pool may be derived from a variety of sources and constitute material from dietary residue: endogenous losses through the ileocaecal valve (digestive enzymes, mucus and sloughed cells), and colonic-derived endogenous material either as protein or non-protein material. Wrong *et al.* (1985) found that intravenously administered urea which passes into the colon might well be diluted by a factor of five, based upon analysis of faecal nitrogen enrichment. These values are of the same order of magnitude as those derived by Jackson *et al.* (1984), who found that labelled urea nitrogen appeared to trace the movement of nitrogen through a pool associated with the bowel, of the order of 16 g nitrogen per day (Jackson *et al.,* 1984; Jackson *et al.,* 1987). The approximate amounts of urea nitrogen movement through the colon, in relation to the intake of amino acids and the production and excretion of urea nitrogen, are outlined in Figure 4.2.

The data of necessity imply that either nitrogen enters the colon through the ileocaecal valve or it passes through the wall of the colon in some form. Gibson *et al.* (1976a) found that the nitrogen output in an ileostomy was less than 2 g of nitrogen per day. They estimated, from ileal urea concentrations, that only 4%, at most, was urea with the rest being dietary residue. They noted that ileal effluent contained more nitrogen than normal stools and that this was exaggerated by a higher protein diet. This suggests that net nitrogen absorption takes place in the colon. Other investigators have confirmed that insufficient nitrogen to account for colonic ammonia production entered from the ileum both in ileostomy studies (Kanaghinis *et al.,* 1963) and by *in vivo* direct sampling of ileal effluent (Chadwick *et al.,* 1977). Summerskill and Wolpert (1970) estimated that 73% (3,080 mg per day) of the daily ammonia production in the gastrointestinal tract of man was in the colon. They also estimated that 3,500 mg of ammonia would be produced by the known hydrolysis of urea and therefore concluded that it was likely that a major source of ammonia in the colon might well be the

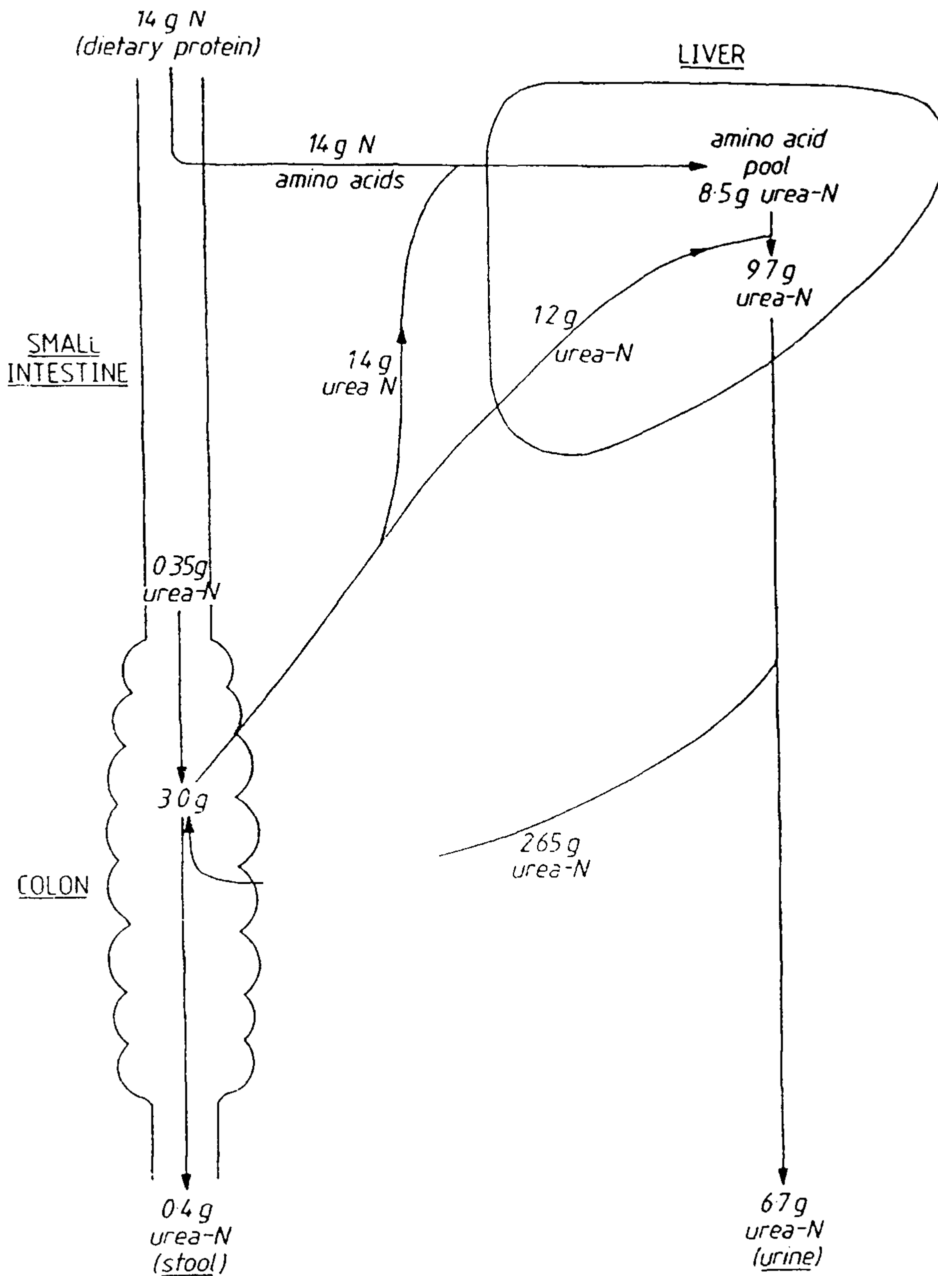


Figure 4.2. Diagrammatic representation of the movement of urea nitrogen in the body. Urea nitrogen is formed from amino acids in the liver, and on an intake of 14 g N about 9.7 g urea is produced each day, 8.5 g from amino acids and 1.2 g coming from the hydrolyzed urea in the bowel. Only 6.7 g of the urea produced is excreted in the urine with the remainder passing to the colon, 0.35 g through the ileocaecal valve and 2.65 g through the colonic wall. The urea is hydrolyzed and of the 3.0 g nitrogen, 0.4 g is excreted in stool, 1.2 g returning to urea synthesis and 1.4 g going to the synthesis of amino acids (Moran and Jackson, 1990a)

hydrolysis of urea (all N taken down to ammonia before being reused). In support of this, Weber and Veach (1979) found that, in the fasting dog, urea was taken up from arterial blood only by the colon and that 51% of portal venous ammonia was produced in the colon by hydrolysis of urea.

4.5.1 Source of colonic urea

If urea hydrolysis occurs in the lumen of the colon, there are only two possible sources of colonic urea; either urea enters from the ileum via the ileocaecal valve or it passes through the wall of the colon. From the work of Gibson *et al.* (1976a) it is possible to calculate a flux of urea nitrogen from the ileum to the colon of 390 mg per day. Chadwick *et al.* (1977), using long intestinal tubes for direct sampling, measured the flux of urea nitrogen from the ileum as 350 mg per day, remarkably similar to the previous study. They concluded that, as the 350 mg was only a fraction of the daily hydrolysis of urea (2,900–5,100 mg per day), substantial quantities must cross the colonic wall.

The general perception of the metabolism of urea in the colon has been dominated by the observations of Wolpert *et al.* (1971) and Bown *et al.* (1975). Wolpert *et al.* (1971), using a steady-state perfusion of the intact colon, found that only 2% of plasma urea was recovered from the lumen. Using the same model they found that only 5% of urea perfused through the lumen of the colon was recovered in the plasma. They concluded that the colon was virtually impermeable to urea and that urea hydrolysis must be occurring juxtamucosally and not in the lumen. Bown *et al.* (1975) perfused the excluded colon (the colon had been excluded from the faecal stream by ileorectal anastomosis and proximal and distal end colostomies) and were unable to demonstrate either luminal or juxtamucosal hydrolysis, and found very limited permeability of the colon mucosa to urea. However, they did allow that exclusion of the colon might abolish the juxtamucosal hydrolysis proposed by Wolpert *et al.* (1971).

Gibson *et al.* (1976b) gave urea labelled with stable isotope and measured the recovery of the label in the urine. They found evidence of urea hydrolysis in patients who had a total colectomy. They concluded that:

(a) Urea hydrolysis could occur in the absence of the colon.
(b) The amount of urea undergoing total hydrolysis appeared to be greater than that which could be accounted for by that entering the colon or diffusing through the wall. They speculated that the colon lumen might not necessarily be the main site for urea hydrolysis, as significant hydrolysis appeared to be taking place elsewhere, and they considered the most likely location might be the terminal ileum.

However, there are problems with the interpretation of these studies, particularly as investigators had used a cleansed (Wolpert *et al.,* 1971) or a defunctional colon (Bown *et al.,* 1975). Furthermore, both groups perfused the colon at 10 ml per minute (extrapolated to 24 hours equals 14,000 ml per day). Thus these key studies are unlikely to represent normal physiological function. Furthermore, it is likely that urea hydrolysis does indeed occur in the terminal ileum in patients with an ileostomy, as suggested by Gibson *et al.* (1976b), and that the development of a ‘functional colon’ associated with a marked increase in resident microflora in the terminal ileum, when the ileum is exteriorized as an ileostomy, is an important part of accommodation to life without an intact colon (Gorbach *et al.,* 1976; Finegold *et al.,* 1970; Moran and Jackson, 1992).

4.5.2
Colonic permeability to urea

Many of the anomalies and apparent controversy in the data could be explained if the colon were permeable to urea. The colon is particularly inaccessible for direct observation and there are only a limited number of approaches with which reliable information might be obtained. Diagnostic endoscopy offers one possible opportunity for direct access. Moran and Jackson followed the fate of $^{15}N^{15}N$-urea instilled into either the right or left colon at colonoscopy. They were able to demonstrate that the colon was directly permeable to urea, that luminal hydrolysis could take place to a significant extent and that much of the nitrogen derived from urea hydrolysis was retained by the host (Moran and Jackson, 1990a). Colonoscopy is seldom carried out in individuals without any symptoms or signs and the colon itself has to be cleansed in preparation for the procedure. Therefore it cannot be assumed that the findings are necessarily representative of the normal state. An alternative approach with which the uncleansed colon could be studied was to utilize colostomy stomata to gain access to the colon. In this way it was possible to study individuals with defunctioning colostomy, and therefore to determine the difference between the functioning intact colon, with its florid active microflora and a defunctioned colon in the same individual (Moran and Jackson, 1990b). The essential findings from the study in which isotope had been placed by colonoscopy were confirmed. In addition it was possible to demonstrate statistically significant differences in the handling of the labelled urea between the functioning and the defunctioned colon in the same individual (Figure 4.3). As all previous studies had been carried out on either the defunctioned or the cleansed colon, and using perfusion techniques equivalent to 14,400 ml per day, it was possible to explain the discrepancies in the findings. Most importantly the results emphasized the care needed in extrapolating from an artificial experimental situation and the potential importance of an intact microflora for the normal function of the colon.

The most extreme form of a defunctioned colon is in patients on total parenteral nutrition, who may be deprived of any external input to the gastrointestinal tract for periods of years. The colonic inflow, and therefore part of the normal bacterial substrate, is reduced. The metabolism of urea was measured in a series of patients on total parenteral nutrition (TPN) who had no oral intake. Surprisingly, patients on TPN hydrolyzed and retained significantly greater proportions of urea nitrogen than normals; this might have been because they were in the postoperative state and therefore actively retaining nitrogen as a part of the recuperation. There was a tendency for antibiotics to abolish the enhanced retention of nitrogen (Moran *et al.,* 1991; Jackson and Moran, 1992). Data of this kind lead to the conclusion that the colon is not merely a conduit, but together with its microflora functions as an important metabolic organ of critical significance to nitrogen metabolism (Moran and Jackson, 1992).

4.6
Destiny of hydrolyzed urea nitrogen in the colon

In their studies, Moran and Jackson found that on average more than 70% of the urea nitrogen placed in the colon was retained within the body pool. This was so for both the functioning and the defunctioned colon (Moran and Jackson, 1990b). These observations bear similarity to the fate of urea-nitrogen salvaged in the normal person. Only 20–30% of the nitrogen which is salvaged returns to urea formation. The majority is retained within the metabolic nitrogen pool. It has generally

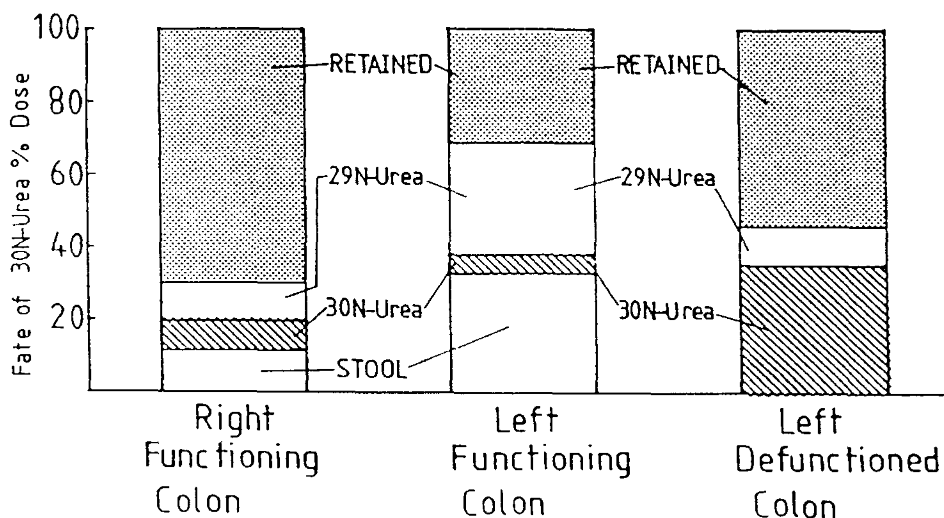


Figure 4.3. The fate of ^{15}N was determined after the installation of a tracer dose of $[^{30}N]$ urea through a colostomy into the right functioning colon, the left functioning colon or the left defunctioned colon. Although ^{15}N was recovered in stool and urine, as both $[^{30}N]$ urea and $[^{29}N]$ urea, a large proportion of the dose was retained in the body. There were significant differences between the right and the left colon and between the functioning and the defunctioning left colon

been observed that when ammonia is taken orally, or placed in the portal vein, very little escapes the preferential conversion to urea in the liver. Therefore, the observation that nitrogen derived from urea hydrolysis is retained implies that it is not reaching the liver as ammonia, but is being fixed in some other functionally useful form. We have hypothesized that urea nitrogen could be utilized by bacteria, and converted into amino acids which might then be absorbed across the wall of the colon and used by the host. There are human (Kies and Fox, 1978; Tanaka *et al.,* 1980; Heine *et al.,* 1987) and animal (Tanaka *et al.,* 1982; Torrallardona *et al.,* 1993) data in favour of this suggestion, but they are not considered sufficiently strong to carry general opinion at the present time. The resolution of this point is important for establishing the adequacy of diets to satisfy the requirements for nitrogen, amino acid and proteins. The possibilities are not mutually exclusive, and either one allows for a significant role of urea salvage in maintaining normal nitrogen metabolism.

4.7
Adaptation to suboptimal protein intakes is dependent on an intact urea-nitrogen salvage mechanism

We have sought to define the extent to which the process of salvage of urea nitrogen is determined by the intensity of the metabolic activity of the microflora itself. Dietary non-starch polysaccharides are known to increase the mass and numbers of bacteria in the faeces, and this is associated with an increase in faecal nitrogen. It seemed possible that this increased intensity of microbial activity might be associated with enhanced hydrolysis of urea and salvage of urea-nitrogen. In neither adults nor children was it possible to demonstrate any effect of either soluble or insoluble dietary non-

starch polysaccharide on urea-nitrogen salvage (Jackson *et al.,* 1990; Doherty and Jackson, 1992; Langran *et al.,* 1992). The only factor which demonstrably influenced the salvage system was the relationship between the metabolic demand for amino acid or nitrogen relative to that taken in the diet. Thus, the increased hydrolysis and salvage of urea-nitrogen in patients on total parenteral nutrition suggests that the protein supply is suboptimal (Moran *et al.,* 1991). However, as all were in positive nitrogen balance at the time, it is possible that this is a qualitative rather than a quantitative deficit. There is an increasing awareness that amino acids which are generally considered to be non-essential (dispensable) under normal circumstances might become conditionally essential. For example, some patients on TPN appear to be deficient in glutamine (Souba, 1988), cysteine or tyrosine (Rudman *et al.,* 1982), histidine (Kopple and Swendseid, 1975), proline (Jaksic *et al,* 1991), or glycine (Moran *et al.,* 1989). Antibiotics significantly reduce urea hydrolysis, presumably by their effect on the colonic flora.

In normal adults it is possible to define three states of metabolism in relation to normal intake, based upon the rate at which urea is produced and salvaged. When the intake of protein exceeds 70 g per day, urea production is about 100–120% of intake, and about 70–75% of the urea produced is excreted in urine with 25–30% of the nitrogen being salvaged in the colon (Jackson, 1993). The physiological minimum requirement for protein is about 35 g per day. As the intake of protein falls from 70 g to 35 g per day, there is a small, non-significant fall in urea production of about 10% (Langran *et al.,* 1992). However, there is a progressive increase in the rate at which urea-nitrogen is salvaged in the colon such that the increase in salvage almost matches the decrease in intake. As urea salvage increases, urea excretion falls, and thus nitrogen balance is maintained. On 35 g protein per day, about 30% of urea production is excreted with 70% of the nitrogen being salvaged. An intake of protein below about 35g per day is less than the physiological minimum required to maintain nitrogen balance. Below this level there is a significant reduction in the rate at which urea is produced, and a concomitant fall in the rate at which urea is salvaged in the colon, leading to increased urinary loss of urea and negative nitrogen balance (Danielsen and Jackson, 1992).

Kies and Fox (1978) have shown that when normal adults are given a corn diet providing 25 g protein per day (4 g nitrogen per day), they are in negative nitrogen balance. When the same diet is provided with the addition of a further 8 g nitrogen per day as urea, all the men were in positive balance. In a repeat of a similar study, we have provisional data which confirm that the move from negative to positive nitrogen balance is related to the enhanced hydrolysis of urea and retention of the nitrogen (Meakins and Jackson, unpublished data: Figure 4.4).

4.8
Clinical implications of the salvage of urea nitrogen

It is universally accepted in clinical practice that the microflora in and around the body represent a potential threat to well-being. Bacteria in the wrong location inevitably increase the risk of disease, thus pathological infestation or bacterial overgrowth are a common source of morbidity and can be lethal. The commensal or symbiotic role of the normal flora and its contribution to maintaining a healthy state is less widely appreciated and less well accepted. It is normal for the colon to have an active microflora, which plays an important part in establishing and maintaining the normal function (Moran and Jackson, 1992). The ability to salvage urea nitrogen and the role this may play in normal growth or in the adaptive response to low intakes of protein is outlined above. The wide array and large numbers of organisms in the colon undoubtedly have different and complementary

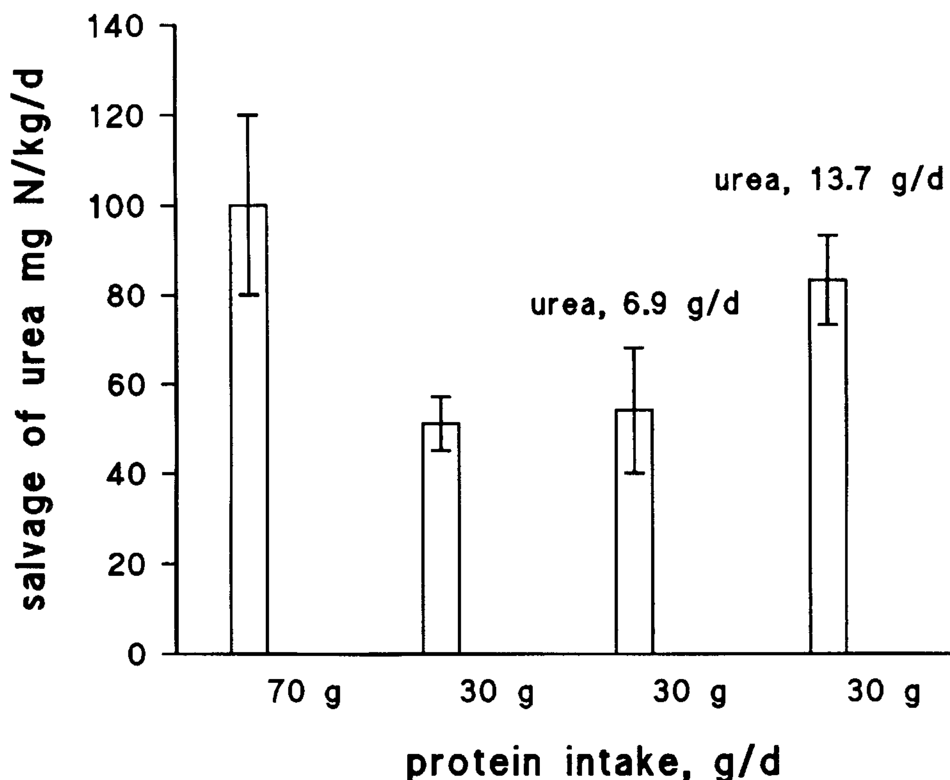


Figure 4.4. The salvage of urea nitrogen was determined in six young men taking a diet which provided 70 g protein, or 30 g protein, or 30 g protein with a supplement of either 6.9 g urea or 13.7 g urea, values are mean ± SE and units are mg N/kg/d (Meakins and Jackson, unpublished data)

metabolic activities. The probability that some organisms predominantly hydrolyze urea into ammonia but may be unable to utilize ammonia has practical implications. We found that in the defunctioned colon the utilization of ammonia from hydrolyzed urea appeared to be reduced (Moran and Jackson, 1990b), and there is evidence from studies with animals which shows that one of the important functions of dietary complex carbohydrate might be to increase the capacity of the microflora to fix ammonia in a non-toxic form. For many years, Visek has provided evidence which shows that colonic mucosal damage can be induced by luminal concentrations of ammonia in the high physiological range (Visek, 1972; Lin and Visek, 1991). He has argued that this may represent one of the important aetiological factors for carcinoma of the colon. If this were the case then the interaction amongst urea hydrolysis, fermentation of dietary complex carbohydrates and the fate of ammonia in the descending and sigmoid colon in particular would be of considerable practical significance for cancer of the colon in the human.

Intraluminal ammonia which is not fixed by the bacteria will be absorbed and in susceptible individuals has the potential to be toxic to the host. The introduction of therapeutic interventions (which, by altering the overall metabolic behaviour of the microflora, such that ammonia was utilized by the bacteria) could have major implications in clinical medicine, especially in the fields of

liver and renal disease. In hepatic encephalopathy practically all the current approaches to management involve manoeuvres designed to alter bacterial metabolism of nitrogen compounds in the colon (Schafer, 1987). Superficially, the two main approaches, namely the use of antibiotics and oral lactulose, appear to have conflicting effects. The objective of treatment with antibiotics is to kill or inhibit the growth of the bacteria, whereas lactulose (a non-absorbable polysaccharide) promotes bacterial proliferation. However, both treatments when used individually have been shown to benefit patients with encephalopathy (Weber *et al.,* 1987). Furthermore, when they have been used in combination, their individual effects have been found to be enhanced, which suggests that the mechanism through which the individual effects are mediated might relate to different populations of bacteria. Given the limited understanding of the mechanisms through which the beneficial effects might be mediated, it is likely that further evaluation of detailed involvement of the colonic microflora might provide information of use in the management of liver diseases. The rationale behind the use of low protein diets in renal failure is based upon the assumption that the salvage and utilization of nitrogen derived from urea will decrease the load presented to the kidney for excretion. Whilst early experience gave encouragement to the application of low protein diets, the particular value of these diets has been less clear in recent years. As outlined above the factors which influence the effective utilization of urea-nitrogen may be complex. Therefore, these complexities have to be borne in mind if dietary manipulation is to be most effective. Advances in this area have probably been hampered by limitations in our knowledge of, and attitudes towards, the role of the flora in nitrogen metabolism.

If, as has been suggested, the intestinal flora plays a key role in the overall function of the gastrointestinal tract, which is the port of entry for most nutrients, then possible effects of more general interventions, such as the use of broad-spectrum antibiotics have to be considered seriously. One good example of the extent to which alterations in bacterial activity brought about by broad-spectrum antibiotics can have major effects on intestinal function and general well-being is in antibiotic-associated diarrhoea. For this reason, in clinical medicine, antibiotic usage should be targeted, appropriate and discontinued as soon as possible. There is the need to know more about the potential effects in nutrient delivery and salvage, especially in children who are susceptible to growth failure.

These simple examples represent some clinically relevant problems, the detailed aspects of which are undoubtedly much more complex. The vital interaction of the metabolic activity of the intestinal flora with the host is an area of investigation which has been relatively neglected, and warrants further study.

4.9
Conclusion

The hydrolysis of urea by the colonic microflora and the making available of the nitrogen derived in a form which is functionally useful to the host plays a central part in adaptation to diets which are relatively low in protein. It may also be of importance in individuals on normal levels of protein intake. It is now clear that an understanding of urea metabolism is of importance for appreciating two aspects of protein/energy interactions during early life. First, the utilization of amino acids as a major source of energy is inevitably associated with the formation of large amounts of urea. Secondly, the nitrogen derived from urea hydrolysis might contribute directly to the formation of amino acids, needed to satisfy the demands for normal growth and development. It remains for

future work to determine the extent to which this complex interaction functions to modulate the pattern of amino acids available to the infant. When this information is available it will be possible to determine the extent to which the functional role of the colon is critical for normal health (Moran and Jackson, 1992), the particular effect of gastrointestinal disorders such as diarrhoeal disease and the implications for the design of infant formulas (Fomon, 1991).

Alterations in urea hydrolysis have been demonstrated where the intake is inadequate in either quantity, or quality, to meet the metabolic demand. As urea-nitrogen salvage is an adaptive mechanism, urea kinetic measurements may be an accurate marker of the adequacy of a protein intake. The recent proposals by Young and Pellett (1990) that the human requirements for essential amino acids are substantially greater than the FAO/WHO/UNU recommendations (1985), together with the important observations by Millward and colleagues (Millward and Rivers, 1988; Millward *et al.,* 1989) suggest that there are unresolved areas in protein metabolism. Urea-nitrogen salvage in the colon (and in the ileum of those who have had a total colectomy) appears to be the mechanism for conserving body nitrogen in health and disease.

References

Allison, J.B. and Bird, J.W.C., 1964, Elimination of nitrogen from the body, in Munro, H.B., Allison, J.B., (Eds) *Mammalian Protein Metabolism,* Vol. 1, pp. 483–512, New York: Academic Press.

Aoyagi, T., Egstrom, G.W., Evans, W.B. and Summerskill, W.H.J., 1966, Gastrointestinal urease in man. Part 1. Activity of the mucosal ureas, *Gut,* 7, 631–39.

Bloch, K., 1946, The metabolism of l(+)arginine in the rat, *J. Biological Chem.,* **165,** 469–84.

Bollman, J.J., Mann, F.C. and Magath, T.B., 1926, Studies on the physiology of the liver. XV. Effect of total removal of the liver on deaminization, *Am. J. Physiol.,* **78,** 258–69.

Bown, R.L., Gibson, J.A., Fenton, J.C.B., Sneddon, W., Clark, M.L. and Salden, G.E., 1975, Ammonia and urea transport by the excluded human colon, *Clin. Sci.,* **48,** 279–87.

Brown, C.L., Hill, M.J. and Richards, P., 1971, Bacterial ureases in ureamic men, *Lancet,* **ii,** 407–9.

Carpenter, K.J., 1992, Protein requirements of adults from an evolutionary perspective, *Am. J. Clin. Nutr.,* **55,** 913–17.

Cathcart, E.P., 1921, *The Physiology of Protein Metabolism,* London: Longmans, Green and Co.

Chadwick, V.S., Jones, J.D., Debongnie, J.C., Gaginella, T. and Phillips, S.F., 1977, Urea, uric acid, and creatinine fluxes through the small intestine in man, *Gut,* **18,** A944.

Chao, F-C. and Tarver, H., 1953, Breakdown of urea in the rat, *Proceedings Soc. Experimental Bio. Med.,* **84,** 406–9.

Danielsen, M. and Jackson, A.A., 1992, Limits of adaptation to a diet low in protein in normal man: urea kinetics, *Clin. Sci.,,* **83,** 103–8.

Das, T.K. and Waterlow, J.C., 1974, The rate of adaptation of urea cycle enzymes, aminotrans-ferases and glutamic dehydrogenase to changes in dietary protein intake, *Brit. J. Nutr.,* **23,** 353–73.

Denne, S.C. and Kalhan, S.C., 1991, Leucine kinetics during feeding in normal newborns, *Pediat. Res.,* **30,** 23–7.

Dintzis, R.Z. and Hastings, A.B., 1953, The effect of antibiotics on urea breakdown in mice, *Proceedings Nat. Acad. Sci.,* **39,** 571–78.

Doherty, J. and Jackson, A.A., 1992, The effect of dietary pectin on rapid catch-up weight gain and urea kinetics in children recovering from severe undernutrition, *Acta Pediat.,* **81,** 514–17.

FAO/WHO/UNU, 1985, Energy and protein requirements, Report of an expert consultation, Technical Report Series No. 724, Geneva: WHO.

Finegold, S.M., Sutter, V.L., Boyle, J.D. and Shimada, K., 1970, The normal flora of ileostomy and transverse colostomy effluents, *J. Infect. Dis.,* **122,** 376–81.

Fomon, S.J., 1991, Requirements and recommended dietary intakes of protein during infancy, *Pediat. Res.,* **30,** 391–95.

Gibson, J.A., Sladen, G.E. and Dawson, A.M., 1976a, Protein absorption and ammonia production: the effects of dietary protein and removal of the colon, *Brit. J. Nutr,* **35,** 61–65.

Gibson, J.A., Park, J.N., Sladen, G.E. and Dawson, A.M., 1976b, The role of the colon in urea metabolism in man, *Clin. Sci.,* **50,** 51–59.

Gorbach, S.L., 1971, Intestinal microflora, *Gastroenterol.,* **60,** 1110–29.

Gorbach, S.L., Nahas, L. and Weinstein, L., 1976, Studies of intestinal microflora. The microflora of ileostomy effluent: a unique microbial ecology, *Gastroenterol.,* **54,** 874–80.

Graham, D.Y., Klein, P.D., Evans, D.J., Albert, L.C., Opekun, A.R. and Boutton, T.W., 1987, *Campylobacter pylori* detected non-invasively by the ^{13}C urea breath test, *Lancet,* **i,** 1171–77.

Gustafsson, B.E., 1982, The physiological importance of the colonic microflora, *Scand. J.Gastroenterol.,* **77** (Suppl), 117–31.

Heine, W., Wutzkue, K.D., Richter, I., Walther, F. and Plath, C., 1987, Evidence for colonic absorption of protein nitrogen in infants, *Acta Pediat. Scand.,* **76,** 741–44.

Hibbert, J.M. and Jackson, A.A., 1991, The intra-individual variation in urea kinetics measured in a single individual over a period of four years, *Eur. J. Clin. Nutr.,* **45,** 347–51.

Hibbert, J.M., Forrester, T., and Jackson, A.A., 1992, Urea kinetics: comparison of oral and intravenous dose regimens, *Eur. J. Clin. Nutr.,* **46,** 405–9.

Jackson, A.A., 1983, Amino acids: essential and non-essential, *Lancet* **ii,** 1034–37.

Jackson, A.A., 1986, Dynamics of protein metabolism and their relation to adaptation, in Taylor, T.G. and Jenkins, N.K. (Eds) *Proceedings of the XIII International Congress of Nutrition, 1985,* pp. 403–9, London: John Libbey.

Jackson, A.A., 1992a, How can early diet influence later disease? *Brit. Nutr. Found. Nutr. Bull.,* **17** (Suppl), 23–30.

Jackson, A.A., 1992b, Critique of protein-energy interactions *in vivo:* urea kinetics, in Scrimshaw, N.S. and Schurch, B. (Eds) *Protein-Energy Interactions,* pp. 63–79, Switzerland: International Dietary Energy Consultative Group.

Jackson, A.A., 1993, Chronic malnutrition: protein metabolism, *Proceedings Nutr. Soc.,* **52,** 1–10.

Jackson, A.A., 1994, Urea as a nutrient, bioavailability and role in nitrogen economy, *Arch. Dis. Childhood,* **70,** 3–4.

Jackson, A.A., Picou, D. and Landman, J., 1984, The non-invasive measurement of urea kinetics in normal man by a constant infusion of ^{15}N^{15}N-urea, *Hum. Nutr: Clin. Nutr.,* **38C,** 339–54.

Jackson, A.A., Persaud, C., Badaloo, V. and de Benoist, BJ., 1987, Whole body protein turnover in man determined in three hours with oral or intravenous ^{15}N-glycine and enrichment in urinary ammonia, *Hum. Nutr: Clin. Nutr.,* **41C,** 263–76.

Jackson, A.A., Doherty, J., de Benoist, M.H., Hibbert, J. and Persaud, C., 1990, The effect of the level of dietary protein, carbohydrate and fat on urea kinetics in young children during rapid catch-up weight gain, *Brit. J. Nutr.,* **64,** 371–85.

Jackson, A.A. and Wootton, S.A., 1990, The energy requirements of growth and catch-up growth, in Schurch, B. and Scrimshaw, N.S. (Eds) *Activity Energy Expenditure and Energy Requirements of Infants and Children,* pp. 185–214, Switzerland: International Dietary Energy Consultative Group, Nestle Foundation.

Jackson, A.A. and Moran, B.J., 1992, Urea kinetics and total parenteral nutrition, *Am. J. Clin. Nutr.,* **55,** 481.

Jackson, A.A., Danielsen, M.S. and Boyes, S., 1993, A non-invasive method for measuring urea kinetics with a single dose of [^{15}N^{15}N]-urea in free-living people, *J. Nutr.,* **123,** 2129–36.

Jackson, A.A. and Forrester, T., 1994, Human requirements for protein, amino acids and nitrogen: the role of urea salvage in adaptation to low intake. Nestle Foundation, Annual Report, 1993, pp. 58–81, Switzerland, Nestle.

Jaksic, T., Wagner, D.A., Burke, J.F. and Young, V.R., 1991, Proline metabolism in adult male burned patients and healthy control subjects, *Am. J. Clin., Nutr.,* **754,** 408–13.

Jones, E.A., Smallwood, R.A., Craigie, A. and Rosenoer, V.M., 1969, The enterohepatic circulation of urea nitrogen, *Clin. Sci.,* **37,** 825–85.

Kalhan, S.C., 1994, Rates of urea synthesis in the human newborn: effect of maternal diabetes and gestational age, *Pediat. Res.,* **34,** 801–4.

Kanaghinis, T., Lubram, M. and Coghill, N.F., 1963, The composition of ileostomy fluid, *Gut,* **4,** 322–38.

Kies, C., 1972, Nonspecific nitrogen in the nutrition of human beings, *Fed. Proceedings,* **531,** 1172–77.

Kies, C. and Fox, H.M., 1978, Urea as a dietary supplement for humans, *Advances Exper. Med. Biol.,* **105,** 103–18.

Kopple, J.D. and Swendseid, M.E., 1975, Evidence that histidine is an essential amino acid in normal and chronically uremic man, *J. Clin. Invest.,* **55,** 881–91.

Kornberg, H.L. and Davies, R.E., 1952, The metabolism of subcutaneously injected [^{15}N] urea in the cat, *Biochem. J.,* **52,** 345–50.

Kornberg, H.L. and Davies, R.E., 1955, Gastric urease, *Physiol. Rev.,* **35,** 169–77.

Laidlaw, S.A. and Kopple, J.D., 1987, Newer concepts of indispensable amino acids, *Am. J. Clin. Nutr.,* **46,** 593–605.

Langran, M., Moran, B.J., Murphy, J.L. and Jackson, A.A., 1992, Adaptation to a diet low in protein: the effect of complex carbohydrate upon urea kinetics in normal man, *Clin. Sci.,* **82,** 191–98.

Leifer, E., Roth, L.J. and Hempelmann, L.H., 1948, Metabolism of C^{14}-labelled urea, *Science,* **108,** 748.

Levenson, S.M., Crowley, L.V., Horowitz, R.E. and Malm, O.J., 1959, The metabolism of carbon-labelled urea in the germ-free rat, *J. Biol. Chem.,* **234,** 2061–62.

Lin, H-C. and Visek, W.J., 1991, Colon mucosal cell damage by ammonia in rats, *J. Nutr.,* **121,** 887–93.

Liu, C.H., Hays, V.W., Svec, H.J., Catron, D.V., Ashton, G.C. and Speer, V.C., 1955, The fate of urea in growing pigs, *J. Nutr.,* **57,** 241–47.

Long, C.L., Jeevenandam, M. and Kinney, J.M., 1978, Metabolism and recycling of urea in man, *Am. J. Clin. Nutr.,* **25,** 1367–82.

McGilverry, R.W., 1970, *Biochemistry: A Functional Approach,* p. 661, Philadelphia: Saunders.

Meijer, A.F., Wouter, H.L. and Chamuleau, A.F.M., 1990, Nitrogen metabolism and ornithine cycle function, *Physiol. Rev.,* **70,** 701–48.

Millward, D.J., 1992, The influence of amino acid composition of protein on protein-energy interactions: the metabolic basis of amino acid requirements, in Schurch, B. and Scrimshaw, N.S. (Eds) *Protein Energy Interactions,* Switzerland: International Dietary Energy Consultative Group.

Millward, D.J. and Rivers, J.P.W., 1988, The nutritional role of indispensable amino acids and the metabolic basis for their requirements, *Eur. J. Clin. Nutr.,* **42,** 367–93.

Millward, D.J., Jackson, A.A., Price, G. and Rivers, J.P.W., 1989, Human amino acid and protein requirements: current dilemmas and uncertainties, *Nutr. Res. Rev.,* **2,** 109–32.

Moore, F.D., Olesen, K.H., McMurray, J.D., Parker, H.V., Ball, M.R. and Boyden, C.M., 1963, *The Body Mass and its Supporting Environment,* Philadelphia: Saunders.

Moran, B.J. and Jackson, A.A., 1990a, ^{15}N-urea metabolism in the functioning human colon: luminal hydrolysis and mucosal permeability, *Gut,* **31,** 454–57.

Moran, B.J. and Jackson, A.A., 1990b, Metabolism of ^{15}N-labelled urea in the functioning and defunctioned human colon, *Clin. Sci.,* **79,** 253–58.

Moran, B.J. and Jackson, A.A., 1992, Function of the human colon, *Brit. J. Surg.,* **79,** 1132–37.

Moran, B.J., Karran, S.J. and Jackson, A.A., 1991, Enhanced retention of urea nitrogen in patients on total parenteral nutrition for intestinal failure, *Clin. Nutr.,* **10,** 67–70.

Moran, B., Persaud, C. and Jackson, A.A., 1989, Urinary excretion of 5-oxoproline in severe inflammatory illness, *Proceedings Nutr. Soc.,* **48,** 75A.

Munro, H.N. and Allison, J.B., 1964, *Mammalian Protein Metabolism,* Vol. 1, New York and London: Academic Press.

Oomen, H.A.P.C., 1970, Interrelationship of the human intestinal flora and protein utilization, *Proceedings Nutr. Soc.,* **29,** 197–205.

Owens, J.A., Owens, P.C. and Robinson, J.S., 1989, Experimental fetal growth retardation: metabolic and endocrine aspects, in Gluckman, P.D., Johnston, B.M. and Nathanielsz, P.W. (Eds) *Advances in Fetal Physiology: Reviews in Honour of GC Liggins,* pp. 263–86, Ithaca: Perinatology Press.

Picou, D. and Phillips, M., 1972, Urea metabolism in malnourished children receiving a high or low protein diet, *Am. J. Clin. Nutr.,* **25,** 1261–66.

Prior, R.L., Hintz, H.F., Lowe, J.E. and Visek, W.J., 1974, Urea recycling and the metabolism of ponies, *J. Animal Sci.,* **38,** 565–71.

Rose, W.C., 1957, The amino acid requirements of adult man, *Nutr. Abstract Rev.,* **27,** 631–47.

Rudman, D., Chawla, R.K. and Bleier, J.C., 1982, Cystine and tyrosine requirements during nutritional repletion of cirrhotic patients, in Blackburn, G.L., Grant, J.P. and Young, V.R. (Eds) *Amino Acids. Metabolism and Medical Applications,* pp. 484–96, Massachusetts: John Wright.

Schafer, D.F., 1987, In hepatic failure the problems come from the colon, but will the answers come from there? *J. Lab. Clin. Med.,* **110,** 253–54.

Schoenheimer, R., 1942, The Dynamic State of the Body Constituents, Cambridge: Harvard University Press.

Silen, W., Harper, H.A., Mawdsley, D.L. and Weirich, W.L., 1955, Effect of antibacterial agents on ammonia production within the intestine, *Proceedings Soc. Exp. Biol. Med.,* **288,** 138–40.

Souba, W.W., 1988, The gut as a nitrogen-processing organ in the metabolic response to critical illness, *Nutr. Support Services,* **8,** 15–22.

Summerskill, W.H.J. and Wolpert, W., 1970, Ammonia metabolism in the gut, *Am. J. Clin. Nutr.,* **23,** 633–39.

Stephen, J.M.L. and Waterlow, J.C., 1968, Effect of malnutrition on activity of two enzymes concerned with amino acid metabolism in human liver, *Lancet* **ii,** 118–19.

Tanaka, N., 1982, Urea utilization in protein deficient rats, *J. Jap. Soc. Nutr. Food Sci.,* **35,** 175–80.

Tanaka, N., Kubo, K., Shiraki, K., Koishi, H. and Yoshimura, H., 1980, A pilot study on protein metabolism in Papua New Guinea Highlanders, *J. Nutr. Sci., Vitaminol.,* **26,** 247–59.

Torrallardona, D., Harris, C.I., Milne, E. and Fuller, M.F., 1993, Contribution of intestinal microflora to lysine requirements of non-ruminants, *Proceedings Nutr. Soc.,* **52,** 153A.

Visek, W.J., 1972, Effects of urea hydrolysis on cell life-span and metabolism, *Fed. Proceedings,* **31,** 1178–93.

Walser, M. and Bodenlos, L.J., 1959, Urea metabolism in man, *J. Clin. Invest.,* **38,** 1617–26.

Wang, Z.M., Pierson, R.N. Jr. and Heymsfeild, S.B., 1992, The five-level model: a new approach to organising body-composition research, *Am. J. Clin. Nutr.,* **56,** 19–28.

Waterlow, J.C., 1968, Observations on the mechanism of adaptation to low protein intakes, *Lancet,* **ii,** 1091–97.

Waterlow, J.C., 1984, Protein turnover with special reference to man, *Quart. J. Exp. Physiol.,* **69,** 409–38.

Weber, F.L. and Veach, G.L., 1979, The importance of the small intestine in gut ammonia production in the fasting dog, *Gastroenterol.,* **77,** 235–40.

Weber, F.L., Banwell, J.G., Fresard, K.M. and Cummings, J.H., 1987, Nitrogen in faecal bacterial, fiber, and soluble fractions of patients with cirrhosis: effects of lactulose and lactulose plus neomycin, *J. Lab. Clin. Med.,* **110,** 259–63.

Wheeler, R.A., Griffiths, D.M. and Jackson, A.A., 1993a, Urea kinetics in neonates receiving total parenteral nutrition, *Arch. Dis. Childhood,* **69,** 24–27.

Wheeler, R.A., Griffiths, D.M. and Jackson, A.A., 1993b, Urea salvage in a neonate with cloacaexstrophy, *Arch. Dis. Childhood,* **69,** 83–84.

Wheeler, R.A., Jackson, A.A. and Griffiths, D.M., 1991, Urea production and recycling in the neonate, *J. Pediat. Surg.,* **26,** 575–77

Wolpert, E., Phillips, S.F. and Summerskill, W.M.J., 1971, Transport of urea and ammonia production in the colon, *Lancet,* **ii,** 1387–90.

Wrong, O.M., Vince, A.J. and Waterlow, J.C., 1985, The contribution of endogenous urea to faecal ammonia in man, determined by ^{15}N labelling of plasma urea, *Clin. Sci.,* **68,** 193–99.

Young, V.R., 1992, Commentary on the paper by D.J.Millward, The metabolic basis of amino acid requirements, in Scrimshaw, N.S. and Schurch, B. (Eds) *Protein Energy Interactions,* pp. 57–61, Switzerland: International Dietary Energy Consultative Group.

Young, V.R. and Marchini, J.S., 1990, Mechanisms and nutritional significance of metabolic response to altered food intake of protein and amino acids, with reference to nutritional adaptation in humans, *Am. J. Clin. Nutr,* **51,** 270–89.

Young, V.R. and Pellett, P.L., 1990, Current concepts concerning indispensable amino acid needs in adults and their implications for international nutrition planning, *Food Nutr. Bull,* **12,** 289–300.

Chapter 5
Nitrate pharmacology and toxicology

P.J.Packer

5.1
Introduction

Nitrate is a fundamental component of the global nitrogen cycle and is therefore found throughout the environment. Since the postulated health effects of nitrate relate to its metabolites nitrite and the N-nitroso compounds, it is very important in risk assessment to have a clear understanding of mammalian nitrate pharmacology and metabolism.

5.2
Pharmacology

Nitrate entering the mouth in the form of food and water has a relatively short residence time there. Nitrate then passes into the stomach where it has a much longer residence time since significant absorption of nitrate does not occur and the residence time of food in the stomach is about 40–80 minutes (Malagelada *et al.,* 1984; Mayer *et al.,* 1984). Once nitrate enters the proximal small intestine it is rapidly and actively absorbed into the blood (Hartman, 1982). Serum nitrate is then actively secreted into the saliva (Spiegelhalder *et al.,* 1976) and by more passive mechanisms into several other body fluids including sweat (Bartholomew and Hill, 1984), gastric mucin (Mueller and Henninger, 1985), gastric juice (Ruddell *et al.,* 1978) milk (Hartman, 1982), ileostomy fluid (Dolby *et al.,* 1984) and probably vaginal secretions (Alsobrook *et al.,* 1974). Levels of apparent total N-nitroso compounds in the faeces have been shown to be dependent upon levels of nitrate in the diet (Rowland *et al.,* 1991), suggesting that nitrate is also secreted into the large intestine from the blood stream.

The process of salivary recirculation continuously circulates nitrate through the oral cavity and it has been estimated that 25% of ingested nitrate is in fact recirculated in the saliva (Spiegelhalder *et al.,* 1976; Bartholomew and Hill, 1984). Parotid duct saliva has been shown to be free of nitrite (Tannenbaum *et al.,* 1974; Granli *et al.,* 1989) whereas it is present in whole saliva. The origin of nitrite in saliva appears to be predominantly a result of bacterial reduction of nitrate to nitrite. Salivary nitrite is swallowed along with salivary nitrate, and is in fact the primary source of nitrite in the normal acid stomach. Nitrite, by contrast with nitrate, has a very short half-life in the normal stomach (Licht *et al.,* 1986). Three processes have been shown to contribute to this: these are predominately gastric absorption, with minor contributions from the dilution of stomach contents and the chemical reactions of nitrite.

A major clearance of nitrate from the blood occurs in the kidneys. Estimates of renal clearance of discrete nitrate challenges range from 50% to 90%, with more recent studies using ^{15}N-labelled nitrate showing the recovery to be nearer 50–60% (Green *et al.*, 1981; Wagner *et al.*, 1983; Bartholomew and Hill, 1984; Leaf *et al.*, 1987). We have recently verified that urinary nitrate excretion over 24 hours is closely correlated with challenge. Regression analysis further demonstrated a background excretion of nitrate of 0.22 mmol/day which was not related to the recent nitrate exposure and possibly represented endogenous nitrate synthesis (Packer *et al.*, 1989). Following doses of 0.3–2.1 mmol, average actual recoveries of 55% were observed (Packer *et al.*, 1989). Similar recoveries of 58% and 51% were observed for nitrate doses of 2.5 mM and 5.0 mM (Packer *et al.*, 1988). Normally the urine contains no or negligible amounts of nitrite. However, if the urinary tract becomes bacterially infected as in the cases of Bilharzia (Tricker *et al.*, 1989) and paraplegia (Ohshima *et al.*, 1987) patients or as a result of disease (Hicks *et al.*, 1977), then nitrite is readily detected in the urine.

5.2.1 Consequences for epidemiology

Twenty-four-hour urine collections are therefore an accurate and reliable measure of nitrate exposure for use in epidemiological studies. Various other methods have been used but they are prone to larger errors and inaccuracies. Dietary questionnaires and food tables are notoriously unreliable and time-consuming (Bingham, 1987). They are confounded largely by poor dietary recall and the unreliability of food tables since nitrate levels in drinking water and vegetables vary considerably from region to region and with the time of the year (White, 1983; Knight *et al.*, 1987). Salivary nitrate/nitrite concentrations have been used as a measure of nitrate/nitrite exposure (Forman *et al.*, 1985); however, they fluctuate markedly throughout the day and there is little evidence that such determinations are likely to reflect dietary levels reliably (Packer *et al.*, 1989).

5.3 Toxicological effects

However, it is not nitrate but its metabolites nitrite and the N-nitroso compounds that are potentially detrimental to normal health. While exposure to nitrite itself either by ingestion or by endogenous formation (either by bacterially mediated host processes) within the body has important health consequences in relation to methaemoglobin formation, its most controversial health risk, cancer, relates to its role as a precursor to genotoxic N-nitroso compound formation.

5.3.1 Short-term/acute effects

Methaemoglobinaemia

The metabolism of methaemoglobinaemia has been well documented. Comley's (1945) article of nitrate-contaminated well-water was the first report of non-congenital methaemoglobinaemia with significant public health implications. Acquired methaemoglobinaemia usually manifests as an acute

illness with symptoms of hypoxia appearing at a methaemoglobin concentration in the order of 35% of total haemoglobin. Single oral doses that have been shown to produce toxic methaemoglobinaemia in humans are 2–4 g nitrate and 60–500 g nitrite (Mirvish, 1992). Methaemoglobin toxicity has been an ongoing problem particularly in children. There are four major reasons for this: first, fetal haemoglobin, which accounts for 70% of total haemoglobin at birth and 30% at three months of age, is more readily oxidized to methaemoglobin than is adult haemoglobin (Mirvish, 1992); secondly, infants have a deficiency of methaemoglobin reductase or NADH in their blood cells (Choury *et al.,* 1983); thirdly, infants have a relatively achlorhydric (high pH) stomach, which allows the growth of bacteria, some of which have the ability to reduce nitrate to nitrite, and fourth, infants drink ten times more than adults on a body weight basis.

Other short-term health effects

Various other health effects have been reported to be associated with nitrate contamination of ground water and include hypertension (Morton, 1971, Malberg *et al.,* 1978), clinical methaemoglobinaemia in older children (Petukhov and Ivanov, 1970), increased infant mortality (Super *et al.,* 1981) and central nervous system birth defects (Dorsch *et al.,* 1984), although none of these reports have been confirmed by others (Gelperin *et al.,* 1975; Arbuckle *et al.,* 1988; Weisenburger, 1992).

5.3.2
Long-term/chronic effects

Long-term health effects are related to the further metabolism of nitrite. There are two mechanisms by which N-nitrosation can occur: these are acid-catalyzed and the bacterially mediated nitrosation reactions of nitrite, the former being particularly relevant to the normal acid stomach and the latter to most of the other sites and particularly the achlorhydric stomach. The acid-catalyzed reactions of nitrite are well understood and are largely dependent upon well-characterized factors such as pH, the presence of inhibitors (e.g. urea, ammonia and vitamin C) and catalysts (e.g. thiocyanate, chloride and some phenolics) (Archer, 1984; Shuker, 1988).

Under the physiological conditions that prevail at sites such as the achlorhydric stomach that have a pH in the range of 6–8, acid-catalyzed reactions of nitrite are likely to be of little significance. These sites are likely to be colonized by nitrate-reducing bacteria which will provide elevated levels of the substrate, nitrite, and are therefore of particular interest with regard to N-nitroso compound formation (Calmels *et al.,* 1985; Leach *et al.,* 1987; Leach, 1988).

Bacterially mediated N-nitrosation reactions will depend to a large degree on the precise composition of the bacterial flora, which will vary considerably from person to person. To date the mouth and the stomach are the two major sites that have had their flora composition and metabolism evaluated to any degree.

The mouth

The microbial flora of the mouth is very complex and in most instances structured into communities with species with a wide range of distinct genera tending to occupy their own particular niche (Theilade, 1989). Several of the species and strains of bacteria in these communities will possess

Table 5.1 Bacterial genera found in the mouth. (Number of species indicated in brackets)

Aerobest facultative anaerobes, microaerophils		*Anaerobes*	
Streptococcus	(11)	*Peptostreptococcus*	(2)
Stomatococcus	(1)	*Actinomyces**	(2)
*Actinomyces**	(3)	*Propionibacterium**	(1)
*Arachnia**	(1)	*Bifidobacterium*	(1)
*Corynebacterium**	(1)	*Eubacterium**	(5)
*Rothia**	(1)	*Veillonella**	(3)
*Lactobacillus**	(7)	*Bacteroides**	(19)
*Neisseria**	(4)	*Mitsuokella*	(1)
*Branhamella**	(1)	*Fusobacterium**	(3)
*Haemophillus**	(5)	*Leptotrichia*	(1)
*Actinobacillus**	(1)	*Wolinella*	(2)
Eikenella	(1)	*Selenomonas**	(1)
Capnocytophaga	(3)	*Centipeda*	(1)
*Campylobacter**	(2)		
Simonsiella	(1)		

Adapted from Theilade (1989), where full species list is cited. *Genera with nitrate reducing species (several sources).

nitrate reductase activity (Table 5.1) and, depending on such factors as their level of carriage within the flora, their spatial distribution in relation to salivary nitrate flow, and their specific nitrate reductase activities in relation to those environmental factors modulating expression, will lead to a particular level of conversion of nitrate to nitrite in the saliva of a particular individual.

In fact, our own studies have demonstrated that subjects tend to be consistently high, medium or low nitrate converters (Packer *et al.*, 1989). Knowing that in humans 20% of the 25% of dietary nitrate undergoing salivary circulation is converted to nitrite (Speigelhalder *et al.*, 1976), and that the two extremes for the median percentage of nitrite are 22% and 57%, then this represents a range of 5.5–22 mol.% of total dietary nitrate intake (average 11.5 mol.%). In other studies different averages for mol.% of overall nitrate intake have been reported, e.g. 5 mol.% in Germany (Speigelhalder *et al.*, 1976), and 6.3 mol.% for reviewed data from The Netherlands, Japan and Germany (Stephany and Schuller, 1980). Such differences in the ability of the oral flora of different individuals to reduce nitrate to nitrite may have important epidemiological consequences since nitrite not nitrate is the ion of lexicological significance. To date these factors have been given little consideration in the mouth. However, more consideration has been given to these processes in the stomach and particularly in the achlorhydric stomach.

5.4
N-nitrosation in the stomach

5.4.1
Nitrate metabolism

In the normal acid stomach intragastric nitrite is largely of salivary origin. However, in those individuals who have impaired gastric acid secretion (hypochlorhydria and achlorhydria) a resident

bacterial flora develops (Charnley *et al.*, 1982; Walters *et al.*, 1978). The intragastric interconversion of nitrate to nitrite by bacteria provides a further and probably dominant source of nitrite. The normal acid stomach is normally sterile with no more than 10^3/ml. transient bacteria present, with little or no metabolic activity. Once gastric acid secretion is sufficiently impaired and the resting gastric pH is consistently above 4, a resident bacterial flora develops with counts of up to 10^9/ml (Drasar *et al.*, 1964; Leach and Hill, 1990). A considerable range of genera and species have been isolated (see chapter 6) and nitrate reductase activity is a feature of several of these. Overall the counts of nitrate-reducing bacteria increase as the intragastric pH increases, in parallel with the total counts of bacteria, resulting in nitrite concentrations increasing from 3 μM to 30–300μM (Ruddell *et al.*, 1978, Tannenbaum *et al.*, 1979, Mueller *et al.*, 1986). In addition, inter- and intra-individual variation in the species composition and metabolic activity of the gastric flora will play a dominant role in determining the intragastric nitrite concentration. These in turn will be influenced by environmental factors such as dietary nitrate load.

We have shown further evidence of bacterial reduction of dietary nitrate to nitrite in a group of pernicious anaemia patients (extreme achlorhydrics) whose urinary excretion of a known nitrate challenge was 23% less than that observed in a group of normochlorhydrics (Packer *et al.*, 1990). It would be expected that a similar effect would be seen in other achlorhydric and hypochlorhydric groups, the levels of loss differing only in degree dependent upon the precise levels of intratgastric bacterial nitrate reductase activity which begins to increase above pH 4 (Milton-Thompson *et al.*, 1983).

5.4.2
Nitrite metabolism

The reactions to which this nitrite would be prone would include further bacterial reduction to ammonia and/or gaseous nitrogen oxides and, theoretically, bacterially catalyzed N-nitrosation. Although there is some epidemiological evidence to support Correa's (1983) proposal that achlorhydria (Caygill *et al.*, 1987) and therefore by analogy bacterial N-nitrosation, is a fundamental factor in the development of gastric cancer, there have been many conflicting reports as to whether N-nitroso levels are in fact elevated in the stomach (Barnard *et al.*, 1982; Dang *et al.*, 1983; Hall *et al.*, 1987; Keithley *et al.*, 1984; Krytopoulos *et al.*, 1985; Meyrick-Thomas *et al.*, 1987; Milton-Thompson *et al.*, 1983; Reed *et al.*, 1981, 1982; Schlag *et al.*, 1980, 1981; Stockbrugger *et al.*, 1982; Walters *et al.*, 1982). However, the review by Pignatelli *et al.* (1987), and reappraisal of the methods used to determine total N-nitroso compounds has cast doubt on findings reported by some workers in which increased levels of N-nitroso compounds were not found in achlorhydric gastric juice, and further showed increased levels of N-nitroso compounds in such gastric juice using a revised method. The fact that bacterial N-nitrosation ability may be restricted to only a few species (Leach, 1988) and that the metabolic processes by which N-nitrosation occurs varies considerably between species (Leach, 1988) is another confounding factor with regard to increased N-nitroso compounds formation in the achlorhydric stomach. Bacterial N-nitrosation is discussed more fully in Chapter 6.

5.5 Conclusions

Ingested nitrate and presumably endogenously-formed nitrate are distributed to various degrees throughout different body compartments. In several of these, bacterial colonization and infection can result in the transformation of nitrate to nitrite by the action of bacterial nitrate reductases. Thus in the mouth, the achlorhydric stomach and the infected urinary bladder, the bacterial metabolism of nitrate provides a dominant reservoir of nitrite exposure, since dietary nitrite levels are exceptionally low by comparison with the levels in bacterially colonized sites. In health, approximately 55% of both dietary and endogenously-formed nitrate are excreted in the urine. Of the 45% of nitrate not recovered in the urine, about 20% appears to be lost to bacterial metabolism in the lower gastrointestinal tract. The nitrate entering the lower bowel arrives by secretion across the gut wall and not by bulk flow. The remainder of the nitrate not excreted in urine is thought to be lost as a result of other 'mammalian processes' such as secretion in tears and sweat, etc. In achlorhydric individuals an average of 23% of ingested nitrate is lost to bacterial metabolism in the stomach.

The formation of nitrite from nitrate within bacterially colonized sites of the body may in itself have considerable health implications, specifically from the point of view of nitrite formation in the stomach of infants, thus enhancing methaemoglobinaemia. Although other health effects such as defects of the central nervous system have been proposed to be associated with nitrate exposure in infants the evidence is not as convincing. A further risk from nitrate metabolism comes from bacterial N-nitroso compound formation in various sites of the body, particularly the achlorhydric stomach. All of these reactions will depend on a number of factors such as pH, the level of nitrate ingestion and the species and strains of bacteria present.

Under ordinary circumstances, nitrate exposure would appear to be inevitable. The degree to which different populations and groups are exposed can differ significantly, however, depending on factors such as the type of diet, water nitrate content, soil type, and agricultural practices. Where there may be a health risk associated with some populations and individuals, control of the extent of nitrate exposure should be possible. However, there is still considerable controversy regarding the role of nitrate in gastric cancer with epidemiological studies reporting conflicting findings. To date various methods have been used to determine nitrate exposure, several of which have now been shown to be flawed. Realistically, a total reassessment of the role of dietary nitrate in the epidemiology of gastric cancer is needed, determining nitrate exposure by means of 24-hour urine samples.

References

Alsobrook, A.J., Duplessis, L.S., Harrington, J.C., Nunn, A.J. and Nunn, J.R., 1974, Nitrosamines in the human vaginal vault, in Bogovski, P. and Walker, E. (Eds) *N-nitroso Compounds in the Environment,* pp. 197–201, Lyon: IARC Scientific Publication.

Arbuckle, T.E., Sherman, G.J., Corey, P.N., Walters, D. and Lo, B., 1988, Water nitrates and CNS birth defects: a population-based case-control study, *Arch. Environ. Health,* **43,** 162–67.

Archer, M.C., 1984, Catalysis and inhibition of N-nitrosation reactions, in O'Neil, I.K., von Borstel, R.C., Long, J.E., Miller, C.T. and Bartsch, H. (Eds) *N-nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer,* pp. 263–74, Lyon: IARC Scientific Publication.

Barnard, J., Bavin, P.M.G., Brimblecombe, R.W., Darkin, D.W., Durant, G.J. and Keithley, M.R.B., 1982, Gastric juice, nitrite and N-nitroso compounds, in Barnes, P.E. (Ed.) *Nitrosamines in Human Cancer,* Banbury Report No. 12, pp. 369–77, New York: Cold Spring Harbor.

Bartholomew, B. and Hill, M.J., 1984, The pharmacology of dietary nitrate and the origin of urinary nitrate, *Food Chem. Toxicol.,* **19,** 297–310.

Bingham, S., 1987, The dietary assessment of individuals: methods, accuracy, new techniques and recommendations, *Nutr. Abs. Rev.,* **57,** 705–42.

Calmels, S., Ohshima, H., Vincent, P., Gounot, A-M. and Bartsch, H., 1985, Screening of microorganisms for nitrosation catalysis at pH 7 and kinetic studies on nitrosamine formation from secondary amines by *E. coli* strains, *Carcinogenesis,* **6,** 911–15.

Caygill, C.P.J., Leach, S.A., Kirkham, J.S., Northfield, T.C., Hall, C.N. and Hill, M.J., 1987, Gastric hypochlorhydria as a risk factor for gastric and other cancers, in Bartsch, H., O'Neill, I. and Schulte-Hermann, R. (Eds) *The Relevance of N-nitroso Compounds to Human Cancer: Exposures and Mechanisms.* IARC Scientific Publication No. 84, pp. 524–26.

Charnley, G., Tannenbaum, S.R. and Correa, P., 1982, in Magee, P.N., *Nitrosamines and Human Cancer,* Banbury Report No. 12, Cold, pp. 503–22, New York: Spring Harbor Laboratory Press, Cold Spring Harbor.

Choury, D., Reghis, A. and Pichard, A.L., 1983, Endogenous proteolysis of membrane-bound red cell cytochrome b5 reductase in adults and newborns: its possible relevance to the generation of soluble methaemoglobin reductase, *Blood,* **61,** 894–98.

Comley, H.H., 1945, Cyanosis in infants caused by nitrate in well water, *JAMA,* **129,** 112–16.

Correa, P., 1983, The gastric precancerous process, *Cancer Surveys,* **2,** 437–50.

Dang Vu, B., Paul, J.R., Ekindjian, O.G., Younger, J., Gaudric, M. and Guerre, J., 1983, Chemiluminometry of nitrite and N-nitroso compounds in gastric juice, *Clin. Chem.,* **29** (**10**), 1861.

Dolby, J.M., Webster, A.D.B., Borriello, S.P., Barclay, F.E., Bartholomew, B. and Hill, M.J., 1984, Bacterial colonisation and nitrite concentration in the achlorhydric stomach of patients with primary hypogammaglobinaemia and classical pernicious anaemia, *Scand. J. Gastero.,* **19,** 105–10.

Dorsch, M.M., Scragg, R.K.R., McMichael, A.J., Baghurst, P.A. and Dyer, K.F., 1984, Congenital malformations and maternal drinking water supply in rural South Australia: A case control study, *Am. J. Epidemiol.,* **119,** 473–86.

Drasar, B.S., Shiner, M. and McCleod, G.M., 1964, Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons, *Gastroenterology,* **56,** 71–79.

Forman, D., Al-Dabbagh, S. and Doll, R., 1985, Nitrates, nitrites, *Nature,* **313,** 620–25.

Gelperin, A., Moses, V.K. and Bridger, C., 1975, Relationship of high nitrate community water supply to infant and fetal mortality, *Illinois Med.,* **147,** 155–57.

Granli, T., Dahl, A., Brodlin, P. and Bockman, O.C., 1989, Nitrate and nitrite concentration in human saliva: variations with salivary flow rate , *Fd. Chem. Toxicol.,* **27,** 675–80

Green, L.C., Wagner, D.A., Ruiz de Luzuriaga, K., Istfan, N., Young, V.R. and Tannenbaum, S.R., 1981, Nitrate biosynthesis in man, *Proc. Nat. Acad. Sci.,* **678,** 7764–68.

Hall, C.N., Darkin, D., Viney., Cook, A., Kirkham, J.S. and Northfield, T.C., 1987, Evaluation of the Nitrosamine hypothesis of gastric carcinogenesis in man, in Bartsch, H., O'Neill, I. and Schulte-Hermann, R. (Eds) *The Relevance of N-nitroso Compounds to Human Cancer: Exposures and Mechanisms,* IARC Scientific Publication No. 84, pp. 527–30, Lyon: IARC.

Hartman, P.E., 1982, Nitrate and nitrites: ingestion, pharmaco-dynamics and toxicology, in de Serres, F.J. and Hollander, A. (Eds) *Chemical Mutagens,* Vol. 7, pp. 211–94, Plenum Publishing Corporation.

Hicks, R,M., Walters, C.L., Elsebai, I., El Aasser, A.B., El Merzabini, M. and Gough, T.A., 1977, Demonstration of nitrosamines in human urine: preliminary observations on possible etiology of bladder cancer in association with chronic urinary tract infection, *Proc. Roy. Soc. Med.,* **70,** 413–17,

Keithley, M.R.D., Young, D., Poxon, D., Morris, D., Muscroft, T.J., Burdon, D.W., Barnard, J., Bavin, P.M.G., Brimblecombe, R.W., Darkin, D.W., Moore, P.J. and Viney, N., 1984, Intragastric nitrosation is unlikely to be responsible for gastric carcinoma developing after operations for duodenal ulcer; *Gut,* **5,** 238–45.

Knight, T.M, Forman, D., Al-Dabbagh, S.A. and Doll, R., 1987, Estimation of dietary intake of nitrate and nitrate in Great Britain, *Fd. Chem. Toxicol.,* **5,** 277–85.

Krytopoulos, S.A., Daslalakis, G., Legakis, N.I., Kondiaris, N., Psarrou, E., Bonatos, G., Golematis, B., Lakootis, G., Bhouras, N. and Outram, J.R., 1985, Studies in gastric carcinogenesis. II. Absence of elevated concentrations of N-nitroso compounds in gastric juice of Greek hypochlorhydric individuals, *Carcinogenesis,* **6 (8),** 1141–45.

Leach, S.A., 1988, Mechanisms of endogenous nitrosation, in Hill, M.J. (Ed.), *Nitrosamines: Toxicology and Microbiology,* pp. 69–87, Chichester: Ellis Horwood.

Leach, S.A. and Hill, M.J., 1990, The effects of sustained and profound inhibition of gastric acid production on gut flora, in Elder, J.B. (Ed.), *Profound Gastric Acid Suppression: A Long Term Safety Risk?,* pp. 139–51, Research and Clinical Forums 12.

Leach, S.A., Thompson, M.H. and Hill, M.J., 1987, Bacterially catalysed N-nitrosation reactions and their relative importance in the human stomach, *Carcinogenesis,* **8,** 1907–12.

Leaf, C.D., Vecchio, A.J., Roe, D.A. and Hotchkiss, J.H., 1987, Influence of ascorbic acid dose on N-nitroso compound formation in humans, *Carcinogenesis,* **8,** 791–95.

Licht, W.R., Schultz, D.S., Fox, J.G., Tannenbaum, S.R. and Deen, W.M., 1986, Mechanisms for nitrite loss from the stomach, *Carcinogenesis,* **7,** 1681–87.

Malagelada, J-R., Robertson, J.S., Brown, M.L., Remington, M., Duenes, J., Thonforde, G.M. and Carryer, P.W., 1984, Intestinal transit of solid and liquid components of a meal in health, *Gastroenterol,* **87,** 1255–63.

Malberg, J.W., Savage, E.P. and Osteryoung, J., 1978, Nitrate in drinking water and the early onset of hypertension, *Environ. Pollut.,* **15,** 155–60.

Mayer, E.A., Thomson, J.B., Jehn, D., Reedy, T., Elashoff, J., Deveny, C. and Meyer, J.H., 1984, Gastric emptying and sieving of solid food and pancreatic and biliary secretions after solid food in patients with non-resective ulcer surgery, *Gastroenterol.,* **87,** 1264–71.

Meyrick-Thomas, J., Misiewicz, J.-J., Cook, A.R., Hill, M.J., Smith, P.L.R., Walters, C.L., Forster, J.K., Martin, L.E. and Woodings, D.F., 1987, Effect of one year's treatment with Ranitidine and of truncal vagotomy on gastric contents, *Gut,* **28,** 726–38.

Milton-Thompson, G.J., Ahmet, Z., Lightfoot, N.F., Hunt, R.H., Barnard, J., Brimblecombe, R.W., Moore, P.J., Bavin, P.M.G., Darkin, D.W. and Viney, N., 1983, Intragastric acidity, bacteria, nitrite and N-nitroso compounds, before, during and after Cimetidine treatment, *Lancet,* **I,** 1091–95.

Mirvish, S.S., 1992, The significance for human health of nitrate, nitrite and N-nitroso compounds, in Bogardi, I. and Kuzelka, R.D. (Eds) *Nitrate Contamination: Exposure, Consequence and Control,* pp. 253–66, Berlin, Heidelberg: Springer-Verlag.

Morton, W.E., 1971, Hypertension and drinking water constituents in Colorado, *Am. J. Publ. Health,* **61,** 1371–78.

Mueller, R.L. and Henninger, H., 1985, The human gastric mucous gel-an endogenous nitrate recevoir, *Zbl. Bakt.,* **b,** 181.

Mueller, R.L., Hagal, H.-J., Greim, G., Ruppin, H. and Domschke, W., 1986, Nitrate and nitrite in normal gastric juice: precursors of endogenous N-nitroso compound synthesis, *Oncology,* **43,** 50–3.

Ohshima, H., Calmels, S., Pignatelli, B., Vincent, P. and Barstch, H., 1987, Nitrosamine formation in urinary tract infection, in Barstch, H., O'Neill, I. and Schulte-Hermann, R. (Eds) *The Relevance of N-nitroso Compounds to Human Cancer: Exposures and Mechanisms,* Scientific Publication No. 84, pp. 384–90, Lyon: IARC.

Packer, P.J., Leach, S.A., Randell, P.R. and Hill, M.J., 1988, Further evaluation of urinary nitrate as a measure of nitrate exposure in relation to human cancer, *Cancer Lett.*, **39 (Suppl.),** S37.

Packer, P.J., Leach, S.A., Duncan, S.N., Thompson, M.H. and Hill, M.J., 1989, The effect of different sources of nitrate exposure on urinary nitrate recovery in humans and its relevance to the methods of estimating nitrate exposure in epidemiological studies, *Carcinogenesis,* **10,** 1989–96.

Packer, P.J., Van Acker, B., Reed, P.I., Haines, K., Thompson, M.H., Hill, M.J. and Leach, S.A., 1990, The effect of gastric achlorhydria on the urinary recovery of nitrate in man: relevance of urinary nitrate as a measure of dietary nitrate exposure, *Carcinogenesis,* **11,** 1373–76.

Petukhov, N.I. and Ivanov, A.V., 1970, Investigation of certain psychophysiological reactions in children suffering from methaemoglobinaemia due to nitrates in water, *Hyg. Sanit.,* **35,** 29–32.

Pignatelli, B., Richard, I., Bourgade, M.-C., and Bartsch, H., 1987, An improved method for analysis of total N-nitroso compounds in gastric juice, in Bartsch, H., O'Neill, I. and Schulte-Hermann, R. (Eds) *The Relevance of N-nitroso Compounds to Human Cancer: Exposures and Mechanisms,* IARC Scientific Publication No. 84, pp. 527–30, Lyon: IARC.

Reed, P.I., Haines, K., Smith, P.L.R., House, F.R. and Walters, C.L., 1981, Effect of Cimetidine on gastric juice N-nitrosamines concentration, *Lancet,* **II,** 553–56.

Reed, P.I., Haines, K., Smith, P.L.R., House, F.R. and Walters, C.L., 1982, The effect of Cimetidine on intragastric nitrosation in man, in Barnes, P.E. (Ed.) *Banbury Report No. 12,* pp. 351–62, New York: Cold Spring Harbor.

Rowland, I.R., Granli, T., Bockman, O.C., Key, P.E. and Massey, R.C., 1991, Endogenous N-nitrosation in man assessed by measurement of apparent total N-nitroso compounds in faeces, *Carcinogenesis,* **12,** 1395–401.

Ruddell, W.S.J., Bone, E.S., Hill, M.J. and Walters, C.L., 1978, Pathogenesis of gastric cancer in pernicious anaemia patients, *Lancet* **I,** 521–22.

Schlag, P., Bockler, R., Peter, M. and Herforth, C.H., 1981, Nitrite and N-nitroso compound in the operated stomach, *Scand. J. Gastro.,* **Suppl. 16,** 63–69.

Schlag, P., Ulrich., H., Merkle, D., Bockler, R., Peter, M. and Herforth, C.H., 1980, Are nitrite and nitroso compounds in gastric juice risk factors for carcinoma in the operated stomach? *Lancet,* **I,** 727–29.

Shuker, D.E., 1988, The chemistry of N-nitrosation, in Hill, M.J. (Ed.) *Nitrosamines: Toxicology and Microbiology,* pp. 48–68, Chichester: Ellis Horwood.

Spiegelhalder, B., Eisenbrand, G. and Pruessman, R., 1976, Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in-vivo* formation of N-nitroso compounds, *Food Cosmet. Toxicol.,* **14,** 545–48.

Stephany, R.W. and Schuller, P.L., 1980, Daily intakes of nitrate, nitrite and N-nitrosamines in The Netherlands using duplicate portion sampling technique, *Oncology,* **37,** 203–10.

Stockbrugger, R.W., Cotton, P.B., Eugenides, N., Bartholomew, B., Hill, M.J. and Walters C.L. 1982, Intragastric nitrites, nitrosamines and bacterial overgrowth during Cimetidine treatment, *Gut,* **23,** 1048–54.

Super, M., Heese, H., MacKenzie, D., Dempster, W.S., duPless, J. and Ferreirra, J.J., 1981, An epidemiologic study of well water nitrates in a group of South West African Namibian infants, *Water Res.,* **15,** 1265–70.

Tannenbaum, S.R., Sinksy, A.J., Weisman, M. and Bishop, W., 1974, Nitrite in human saliva, its possible role in Nitrosamine formation, *J. Natl. Cancer, Inst.,* **53,** 79–84.

Tannenbaum, S.R., Moran, D., Rand, W., Cuello, C. and Correa, P., 1979, Gastric cancer in Columbia IV: nitrite and other ions in gastric contents of residents from a high risk region, *J. Nat. Cancer Inst.,* **69,** 9–12.

Theilade, E., 1989, Factors controlling the microflora of the healthy mouth, in Hill, M.J. and Marsh, P.D. (Eds) *Human Microbial Ecology,* pp. 1–35, Florida: CRC Press, Boca Raton.

Tricker, A.R., Mostafa, M,H., Speigelhalder, B. and Preussmann, R., 1989, Urinary excretion of nitrate, nitrite and N-nitroso compounds in schistosomiasis and Bilharzia bladder cancer patients, *Carcinogenesis,* **10,** 547.

Wagner, D.A., Schultz, D.S., Deen, W.M., Young, V.R. and Tannenbaum, S.R., 1983, Metabolic fate of an oral dose of [^{15}N]-labelled nitrate in humans: effect of diet supplementation with ascorbic acid, *Cancer Res.,* **43,** 1921–25.

Walters, C.L., Downs, M.J., Edwards, M.W. and Smith, P.L.R., 1978, Determination of N-nitrosamines in a food matrix, *Analyst,* **103,** 1127–33.

Walters, C.L., Smith, P.L.R., Reed, P.I., Haines, K. and House, F.R., 1982, N-nitroso compounds in gastric juice and their relationship to gastroduodenal disease, in Barsch, H., O'Neill, I., Castregnaro, M., Okado, M. and Davies, W. (Eds) *N-nitroso Compounds: Occurrence and Biological Effects,* IARC Scientific Publication No. 41, pp. 345–56, Lyon: IARC.

Weisenburger, D.D., 1992, Potential health consequences of contamination of ground water in Nebraska, in Bogardi, I. and Kuzelka, R.D. (Eds) *Nitrate Contamination: Exposure, Consequence and Control,* pp. 309–16, Berlin, Heidelberg: Springer-Verlag.

White, R.J., 1983, Nitrate in British waters, *Aqua,* **2,** 51–57.

Chapter 6
N-nitroso compounds

S.A.Leach

6.1
Introduction

N-nitroso compounds (NNC) have in common the possession of an >N–N=O moiety. They are otherwise a very structurally diverse and heterogeneous group. Of the many investigated, the majority have the ability to bring about the alkylation of DNA and other cellular constituents and thus have mutagenic and carcinogenic potential (Preussmann and Stewart, 1984). Some may also have acute toxic effects or may be teratogenic. Human exposure to N-nitroso compounds would appear to be all but unavoidable, though the levels of exposure in some instances can be minimized (Hill, 1988).

NNC are often considered chemically to be derivatives of amino compounds from which they can be formed by N-nitrosation reactions. Their structural diversity is reflected in marked differences in their lexicological, pharmacological, physical and chemical properties. Based on their chemical structure they can be divided into several broad groups; for example the N-nitrosamines (aliphatic and aromatic), N-nitrosamides, N-nitrosoureas, N-nitrosoguanidines, N-nitrosoamino acids and N-nitrosopeptides are each based on a corresponding parent amine class. Of these, the N-nitrosamines have been the most intensively studied, largely because of their relative ease of analysis, which in turn is related to their relative stability and volatility (Massey, 1988).

Generally, nitrosamines are reasonably stable and require metabolic activation by mammalian enzyme systems before they can exert their mutagenic and carcinogenic effects. N-nitrosamides, nitrosoureas and nitrosoguanidines by contrast are intrinsically more reactive and unstable at neutral and alkaline pH, and can bring about DNA alkylation without metabolic activation (Rowland, 1988). As a consequence they usually cause tumours at their site of application, whereas N-nitrosamines tend to cause tumours remote from their site of application and often show a tropism for a particular organ or tissue. In this case the tumour site may depend on several factors, for example, the species of test animal, the route of administration and the dose. Volatile alkylnitrosamines such as N-nitrosodimethylamine (NDMA) are probably distributed quickly throughout the body once in the blood stream, as has been demonstrated by the intravenous administration of labelled NDMA into mice (Johansson and Tjalve, 1978). Absorption from the gut, however, may be much less uniform, the uptake of NDMA being predominantly from the upper small intestine in the rat with little absorption from the stomach or caecum (Pegg and Perry, 1981). The liver has been demonstrated to be the primary site for the metabolism of many N-nitrosamines

carried in the blood. Here enzyme systems such as the cytochrome p450-dependent mixed-function oxidases, and hepatic demethylases are thought to give rise ultimately to the active alkylating intermediates (Rowland, 1988). In the case of the mixed-function oxidases this is considered to occur through the initial a-hydroxylation of the nitrosamine or, in the case of the longer-chain alkylnitrosamines, also through for example β- and γ-hydroxylation. The hydroxylated product is then thought to yield the diazonium hydroxide and finally the alkylating agent, the alkylcarbonium ion, which attacks DNA and other cellular macromolecules. Whilst the role of the liver in nitrosamine metabolism has been much studied, it is also clear that other tissues have the ability to activate nitrosamines; for example, tissue from the human bladder, colon, bronchus and oesophagus has been demonstrated to have such metabolic activity. Indeed it is suspected that this may explain the organotropic nature of certain nitrosamines in some animal studies.

The metabolism and pharmacokinetics of classes of N-nitroso compounds other than the simpler dialkyl N-nitrosamines are generally less well understood. However, the N-nitrosamides, nitrosoureas etc. and the more recently investigated N-nitroso-peptides are direct-acting carcinogens, as explained previously, and elicit damage at their site of application without the need for metabolic activation. In the more unusual case of the noncarcinogenic N-nitrosoamino acid, N-nitrosoproline (NPRO), it is clear that it is more or less quantitatively recovered in urine without metabolic change. This may also be true for certain of the other N-nitrosoamino acids which appear to have little or no carcinogenic activity and are excreted with little metabolic change. Indeed, the urinary excretion of NPRO has been successfully employed in the NPRO excretion test as a probe to determine the factors involved in modulating endogenous N-nitrosation more generally, both in free-living populations and in experiments with animal models and human volunteers (Ohshima and Bartsch, 1981).

Exposure to NNC occurs through a variety of causes, including use of contaminated cosmetic products, rubber goods, foodstuffs and air (Tricker and Preussmann, 1988). Further, these compounds are also formed endogenously at various sites within the body. With the awareness of the toxicity of N-nitroso compounds, several of the exogenous sources of exposure have been identified and reduced, for example in particular industrial situations, and in those instances where food processing or cosmetic manufacture are responsible. Other factors influencing overall human exposure may be less well identified particularly in relation to endogenously formed NNC. This area will provide the focus for much of the following chapter.

Whilst in many instances it has been difficult to obtain reliable data on NNC levels in body fluids, particularly before the advent of more reliable analytical methods (Massey, 1988), it is clear that NNC are formed endogenously to differing degrees at various sites within the body, including the mouth, the stomach, the lower gastrointestinal (GI) tract, the bladder and the naso-pharynx. Several different chemical mechanisms by which NNC can be formed *in vitro* are known and these have been the subject of several reviews (Mirvish, 1975; Douglass *et al.,* 1978; Challis and Challis, 1982; Williams, 1983; Shuker, 1988). Those mechanisms which are most likely to be of relevance to NNC formation *in vivo* and particularly in the gut will now be considered. In most instances these involve the indirect involvement of nitrate and nitrite and it will be clear from the preceding chapter that these are to be found in most body fluids. The primary source of the nitrate is most usually as a constituent of dietary items, with nitrite being formed locally at bacterially colonized sites, mediated by microbial nitrate reductases.

6.2
N-nitrosation involving nitrite in acidic solution

Nitrite, though not itself a nitrosating agent, in moderately acidic (pH 2–5) aqueous solution produces a range of different chemical species several of which are nitrosating agents (Shuker, 1988). Additional nitrosating agents can also be produced depending on the presence of certain catalysts such as chloride and thiocyanate. The balance between the levels of the different nitrosating agents depends on the pH. At moderately acidic pH and in the absence of catalysts the primary nitrosating agent appears to be N_2O_3, at least for secondary amines, and the subsequent nitrosation reactions of secondary amines usually have pH optima around 3. For the nitrosation of amides and related compounds, which possess moieties that considerably reduce the basicity of the amino nitrogen, the reactive nitrosating agent appears, by contrast, to be $H_2{}^{+}ONO$ and reaction rates increase progressively with decrease in pH without an optimum.

These acid-catalyzed reactions of nitrite are likely to be of significance *in vivo* only in the normal acid stomach since other sites within the body are unlikely to achieve the required low pH values for significant rates of reaction to occur. Nitrite is found in normal gastric juice and certainly gives rise to endogenous NNC formation there. The relatively low concentrations of nitrite, however, probably severely limit the levels of subsequent NNC formation. In health the major source of this intragastric nitrite arises as a consequence of the swallowing of saliva. It will be clear from the preceding chapter that both during its first pass with a meal and subsequently during its salivary recirculation, ingested nitrate will be prone to the action of the bacterial nitrate reductases of many of the members of the oral flora. Differences in the composition and metabolic activity of this flora between individuals result in very different levels of conversion of nitrate to nitrite. Thus, the oral flora of the mouth clearly plays an important role in determining the potential extent of intragastric nitrosation (Shapiro *et al.,* 1991) since it is understood that the levels of nitrosating agent, in this case derived from salivary nitrite, are the limiting factors. The concentrations of amino substrates for these reactions, by contrast, are likely to be in large excess in most body fluids and certainly in gastric contents (Challis *et al.,* 1982; Sheppard *et al.,* 1987).

6.3
N-nitrosation involving gaseous nitrogen oxides

The gaseous nitrogen oxides, including NO_2, N_2O_3, N_2O_4 and NOCl, are efficient nitrosating agents at neutral and alkaline pH in aqueous solution and in anhydrous organic solvents (Challis and Challis, 1982; Shuker, 1988). Indeed gaseous nitrogen oxides have been used in the synthetic organic chemistry of several N-nitroso compounds in organic solvents as a more convenient system than the reactions of nitrite in acidic aqueous mixtures. In aqueous systems it is important to note that water and the hydroxide ion also react with these nitrosating agents. However, many amines are able to compete successfully and produce N-nitroso compounds. This is particularly true of the more basic amines with a pK_a greater than 1. Below this, the poorer reactivities of the amines become increasingly important as they compete less well for the nitrosating agent than hydrolysis by water. Amine substrates with significantly lower pK_a, such as may be more typical of amides, show negligible reaction rates.

N-nitrosation reactions brought about by gaseous nitrogen oxides have received attention because of the importance of the latter as environmental pollutants, and *in vivo* experiments have

demonstrated measurable formation of NDMA in whole mice as a consequence of exposure to environmentally relevant levels of NO_2 (Iqbal, 1984). The primary sites identified as being important for nitroso compound formation from exogenous sources of gaseous nitrogen oxides are probably the skin and the lung. However, such reactions may also have relevance to endogenous NNC formation at bacterially colonized sites within the body including the GI tract. This aspect will be highlighted later in relation to the nitrosation reactions of nitrite in aqueous solution at neutral pH mediated by bacterial enzyme systems, and in relation to reactions mediated by activated macrophage and other mammalian cell lines which can produce NO from L-arginine.

6.4
N-nitrosation involving bacterial enzyme systems

From what has been said previously, bacterial enzyme systems in the mouth are clearly indirectly involved in acid-catalyzed N-nitroso compound formation in the normal acid stomach. The enzyme systems which are most important in this respect are the various nitrate and nitrite reductase complexes, the former producing nitrite and the latter consuming it (Payne, 1973; Knowles, 1982; Hochstein and Tomlinson, 1988; Stewart, 1988). The balance between these two enzyme activities across the various species of the oral flora will clearly determine the extent of nitrite accumulation and thus the levels subsequently available for NNC formation in the stomach. There are other circumstances which can similarly lead to bacterially mediated nitrite formation at other sites within the body which are, or become, bacterially colonized, and several of these relate to the GI tract (Leach and Hill, 1990). For example, with the loss of stomach acidity a resident bacterial flora develops there, in what is ordinarily considered to be an essentially sterile organ. This loss of stomach acidity can arise from a number of different therapeutic, surgical, disease and ageing processes. It is also considered that in the lower GI tract and particularly in the colon there is further potential for the bacterially mediated formation of nitrite. However, as will be appreciated from the preceding chapter, most nitrate is rapidly absorbed from the upper small intestine before it reaches any further down the GI tract. In this instance, therefore, nitrate is most likely to gain access through secretion into colonic contents from the blood and not by bulk flow down the GI tract. Any nitrate gaining access in this manner to the lower GI tract, and the nitrite derived from it, must both have extremely short half-lives, given the reducing conditions in this organ. Thus, the measured concentrations of both these ions in the lower GI tract are usually very low. Despite this, probably the best indication that these ions are gaining access to this region of the GI tract comes from comparisons of apparent total N-nitroso compound (ATNC) levels at different sites down the gut, in experimental studies with germ-free and conventional flora (CF) rats (Massey *et al.,* 1988) and in faeces in humans (Rowland *et al.,* 1991). Elevated ATNC levels were observed in both studies at higher dietary nitrate intakes. This was considered to be as a consequence of *in situ* endogenous NNC formation, presumably indirectly from nitrate which had gained access to this location.

In all of these latter instances of bacterially mediated nitrite formation within body fluids, the potential for further reaction to produce N-nitroso compounds seems limited. These sites have a more-or-less neutral pH which favours bacterial colonization and metabolism, but nitrite is ordinarily unreactive in this respect at such pHs. However, it has been long suspected that bacteria themselves may catalyze N-nitrosation at neutral pH at such sites.

Despite controversy regarding earlier studies (Ralt and Tannenbaum, 1981; Leach, 1988), it has now been conclusively demonstrated that certain bacteria can catalyze the formation of NNC from

nitrite at neutral pH. Earlier studies of bacterial N-nitrosation were hampered largely by the lack of a suitably specific and sensitive method for determining low NNC concentrations in such complex matrices as bacterial incubation mixtures. More recent studies, benefiting from the availability of the Thermal Energy Analyzer (TEA, Waltham Mass.), a specific N-nitrosamine detector (Fine *et al.,* 1976), have been able to demonstrate clearly a number of the important characteristics of these reactions and elucidate the potential mechanisms of NNC formation involved. These will be described briefly below.

First, not all bacteria are capable of catalyzing N-nitrosation reactions. Bacterially catalyzed N-nitrosation appears to be a secondary feature of the enzymes involved in bacterial nitrate and nitrite reduction, and is therefore restricted, in the first instance, to those organisms possessing these enzymes. However, not every bacterial species normally possessing these reductase enzymes would appear to demonstrate significant N-nitrosation activity (Leach and Hill, 1990). Although elementary, these observations are of considerable relevance to the potential for NNC formation in the GI tract. Unlike the acid-catalyzed N-nitrosation reactions taking place in the normal acid stomach, which will depend largely on relatively well-characterized factors such as the nitrite concentration, the pH and the presence of catalysts and inhibitors, the extent of the bacterially mediated reactions will depend to a large degree on the precise composition of the bacterial flora in relation to the N-nitrosation activity of its members. This is likely to be a feature which varies considerably between different individuals, given the great variability in the composition of different gastric floras (Mueller *et al.,* 1984; Leach and Hill, 1990).

At least two distinct mechanisms appear to be employed by different bacterial species in the expression of N-nitrosation activity, one involving *nitrate* reductase in nitrate respirers such as *Escherichia coli* and the second involving *nitrite* reductase in denitrifiers such as *Pseudomonas aeruginosa* (Calmels *et al.,* 1988; Leach *et al.,* 1987b; Ralt *et al.,* 1988). Both mechanisms have been shown to display reaction kinetics which are consistent with bacterial enzyme mediation (Calmels *et al.,* 1985; Leach *et al.,* 1985, 1987a) and this is supported by the fact that the reaction in both cases is dependent on the presence of whole live cells. In the main, the specific N-nitrosation activities displayed by these two groups of organisms are very different (Leach *et al.,* 1987a, 1987b). In standardized *in vitro* tests denitrifiers tend to catalyze much more rapid specific rates of reaction than non-denitrifiers, typically by a factor of between 10 and 100. The major reason for the difference in the magnitude of the N-nitrosation rate between these two groups of organisms probably relates to the differential in their abilities to produce nitric oxide (NO) from nitrite. Nitric oxide itself is not a nitrosating agent. However, it is readily oxidized by traces of molecular oxygen to N_2O_3 and N_2O_4, both of which, as indicated earlier, are potent nitrosating agents at neutral pH in aqueous solution.

Thus, some non-denitrifying organisms such as *E. coli* produce small amounts of NO from nitrite during the course of nitrate respiration, a process which ordinarily leads to the formation of ammonia (Goretski and Hollocher, 1988; Ji and Hollocher, 1988a). This probably occurs as a result of an adventitious activity of nitrate reductase acting on nitrite. Certainly, the enzyme activity most likely to be responsible for nitrosation catalysis in these organisms appears to be nitrate reductase (Calmels *et al.,* 1988; Ralt *et al.,* 1988). Furthermore, the anaerobic incubation of these organisms with nitrite has been shown to result in the production of a gaseous species which is itself not a nitrosating agent, but only becomes so once traces of oxygen have been introduced into the incubation mixture. Further experiments have also shown that the nitrosation activity of these bacteria is closely mirrored by their ability to generate NO from nitrite (Ji and Hollocher, 1988b).

Thus NNC formation by nitrate-respiring organisms probably involves three distinct steps. The first is the adventitious enzymatic reduction of nitrite by nitrate reductase to produce NO. The second is the purely chemical oxidation of NO by traces of dissolved molecular oxygen to N_2O_3 and N_2O_4. The third is the transfer of the nitroso group from these nitrosating agents to suitable amino substrates.

The N-nitrosation reactions of denitrifiers, on the other hand, appear to be more closely associated with their dissimilatory nitrite reductase not their nitrate reductase (Calmels *et al.,* 1988; Garber and Hollocher, 1982). This must certainly be true of those denitrifiers which catalyze N-nitrosation but do not possess a nitrate reductase, for example some *Neisseria* spp. (Leach *et al.,* 1987a). The normal pathway for the reduction of nitrite by denitrifiers differs considerably from that of non-denitrifiers (nitrate respirers) in that the products in this case are certain of the gaseous oxides of nitrogen, and ultimately molecular nitrogen itself, rather than ammonia. Whilst nitrous oxide (N_2O) is known to be a free intermediate in the reduction of nitrite by denitrifiers it is not a nitrosating agent. However some denitrifiers may produce free NO as an earlier product of nitrite reduction. In those denitrifiers in which this is the case, this free NO would then go on to produce the nitrosating agents N_2O_3 and N_2O_4 in the same way as the NO generated by the nitrate respirers; that is, as a consequence of chemical oxidation by traces of molecular oxygen. It is not certain, however, whether all denitrifiers produce free NO (Garber and Hollocher, 1981, 1982). In some instances NO may remain bound to the reductase cytochromes as a ferrous nitrosyl. Such ferrous nitrosyls would be expected to be themselves good nitrosating agents, by analogy with the well-known reaction involving nitroprusside (Averill and Tiedje, 1982). However, the nitrosating agent elaborated by *P. aeruginosa* behaves in a very similar fashion to mixed oxides of nitrogen (N_2O_3 and N_2O_4) in relation to its relative reactivity towards a number of secondary amines of differing pK_a (Leach *et al.,* 1991). In contrast, experiments with the ferrous nitrosyl, nitroprusside, demonstrated a rather different pattern of reactivity towards these same amines.

It is not yet clear what are the dominant factors influencing the levels of N-nitrosation activity of different denitrifying bacteria. However, some insight into this may be provided by a denitrification scheme proposed by Averill and Tiedje (1982). In this scheme a range of free and enzyme-bound intermediates linking the reduction of nitrite to nitrous oxide (N_2O) are proposed, alongside a system of appropriate electron transfers. An important aspect is that certain of the proposed enzyme-bound intermediates would have the capacity for the release of free NO, but that the extent of this release would be determined to a large degree by the flux of nitrite and reductants through the sequence. This in turn would be open to influence from interactions between the denitrification apparatus of the particular bacterial species or strain and important environmental influences such as the external redox potential, oxygen tension, the availability of particular electron donors and the nitrite concentration. Thus, the levels of free NO and nitrosating activity may critically depend upon the precise conditions to which the bacteria are exposed.

Thus the extent to which 'bacterial N-nitrosation' is important at any site within the body is likely to vary greatly between individuals depending, as seems likely, on the species composition of the bacterial flora and the conditions to which the latter is exposed.

6.5
N-nitrosation involving mammalian enzyme systems

It is clear from experiments on whole animals, *in vitro* cell cultures and whole tissue explants that nitric oxide (NO) production is a fundamental part of the metabolism of a range of mammalian cell types and may be modulated by a range of effectors. Such is the case, for example, for macrophages (Stuehr and Marletta, 1985, 1987), endothelial cells (Palmer *et al.,* 1987; Moncada *et al.,* 1988), neural tissue (Knowles *et al.,* 1989), neutrophils (McCall *et al.,* 1989), and possibly hepatocytes and Kupffer cells (Billiar *et al.,* 1989). It has been suggested that the NO produced in each case ordinarily serves an intercellular signalling function, for example, in neural tissue by stimulating soluble guanylate cyclase activity. The free NO produced by these cells has a very short half-life. Nitric oxide, as explained earlier, is rapidly oxidized to NO_2, N_2O_3 and N_2O_4 in the presence of oxygen, as will be the case in oxygenated tissues and in the presence of oxyhaemoglobin. These more oxidized nitrogen oxides are readily hydrolyzed to produce nitrite and ultimately nitrate. It is only the latter which is usually observed in whole animals as an increase in the urinary output of this ion following, for example, immune stimulation (Stuehr and Marletta, 1985; Marletta, 1989). Macrophages in culture also clearly demonstrate nitrate and nitrite formation in culture media when stimulated by either the exogenous stimulant *Escherichia coli* lipopolysaccharide (Stuehr and Marletta, 1985) or the endogenous lymphokine interferon-τ (Stuehr and Marletta, 1987). Similar studies have also revealed that the precursor to nitrate and nitrite was the amino acid L-arginine (Iyengar *et al.,* 1987; Hibbs *et al.,* 1987). Use of ^{15}N- and ^{14}C-labelled L-arginine has demonstrated that the N atoms of both nitrate and nitrite are derived exclusively from one of L-arginine's two equivalent guanidino nitrogens, and that citrulline was the other major product (Iyengar *et al.,* 1987; Leaf *et al.,* 1990). Partially purified enzyme activity which produces NO from L-arginine has subsequently been isolated, after centrifugation, in the 100000g supernatant of macrophages. This enzyme activity was only recoverable from cells which had been previously stimulated with LPS and τ-IFN, being completely absent from unstimulated cells.

Concomitant nitrosation of secondary amines by stimulated macrophages has also been demonstrated (Miwa *et al.,* 1987) and attributed to the formation of the intermediate, nitric oxide, which, as explained previously, on oxidation by molecular oxygen would be expected to give rise to the active nitrosating agents N_2O_3 and N_2O_4 N-nitrosation mediated in this manner by mammalian cells may have relevance to NNC formation in the GI tract, particularly at sites which are responding to immune stimulation. For example, the usually chronic infection of the gastric mucosa by *Helicobacter pylori,* an organism linked with gastric disease and cancer, may elicit mammalian cellular responses which locally mediate NO and NNC formation. More acute infections of the lower GI tract by other organisms may similarly lead to the localized formation of NNC. Indeed, several studies of individuals with gastrointestinal infection and diarrhoea have demonstrated concomitant increases in urinary nitrate output, which would be consistent with such processes occurring as a result of stimulation of the immune system (Wagner *et al.,* 1983; Hegesh and Shiloah, 1982; Wettig *et al.,* 1990).

6.6
Relevance to human health

From what has been said above there is considerable potential for the formation of NNC at several sites within the GI tract in man and in many instances the species composition of the bacterial flora, including the activity of pathogens, at each site may have a profound influence on the extent of formation. Thus, considerable interpersonal differences in this respect would be expected to exist which would be difficult or impossible to predict on the basis of existing knowledge. Such underlying differences are in fact observed which make epidemiological studies of the consequences of endogenous NNC exposure difficult to conduct and evaluate (NATO, 1991). Further, despite improvements in analytical procedures it still remains difficult to identify or characterize the bulk of the NNCs which are formed endogenously (Massey, 1988). It is important to identify these compounds if an evaluation of their lexicological significance is going to be possible.

References

Averill, B.A. and Tiedje, J.M., 1982, The chemical mechanism of microbial denitrification, *FEBS Lett.,* **138,** 8–12.

Billiar, T.R., Curran, R.D., Stuehr, D.J., Ferrari, F.K. and Simmons, R.L., 1989, Evidence that activation of Kupffer cells results in production of L-arginine metabolites that release cell-associated iron and inhibit hepatocyte protein synthesis, *Surgery,* **106,** 364–72.

Calmels, S., Ohshima, H., Vincent, P., Gounot, A.-M. and Bartsch, H., 1985, Screening of microorganisms for nitrosation catalysis at pH 7 and kinetic studies on nitrosamine formation from secondary amines by *E. coli* strains, *Carcinogenesis,* **6,** 911–15.

Calmels, S., Ohshima, H. and Bartsch, H., 1988, Nitrosamine formation by denitrifying and non-denitrifying bacteria: Implication of nitrite reductase and nitrate reductase in nitrosation catalysis, *J. Gen. Microbiol.,* **134,** 221–26.

Challis, B.C. and Challis, J.A., 1982, N-Nitrosamines and N-nitrosoimines, in Patai, S. (Ed.), *The Chemistry of Amino, Nitroso and Nitro-compounds and their derivatives,* pp. 1151–223, New York: Wiley.

Challis, B.C., Lomas, S.J., Rzepa, H.S., Bavin, P.M.G., Darkin, D., Viney, N.J. and Moore, P.J., 1982, in Magee, P.N. (Ed.) *Nitrosamines and Human Cancer,* pp. 243–53, Banbury Report 12: Cold Spring Harbor.

Douglass, M.L., Kabacoff, B.L., Anderson, G.A. and Cheng, M.C., 1978, The chemistry of nitrosamine formation, inhibition and destruction, *J. Soc. Cosmet. Chem.,* **29,** 581–606.

Fine, D.H., Rounbehler, D.P. and Sen, N.P., 1976, A comparison of some chromatographic detectors for the analysis of volatile N-nitrosamines, *J. Agric. Fd. Chem.,* **24,** 980–84.

Garber, E.A.E. and Hollocher, T.C., 1981, ^{15}N Tracer studies on the role of NO in denitrification, *J. Biol Chem.,* **256,** 5459–65.

Garber, E.A.E. and Hollocher, T.C., 1982, ^{15}N, ^{18}O Tracer studies on the activation of nitrite by denitrifying bacteria, *J. Biol. Chem.,* **257,** 8091–97.

Goretski, J. and Hollocher, T.C., 1988, Trapping of nitric oxide produced during denitrification by extracellular hemoglobin, *J. Biol. Chem.,* **223,** 2316–23.

Hegesh, E. and Shiloah, J., 1982, Blood nitrates and infantile methaemoglobinaemia, *Clin. Chim. Acta.,* **125,** 107–15.

Hibbs, J.B., Read, J.R., Taintor, R. and Vavrin, Z., 1987, Macrophage cytotoxicity: role of L-arginine deaminase and imino nitrogen oxidation to nitrite, *Science,* **235,** 473–76.

Hill, M.J., 1988, N-nitroso compounds and human cancer, in Hill, M.J. (Ed.) *Nitrosamines: Toxicology and Microbiology,* pp. 142–62, Chichester: Ellis Horwood.

Hochstein, L.I. and Tomlinson, G.A., 1988, The enzymes associated with denitrification, *Ann. Rev. Microbiol.,* **42,** 231–61.

Iqbal, Z.M., 1984, *In vivo* nitrosation of amines in mice by inhaled nitrogen dioxide and inhibition of biosynthesis of N-nitrosamines, in O'Neill, I.K., von Borstel, R.C., Miller, C.T., Lond, J. and Bartsch, H. (Eds) *N-nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer* (IARC Scientific Publications No. 57), pp. 291–300, Lyon: International Agency for Research on Cancer.

Iyengar, R., Stuehr, D.J. and Marletta, M.A, 1987, Macrophage synthesis of nitrite, nitrate and N-nitrosamines: precursors and the role of the respiratory burst, *Proc. Natl. Acad. Sci.,* **84,** 6369–73.

Ji, X.-B. and Hollocher, T.C., 1988a, Reduction of nitrite to nitric oxide by enteric bacteria, *Biochem. Biophys. Res. Comm.,* **157,** 106–8.

Ji, X.-B. and Hollocher, T.C., 1988b, Mechanism of nitrosation of 2, 3-diaminonaphthalene by *Escherichia coli:* Enzymatic production of NO followed by O_2-dependent chemical nitrosation, *App. Env. Microbiol,* **54,** 1791–94.

Johansson, E.B. and Tjalve, H., 1978, The distribution of [^{14}C] dimethylnitrosamine in mice, *Toxicol. Appl. Pharmacol.,* **45,** 565–75.

Knowles, R., 1982, Denitrification, *Microbiol. Rev.,* **46,** 43–70.

Knowles, R.G., Palacios, M., Palmer, R.M.J. and Moncada, S., 1989, Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for the stimulation of the soluble guanylate cyclase, *Proc. Natl. Acad. Sci.,* **86,** 5159–62.

Leach, S., 1988, Mechanisms of endogenous N-nitrosation, in Hill, M.J. (Ed.) *Nitrosamines: Toxicology and Microbiology,* pp. 69–87, Chichester: Ellis Horwood.

Leach, S.A. and Hill, M.J., 1990, The effects of the sustained and profound inhibition of gastric acid production on gut flora, in Elder, J.B. (Ed.) *Profound Gastric Acid Suppression: A LongTerm Safety Risk? Research and Clinical Forums,* **12,** 139–51.

Leach, S.A., Thompson, M.H. and Hill, M.J., 1987a, Bacterially catalysed N-nitrosation reactions and their relative importance in the human stomach, *Carcinogenesis,* **8,** 1907–12.

Leach S.A., Cook, A.R., Challis, B.C., Hill, M.J. and Thompson, M.H., 1987b, Bacterially mediated N-nitrosation reactions and endogenous formation of N-nitroso compounds, in Bartsch, H., O'Neill, I. and Schulte-Herman, R. (Eds) *The Relevance of N-nitroso Compounds to Human Cancer: Exposure and Mechanisms,* IARC Scientific Publications No. 84, pp. 396–99, Lyon: IARC.

Leach, S.A., Challis, B.C., Cook, A.R., Hill, M.J. and Thompson, M.H., 1985, Bacterial catalysis of the N-nitrosation of secondary amines, *Biochem. Soc. Trans.,* **13,** 381–82.

Leach, S.A., Mackerness, C.W., Hill, M.J and Thompson. M.H., 1991, Inhibition of bacterially mediated N-nitrosation by ascorbate: Therapeutic and mechanistic considerations, in O'Neill, I.K., Chen, J. and Bartsch, H. (Eds) *Relevance to Human Cancer of N-Nitrosocompounds. Tobacco Smoke and Mycotoxins,* IARC Scientific Publications No. 105. pp. 571–78. Lyon: IARC.

Leaf, C.D., Wishnok, J.S., Hurley, J.P., Rosenbald, W.D., Fox, J.G. and Tannenbaum, S.R., 1990, Nitrate biosynthesis in rats, ferrets, and humans. Precursor studies with L-arginine, *Carcinogenesis,* **11,** 855–58.

Marletta, D.J., 1989, Nitric oxide: biosynthesis and biological significance, *TIBS.,* **14,** 488–92.

Massey, R.C., 1988, Analysis of N-nitroso compounds in foods and human body fluids, in Hill, M.J. (Ed.) *Nitrosamines: Toxicology and Microbiology,* pp. 16–47, Chichester: Ellis Horwood.

Massey, R.C., Key, P.E., Mallett, A.K. and Rowland, I.R., 1988, An investigation of the endogenous formation of apparent total N-nitroso compounds in conventional microflora and germfree rats, *Food Chem. Toxicol.,* **26,** 595–600.

McCall, T.B., Boughton-Smith, N.K., Palmer, R.M.J., Wittle, B.J.R. and Moncada, S., 1989, Synthesis of nitric oxide from L-arginine by neutrophils, *Biochem. J.,* **261,** 293–96.

Mirvish, S.S., 1975, Formation of N-nitroso compounds: chemistry, kinetics and *in vivo* occurrence, *Tox. Appl. Pharmacol.,* **31,** 325–51.

Miwa, M., Stuehr, D.J., Marletta, M.A., Wishnok, J.S. and Tannenbaum, S.R., 1987, Nitrosation of amines by stimulated macrophages, *Carcinogenesis,* **8,** 955–58.

Moncada, S., Radomski, M.W. and Palmer, R.M.J., 1988, Endothelium-derived relaxing factor: identification as nitric oxide and role in the control of vascular tone and platelet function, *Biochem. Pharmacol.,* **37,** 2495–501.

Mueller, R.L., Hagel, H.-J., Greim, G., Ruppin, H. and Domschke, W., 1984, The dynamics of the endogenous bacterial nitrite formation in the stomach, *Zbl. Bakt. Hyg. I. Abt. Orig. B.* **179,** 381–96.

NATO, 1991, ASI Series G on Ecological Sciences, Vol. 30, *Nitrate Contamination: Exposure Consequence, and Control,* Berlin, Heidelberg: Springer-Verlag.

Ohshima, Y. and Bartsch, W., 1981, Quantitative estimation of endogenous nitrosation in humans by monitoring N-nitrosoproline excreted in urine, *Cancer Res.,* **41,** 3658–62.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S., 1987, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, *Nature,* **327,** 524–26.

Payne, W.J., 1973, Reduction of nitrogenous oxides by micro-organisms, *Bacterial. Rev.,* **37,** 409–52.

Pegg, A.E. and Perry, W., 1981, Alkylation of nucleic acids and metabolism of small doses of dimethylnitrosamine in the rat, *Cancer Res.,* **42,** 3128–32.

Preussmann, R. and Stewart, B.W., 1984, N-nitroso carcinogens, in Searle, C.E. (Ed.) *Chemical Carcinogens* Vol. 2, pp. 643–828, Washington, DC: ACS Monograph 182, American Chemical Society.

Ralt, D. and Tannenbaum, S.R., 1981, The role of bacteria in nitrosamine formation, in Scanlan, R.A. and Tannenbaum, S.R. (Eds) *ACS Symposium Series 174,* pp. 159–64, Washington, DC: American Chemical Society.

Ralt, D., Wishnok, J.S., Fitts, R. and Tannenbaum, S.R., 1988, Bacterial catalysis of N-nitrosation: Involvement of the nar operon of *Escherichia coli, J. Bacterial.,* **170,** 359–64.

Rowland, I.R., 1988, The toxicology of N-nitroso compounds, in Hill, M.J. (Ed.) *Nitrosamines: Toxicology and Microbiology,* pp. 117–41, Chichester: Ellis Horwood.

Rowland, I.R., Granli, T., Bockman, O.C., Key, P.E. and Massey, R.C., 1991, Endogenous N-nitrosation in man assessed by measurement of apparent total N-nitroso compounds in faeces, *Carcinogenesis,* **12,** 1395–401.

Shapiro, K.B., Hotchkiss, J.H. and Roe, D.A., 1991, Quantitative relationship between oral nitrate-reducing activity and the endogenous formation of N-nitrosoamino acids in humans, *Food Chem. Toxicol.,* **29,** 751–55.

Sheppard, S.E., Schlatter, C. and Lutz, W.K., 1987, Assessment of the risk of formation of carcinogenic N-nitroso compounds from dietary precursors in the stomach, *Food Chem. Toxicol.,* **25,** 91–108.

Shuker, D.E.G., 1988, The chemistry of N-nitrosation, in Hill, M.J. (Ed.) *Nitrosamines: Toxicology and Microbiology* , pp. 48–68, Chichester: Ellis Horwood.

Stewart, V., 1988, Nitrate respiration in relation to facultative metabolism in Enterobacteria, *Microbiol. Rev.,* **52,** 190–232.

Stuehr, D.J. and Marletta, M.A., 1985, Mammalian nitrate biosynthesis: mouse macrophages produce nitrate and nitrite in response to *Escherichia coli* lipopolysaccharide, *Proc Natl. Acad. Sci.,* **82,** 7738–42.

Stuehr, D.J. and Marletta, M.A., 1987, Synthesis of nitrate and nitrite in macrophage cell lines, *Cancer Res.,* **47,** 5590–94.

Tricker, A.R. and Preussmann, R., 1988, N-nitroso compounds and their precursors in the human environment, in Hill, M.J. (Ed.) *Nitrosamines: Toxicology and Microbiology,* pp. 88–116, Chichester: Ellis Horwood.

Wagner, D.A., Schultz, D.S., Deen, W.M., Young, V.R. and Tannenbaum, S.R., 1983, Metabolic fate of an oral dose of [^{15}N]-labelled nitrate in humans: effect of diet supplementation with ascorbic acid, *Cancer Res.,* **43,** 1921–25.

Wettig, K., Dobberkau, H.-J. and Flentje, F., 1990, Elevated endogenous nitrate synthesis associated with Giardiasis , *J. Hyg. Epidemiol. Microbiol. Immunol.,* **34,** 69–72.

Williams, D.L.H., 1983, Nitrosation mechanisms, *Adv. Phys. Org. Chem.,* **19,** 381–84.

Chapter 7
Metabolism of nitrogen compounds: miscellaneous compounds

M.J.Hill

7.1
Introduction

Nitrogen compounds are ubiquitous in the diet and the environment and among xenobiotics. Elsewhere in this section there are chapters on the role of ammonia metabolism in nitrogen conservation, the pharmacology of dietary nitrate and the formation of N-nitroso compounds. In this chapter the topics to be reviewed are azo reductase, nitro reductase, amino acid decarboxylase, amino acid deaminase and the metabolism of some specific amino acids. The major initial bacterial metabolites produced from amino acids in the mammalian colon are the results of decarboxylation and deamination (Barker, 1981).

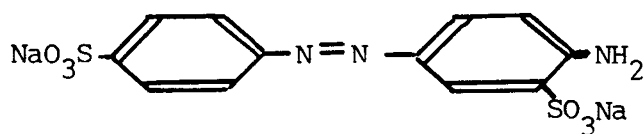
7.2
Diazo compounds

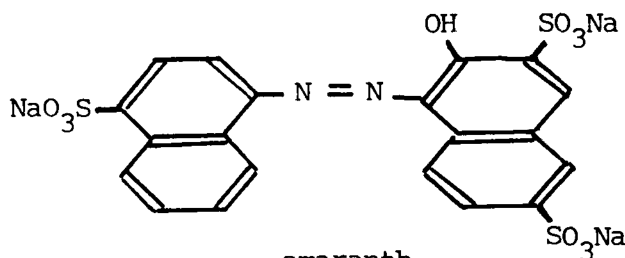
7.2.1
Introduction

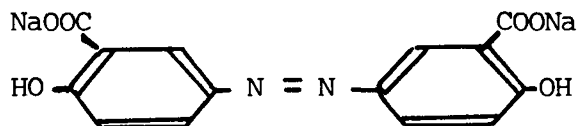
Diazo compounds are formed by the coupling of an aromatic amine or phenol to a diazotized aromatic amine. Because of the intensity and the range of colours available amongst the diazo dyes they are widely used in the textile and printing industries as well as in the food, pharmaceutical and cosmetics industries.

Of the many azo dyes that have been used as food additives in the past 100 years, five are still permitted. These are Tartrazine, Sunset Yellow, Allura Red, Citrus Red No. 2 and Orange B; in total more than 2 million kilograms of the first three of these are used in food products each year. In addition to these permitted food colours a number of other azo dyes have been withdrawn from use because of their carcinogenicity (e.g. Brown FK, Butter Yellow, Amaranth).

Diazo coupling is now widely used in drug delivery systems where it is necessary to release the drug in the colon. An early example of this strategy was salazopyrin (Figure 7.1), used in the treatment of ulcerative colitis, in which an anti-inflammatory agent (5-aminosalicylate) is diazo-coupled to an antimicrobial (sulphapyridine). Interestingly, two of the early widely-used antimicrobials were diazo-coupled sulphanilamide derivatives (prontosil rubrum and neoprontosil);







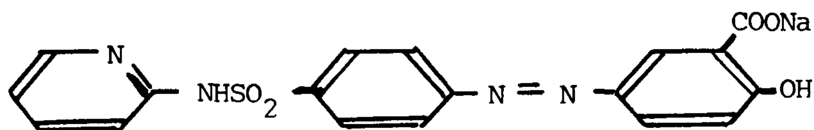


Figure 7.1 Some azo dyes and azo-linked therapeutic agents

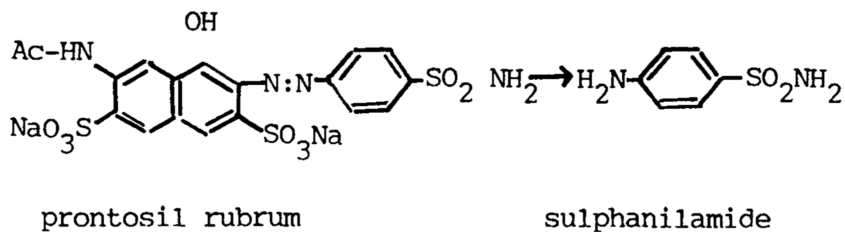


Figure 7.2 Azo reduction of prontosil rubrum to release sulphanilamide

Table 7.1 Bacterial species able to reduce the diazo bond in azo dyes

Reference	*Organisms*
Roxon *et al.* (1967)	*Ps vulgaris* *E. coli*
Scheline and Lonborg (1965)	*Strep. faecalis*
Fouts *et al.* (1967)	*Strep. faecalis*
Drasar and Hill (1974)	Wide range of gut bacterial species
Walker (1970)	Wide range of gut bacterial species
Daniel (1962)	Mixed bacterial genera
Chung (1983)	Wide range of gut bacterial species

Table 7.2 Azoreductase activity in some typical gut bacterial species (data from Drasar and Hill, 1974).

Organism	*Number of strains*	*Azoreductase activity*	
		Mean	*Range*
Facultative			
E. coli	10	0.4	0.1–0.9
Micro–oesophilic			
Strep. faecalis	9	0.9	0.4–2.2
Lactobacillus spp.	10	13.0	0–50.7
Obligate anaerobes			
Bacteroides fragilis	9	0.2	0–0.8
Eubacterium biforme	5	0.3	0.2–0.5
Bif. adolescentis	4	0.8	0–3.1
C1. bifermentans	5	14.6	0–64.6
C1. perfringens	5	26.7	2.2–115.4
Clostridium spp.	9	7.4	0–22.2

however, these agents were introduced in the belief that diazo dyes as such were antimicrobials and only later were recognized as delivery systems for the true antibiotic.

7.2.2 Bacterial azoreductase

The diazo linkage in azo food colours is readily reduced by a wide range of gut bacterial species and by mixed populations of gut bacteria to release the parent aromatic amines (Figure. 7.2); the azo reductase activity is readily detected (and quantified) from the loss of pigment, since the parent amines give colourless solutions. Table 7.1 lists some of the genera and species of gut bacteria demonstrated to have azoreductase activity whilst Table 7.2 shows data on the relative rates of azoreduction when salazopyrin was the substrate.

Production of azoreductases was the subject of many reviews in the 1960s (e.g. Daniel, 1962; Roxon *et al.*, 1967; Walker, 1970). Early studies of bacterial azoreductase assumed the enzymic nature of the reaction (Drasar and Hill, 1974), and it was observed that the reaction required strictly anaerobic conditions and had an optimum pH close to 7 for *Enterococcus faecalis* but

nearer to 5 for *Clostridium perfringens;* with all strains it was noted that the reaction required actively and anaerobically dividing cells and that no reduction took place when bacteria were incubated with diazo substrate in simple buffers, even when oxygen was excluded. Roxon *et al.* (1967) showed that the azoreductase of *Pseudomonas vulgaris* was an NADPH-specific flavoprotein, whilst Scheline *et al.*, (1970) showed that the enzyme from *Enterobacter faecalis* was a flavoprotein requiring NADH.

Gingell and Walker (1971) demonstrated the importance of flavins in the azo reduction, acting as two-electron shuttles between the flavoprotein and the acceptor azo-bond. This led Walker to propose that the reduction was non-enzymic and merely due to the anaerobic production of flavins. Cytochromes are not directly involved in the reduction although all strains with azoreductase had cytochrome c reductase.

7.2.3 Factors affecting *in vivo* activity

The factors controlling the *in vitro* activity of azoreductase include the bile salt concentration, surface active and wetting agents, chelating agents and the rate of growth. Allan and Roxon (1974) demonstrated a fivefold increase in azoreduction of tartrazine in the presence of bile salts#, which were thought to be acting as surface-active agents. Drasar and Hill (1974) reported that the reaction was inhibited by the surface-active agent sodium lauryl sulphate and by the chelating agent EDTA, but not by the lytic agent toluene. Increased glucose in the reaction mixture increased the rate of azoreduction.

The nature of the substrate is important. In a systematic study by Linecar and colleagues, quoted by Drasar and Hill (1974), of the reduction by a wide range of gut bacterial species of 19 azo dyes, which were permitted as food colours at that time in the UK, it was noted that those dyes with sulphonated aromatic amines on both sides of the diazo bond (i.e. the most water-soluble dyes) were the most readily reduced whilst those which yielded at least one unsulphonated product were less readily degraded. The relative activity of the bacterial species was similar to that seen with salazopyrin as the substrate

The *in vivo* studies of Goldin and Gorbach (1976), as part of their investigation of the value of ‘sentinel enzymes’ in detecting changes in the gut bacterial flora, showed that faecal azo reductase activity was higher in rats fed a meat diet than in controls fed a corn-based diet, and these results were confirmed by Wise *et al.* (1983). Table 7.3 shows the results obtained by Mallett *et al.* (1983) on the effects of various types of dietary fibre and of a fibre-free diet. Such results could be due to a change in enzyme activity per cell with no numerical change in the composition of the flora; alternatively they could be due to a change in the composition of the flora with no change in the specific activity of the organisms. This was investigated by Goldin and Gorbach (1977, 1984), who showed that when the composition of the flora was manipulated by the effect of antibiotics or of supplements of *Lactobacillus acidophilus* there were parallel changes in the activity of faecal azoreductase (Table 7.4).

Table 7.3 The effect of diet on azo reductase activity (Data from various sources).

Diet change	*Effect on azoreductase*
Pectin – 5% supplements	20% decrease
Cellulose – 15% supplement	20–fold increase
CMC – 5% supplement	2.3–fold increase
From grain–based to meat–based diet	2–fold increase
From grain–based to meat–based plus tetracycline	No change
Supplements of lactalbumin	No change

Table 7.4 The effect on faecal azo reductase activity of manipulation of the composition of the gut bacterial flora (Goldin and Gorbach, 1977, 1984).

	Azo reductase activity
Basal grain diet	(1.0)
Grain diet plus tetracycline	1.0
Meat diet	1.5–2.0
Meat plus tetracycline	1.0
Meat plus *Lactobacillus* supplements	1.0
Grain plus *Lactobacillus* supplements	1.0

Table 7.5 Some former food colours shown to be carcinogenic because of the products of their azo reduction.

Dye	*Carcinogenic products*	*Reference*
Trypan Blue	*o*–toluidine	Hartman *et al.*, 1978
Ponceau 3R	2,3,5–trimethylaniline	Hartman *et al.*, 1979
Butter Yellow	4–aminodimethylaniline	Chung *et al.*, 1992
Methyl Red	4–aminodimethylaniline	Chung *et al.*, 1992
Orange II	1–amino–2–naphthol	Chung, 1983
Brown FK		Fouts *et al.*, 1967
Red 2G	Aniline	Walker, 1970

7.2.4
Toxicological consequences

The toxicological interest in gut bacterial azoreductase derives from the nature of the reduction products—the aromatic amines—since benzidine, *β*-naphthylamine, and *o*-toluidine are known human bladder carcinogens. Azo compounds enter the digestive tract as food colours and as pharmaceuticals. Most are water-soluble and so are poorly absorbed from the upper digestive tract and consequently they reach the colon in quantitative amounts.

The lower gut is a highly anaerobic and anoxic environment, ideal for azo reductase activity, and it is not surprising that the diazo linkages are quantitatively reduced to release the component aromatic amines (e.g. 1-amino-2-naphthol from Orange II, which is a bladder carcinogen in mice; the human bladder carcinogen *o*-toluidine from Trypan Blue). The carcinogenicity of aromatic amines has been reviewed recently by Crabtree (1992). Many food colours, now no longer used, have been shown to be carcinogenic or teratogenic in animals, as a result of the action of the

reduction products; typical examples of these are shown in Table 7.5. The safety testing of food colours and other additives has been discussed by Lin (1992).

In contrast to these potential hazards associated with azo reduction, the enzyme is put to good use in the design of drug delivery systems needed in the treatment of colorectal disease. The first example of this was the anti-colitis agent salazopyrin, which contains an anti-inflammatory agent—aminosalicylic acid—diazo coupled to an antibiotic—sulphapyridine. If not coupled in this way both components would be absorbed from the upper digestive tract, excreted in the urine, and would never reach the desired site of action. This technique of delivery of drugs to the colon is being widely investigated in the design of new treatments for inflammatory or other disease of the large bowel.

7.3
Nitro reduction

7.3.1
Background

The human body is exposed to a wide range of nitro compounds as drugs (e.g. chloramphenicol, the nitroimidazoles, etc.) and as environmental contaminants produced during the combustion of fossil fuels (e.g. nitrobenzenes, nitrotoluenes, nitropyrenes, etc.). They are readily reduced both by gut bacterial nitro reductase and by the mammalian hepatic enzyme to the corresponding aromatic amino compounds; as with the products of azo reductase activity, the lexicological interest has been mainly directed at the possible carcinogenicity of the amine products rather than the nitro compounds themselves, although the carcinogenicity of the aromatic nitro compounds has been reviewed by Crabtree (1992). The microbial degradation of nitro aromatic compounds has been discussed recently by Higson (1992). The metabolism of the environmental nitro aromatic contaminants has been reviewed by Rickert (1987).

Nitro compounds in general are highly lipophilic and so are easily absorbed from the upper small intestine by passive diffusion. They reach the heavily-colonized regions of the lower gut only if they are either (a) poorly absorbed from the small bowel and so reach the colon directly, or (b) well absorbed but then secreted in bile as hydrophilic metabolites (e.g. chloramphenicol), or (c) well absorbed but then re-enter the colon through passive or active secretion across the gut mucosal barrier (e.g. metronidazole). In most whole animal studies the metabolites seen in the urine or faeces are the products both of hepatic and of gut bacterial metabolism.

7.3.2
Gut bacterial nitro reductase

Nitro reductase enzymes are commonly produced by the anaerobic components of the human large bowel flora, and this led Goldin and Gorbach (1976) to select this enzyme as one of their panel of 'sentinel enzymes' used to monitor the changes in the composition of the faecal flora induced by diet, age, climate and so on. The enzyme requires anoxic conditions for activity *in vitro*—conditions that are readily satisfied in *vivo in* the mammalian colon. Bacterial nitro reductase has many characteristics in common with nitrite reductase; early studies of the enzyme from *Streptococcus*

Table 7.6 Percentage reduction of 1-nitropyrene to 1-aminopyrene during a 6-hour incubation (Howard *et al.*, 1983b)

Bacteroides thetaiotaomicron	95%
Clostridium perfringens	95%
Peptococcus anaerobius	95%
Peptostreptococcus productus	95%
Bifidobacterium infantis	70%
Citrobacter spp.	70%
Lactobacillus acidophilus	20%
E. coli	20%
Salmonella typhimurium	20%

pyogenes suggested that the two activities were expressions of the same enzyme but studies of the *Escherichia coli* product did not confirm this (Saz and Slie, 1954).

The bacterial enzyme has low substrate specificity, acting on a wide range of substituted nitrophenols. The *E. coli* enzyme had a requirement for NAD, a reducing agent and a dicarboxylic acid (e.g. fumarate). The nitro substrate is reduced to an amino product via nitroso and hydroxylamino intermediates (Kiese, 1974); both of these are important in the methaemoglobinaemia produced by nitrobenzene and neither the metabolites nor the methaemoglobin normally produced in conventional rats was seen by Kiese (1974) in germ-free animals.

7.3.3
Reduction of environmental contaminants

Rickert (1987) has reviewed the metabolism of nitrobenzenes, nitrotoluenes and nitropyrenes. Nitrobenzene, fed to rats, was excreted in the urine as the 3- and 4-nitrophenol (as a result of hepatic hydroxylation) and 4-hydroxyacetanilide (resulting from nitroreduction by the gut bacteria combined with ring hydroxylation and amine acetylation by the liver) in the studies of Levin and Dent (1982).

Although nitro reduction can occur in several tissues, the absence of methaemoglobinaemia and significant levels of 4-hydroxyacetanilide in germ-free rats (less than 1% compared with more than 16% in conventional rats) confirms the importance of the gut flora to nitro reduction in the whole animal (Levin and Dent, 1982). Caecal contents from the conventional but not the germ-free rats readily reduced the nitro group of nitrobenzene; the first metabolites produced were nitrosobenzene and phenylhy-droxylamine, reaching a maximum at 60 minutes, whilst the final product, aniline, increased steadily to a plateau at 180 minutes (by which time it was virtually the sole metabolite).

Nitrotoluenes are widespread environmental and occupational contaminants; 2-nitrotoluene is genotoxic whilst 2, 6-dinitrotoluene is a hepatocarcinogen in conventional rats (Doolittle *et al.*, 1983) but not in germ-free rats (Mirsalis *et al.*, 1982). In studies of the toxicology of 2, 4-dinitrotoluene, conventional rats excreted 16% of the dose as 2-amino-4-nitrotoluene or 4-N-acetyl-2-nitrotoluene compared with only 2% in germfree rats.

Isolated rat caecal contents or human ileostomy exudate, when incubated with 2, 4-dinitrotoluene under anaerobic conditions produced first 2-nitroso-4-nitrotoluene and 4-nitroso-2-nitrotoluene and eventually 2, 4-diaminotoluene (Guest *et al.*, 1982) illustrating the importance of the gut bacterial

flora in aromatic nitro reduction. No hydroxylamine metabolites were isolated in the above experiments but the authors suggested that this could have been because active intermediates were formed capable of binding directly to DNA. However, although the genotoxicity of nitrotoluenes may be related in this way to microbial nitro reductase activity, the acute toxicity is known to be due to the formation of nitrobenzyl alcohols.

Nitropyrenes, which are common products of combustion and are widespread in urban air particles and diesel exhaust fumes (Wang *et al.,* 1980), can be reduced by gut bacteria to potent mutagens (Howard *et al.,* 1983a). When incubated with rat gut bacteria, 1-nitropyrene was rapidly reduced to 1-aminopyrene (Table 7.6) by various species of anaerobic bacteria but more slowly by some facultative organisms. When fed to germ-free rats 1-nitropyrene was excreted unreduced, whereas it was excreted in faeces as 1-aminopyrene, illustrating the importance of the gut flora *in vivo* (El-Bahoumy *et al.,* 1983).

7.4
Amino acid decarboxylase

7.4.1
Introduction

In normal healthy persons the digestion of dietary protein is virtually complete and the main sources of amino acids to the gut bacteria are desquamated gut mucosal tissues and enzymes involved in digestion. In earlier decades these sources were thought to be unimportant, quantitatively; however it is established (Bone *et al.,* 1976) that in western persons consuming their normal diet, approximately 100 mg of phenol and *p*-cresol are formed in the colon (by bacterial action on phenylalanine and tyrosine), indicating that at least 170 mg of phenolic amino acid reaches the colon per day. Vince (1986) has argued persuasively that protein metabolism rather than urease is the main source of ammonia in the human gut (this topic is dealt with in detail by Jackson in Chapter 4).

7.4.2
Amino acid decarboxylase

Amino acid decarboxylation to yield primary amines has a long history and was, for example, described by Berthelot (1911, 1918), and by Hanke and Koessler (1924), both using pure bacterial strains *in vitro,* and *in vivo* in humans and in animals.

The decarboxylases were investigated in detail by Gale (1946). They are inducible intracellular enzymes with a requirement for pyridoxal phosphate as a co-factor; an exception to this is histidine decarboxylase which has no co-factor requirements. The decarboxylases studied by Gale (1946) were substrate-specific, acting on only a single amino acid; a less substrate-specific enzyme active on a range of amino acid substrates was described by Haughton and King (1961) and others have since been described by, for example, Morris and Fillingame (1974) and Johnson (1977) for example. Although active over a wide range of pH values (e.g. 4.0–7.5) they are usually most active at acidic pH with a pH-optimum close to 5.0 (Hayes and Hyatt, 1974), leading to the suggestion that these enzymes may have a role in controlling the acidity of the local environment.

Table 7.7 Production of arginine, lysene and ornithine decarboxylases by gut bacteria.

Organisms	*Number of strains tested*	*Amino acid decarboxylases*		
		Lys.	*Orn.*	*Arg.*
Faecal isolates				
Enterobacteria	37	25	13	0
Enterococcus faecalis	25	0	3	8
Strep. viridans	9	0	0	0
Bacteroides fragilis	133	0	1	0
Bifidobacteria	31	0	0	0
Eubacterium spp.	4	0	0	0
Fusobacterium spp.	8	0	0	0
Clostridium perfringens	28	0	0	0
Clostridium lec ve	133	0	0	0
Cl. septicum	7	0	7	0
Mucosal isolates	20	11	13	10
NCTC strains				
Mixed anaerobes	9	0	0	0
Cl. septicum NCTC547		0	+	0

Johnson (1975) tested faecal isolates, strains from the National Collection of Type Cultures (NCTC) and strains isolated from colonic mucosa or tumour tissue (Table 7.7) for decarboxylase activity against lysine, arginine or ornithine. There was considerable activity amongst the facultative organisms—the enterobacteria and enterococci—and all seven of the *Cl. septicum* strains decarboxylated ornithine, but there was little other activity amongst the other 349 strains of faecal anaerobes tested. There was similarly little activity amongst the NCTC strains. The strains isolated from the gut mucosa of normal or tumour tissue, however, were extremely active against all three amino acids.

Amongst the enterobacteria the decarboxylases active on lysine, ornithine and arginine are of taxonomic value since *E. coli* and *Salmonella* are active on all three, *Shigella* only decarboxylate arginine, *Klebsiella* only decarboxylate lysine and *Proteus vulgaris* are inactive against all three.

7.4.3 Toxicological significance

The products of amino acid decarboxylases are aliphatic amines, diamines (from the basic amino acids) or omega-amino carboxylic acids (from the acidic amino acids). Many of the primary amines (e.g. histamine, tyramine, octopamine and tryptamine) have well-documented pharmacological activity. Octopamine, a decarboxylation product of tyrosine, has a pharmacological action similar to that of noradrenaline.

The products of decarboxylation of glycine and alanine, methylamine and ethylamine respectively, are absorbed from the gut and, after portal transport to the liver, are finally excreted in urine. The pharmacologically-active amines, also absorbed from the gut and transported in the portal blood to the liver, are detoxified by conjugation (e.g. histamine as its acetate, as demonstrated by Hanke and Koessler (1924)). This effectively prevents these bacterial metabolites from entering the peripheral blood in an active form. A major complication of hepatic failure is a failure to

prevent ammonia and amines from entering the peripheral blood system from the portal blood, resulting in its extreme form in hepatic coma and death.

7.5 Amino acid deamination

7.5.1 Background

Bacteria are able to deaminate amino acids by four main pathways, namely (a) oxidatively, yielding a 2-keto acid; (b) reductively to yield a saturated acid; (c) hydrolytically to yield a 2-hydroxy fatty acid, and (d) removal of the elements of ammonia to yield an unsaturated acid. In addition, *Clostridium* spp. can carry out a mixed fermentation (Stickland) reaction in which a pair of amino acids undergo deamination, one oxidatively and the other reductively. These may be difficult to disentangle in a reducing environment such as the gut, since the products of (a) and (d) are rapidly reduced to the products of (c) and (b), respectively.

7.5.2 Amino acid deamination

Within the gut the most widely reported deamination mechanism is by the reductive pathway (Vince, 1986), since oxidative deamination is favoured by the presence of molecular oxygen—which is absent from the normal gut. However, other electron-acceptors can be used by anaerobes (e.g. keto acids, unsaturated acids or their coenzyme A thiolesters). In *Clostridium* spp. it has been demonstrated that the deamination sequence for both aromatic and non-aromatic amino acids is amino acid to 2-keto acid to 2-hydroxy acid to 2–3-unsaturated acid to saturated acid (Barker, 1981); thus although the final product apparently suggests the reductive route the initial deamination is in fact oxidative.

An example of hydrolytic deamination is the aspartase produced by *Pseudomonas fluorescens,* which yields malic acid from aspartic acid. An example of removal of the elements of ammonia is the aspartase produced by *E. coli,* which yields fumaric acid from aspartic acid (although the product is rapidly reduced to succinic acid unless inhibitors such as toluene are added to prevent it).

7.5.3 Pharmacological significance

The major product of deamination is ammonia, which is absorbed and returns to the liver where it is either utilized in protein synthesis or is detoxified by urea formation. In hepatic failure the ammonia remains in the peripheral blood system where it reaches toxic concentrations, as is described in more detail in Chapter 4.

7.6
Metabolism of specific amino acids

7.6.1
Basic amino acids

The basic amino acids, lysine and ornithine, are decarboxylated to the diamines putrescine and cadaverine respectively. These then undergo oxidative deamination to yield an omega-amino aldehyde, which spontaneously cyclises and then undergoes reduction to yield the cyclic secondary amines piperidine and pyrrolidine respectively.

The ability to deaminate the diamines is widespread amongst fresh isolates of faecal anaerobes (Table 7.8), with the C-5 substrate being preferred to the C-4 diamine by the numerically dominant non-sporing anaerobes.

The cyclic secondary amines are absorbed from the colon and excreted in urine; the amount of piperidine exceeds that of pyrrolidine twofold, consistent with the differences in rate of deamination suggested by Table 7.8.

7.6.2
Tyrosine metabolism

The phenolic amino acid tyrosine may undergo reductive deamination to phloretic acid. Alternatively it may undergo decarboxylation to tyramine. In addition it may undergo fission by tyrosinase to yield ammonia, pyruvate and phenol. Phloretic acid may undergo further side-chain shortening to p-hydroxyphenylacetic acid then decarboxylation to *p*-cresol.

In the normal human colon 50–100 mg of *p*-cresol and 10–20 mg phenol are produced from tyrosine each day and excreted in the urine as their glucuronide or sulphate conjugates (and referred to as the urinary volatile phenols, or UVP). There is clear evidence of the role of the colonic bacterial flora, acting on protein, as shown by Bone *et al.* (1976), and summarized in Table 7.9. In persons who have undergone total colectomy, and who therefore have no colonic bacteria, the excretion of UVP is very low; similar results are obtained with persons who have had bowel cleansing to remove the gut bacteria. In those who have undergone removal of the distal colon the amount of UVP is undiminished, suggesting that the UVP are produced in the proximal colon. The excretion of UVP is increased by increased protein intake and is related to intestinal transit time (Cummings *et al.,* 1976). It is also increased in persons with small bowel overgrowth (Aarbakke and Sjonsby, 1976).

The lexicological interest in tyrosine metabolism derives from the demonstration by Boutwell and Bosch (1959), and Boutwell (1974), that phenol and *p*-cresol are promoters of the experimental skin cancers induced in mice by 7, 12-dimethylbenzanthracene (DMBA). Bakke and Midtvedt (1970) suggested that UVP produced in the colon and returned to the liver by the portal system are responsible for the spontaneous liver tumours in rats. The population data in Table 7.9 suggest that there is no relation between UVP production and colorectal cancer risk (which is very much lower in black South Africans and Finns than in British and Danish persons).

Table 7.8 The deamination of cadaverine and putrescine by gut bacteria.

Organism	*% able to deaminate*	
	Putrescine	*Cadaverine*
Facultative		
E. coli	0	0
Enterococcus	0	0
Obligate anaerobes		
Bacteroides spp.	75	38
Bifidobacterium spp.	63	25
Sporing anaerobes		
Clostridium perfringens	50	63

Table 7.9 The urinary volatile phenols (UVP) in various populations and patient groups.

Population	*UVP (mg/day)*		
	Total	*Cresol/phenol*	*Sample size*
London	68.0	5.0	20
Copenhagen	94.0	6.0	30
Helsinki	71.0	3.1	30
Johannesburg (Black)	72.0	6.6	20
Diet study			
140g protein/day	108.0		6
60g protein/day	74.0		
Patient groups			
Small bowel disease	140.0	2.0	16
Total colectomy	6.0	0.2	10
Left hemicolectomy	62.0	12.0	10
Bowel cleansing	21.0	8.0	10

7.6.3
Tryptophan metabolism

Tryptophan is metabolized *in vivo* by the gut bacterial flora to a wide range of products (Hill, 1986). The most widely studied pathway is the formation of indole, pyruvate and ammonia by the action of tryptophanase; this reaction is used taxonomically in the identification of the enterobacteriaceae, since *Proteus* and *Escherichia* spp. produce tryptophanase whilst *Salmonella* and *Klebsiella* spp. do not. Production of the enzyme by other genera is summarized in Table 7.10.

Tryptophan is decarboxylated to yield the pharmacologically active tryptamine. It is deaminated to indolepropionic acid, which then undergoes side chain shortening to indole acetic acid which is decarboxylated to methyl indole (skatole). In addition it is metabolized through the kynurenine pathway to quinaldic acid, xanthenuric acid, anthranilic acid and a range of related metabolites, reviewed by Hill (1986).

The pharmacological interest in the tryptophan metabolites produced in the colon is derived from the work of Dunning *et al.*, (1950), who showed that, whilst rats fed 2-acetyl-aminofluorene (AAF)

Table 7.10 Production of tryptophanase by human gut bacteria.

Organisms	*Indole +ve*	*Indole –ve*
Enterobacteria	*Escherichia* spp. *Proteus* spp. *Providencia* spp.	*Salmonella* spp. *P. mirabilis* *Klebsiella* spp. *Aerobacter* spp. *Serratia* spp.
Streptococci		All other species
Lactobacilli		All other species
Enterococci		*SE. faecalis*
Bacilli	*B. alvei*	All other species
Propionibacteria	*Prop. acnes*	All other species
Bifidobacteria		All other species
Eubacteria		All other species
Bacteroides	*B. fragilis* *B. melaninogenicus* *B. coagulans*	All other species
Fusobacteria	7/14 species	7/14 species

do not develop bladder tumours, those fed AAF supplemented with tryptophan develop a high incidence of such tumours. The tryptophan could be replaced by indole and by indoleacetic acid. Bryan (1971), developed a bladder implantation test in which the test compound was mixed with cholesterol and implanted in the bladder wall. This allowed the test substance to be delivered slowly into the bladder and, using this test, he was able to demonstrate tumour promoting activity in kynurenine, quinaldic acid, 8-hydroxyquinaldic acid, xanthenuric acid, 3-hydroxyanthranilic acid and 3-hydroxykynurenine. Later more stringent tests have failed to confirm these findings and there is little support currently for the view that tryptophan metabolites are mutagenic or carcinogenic.

References

Aarbakke, J. and Sjonsby, H., 1976, Value of urinary simple phenol and indican determinations in the diagnosis of stagnant loop syndrome, *Scand. J. Gastro.,* **11,** 409–14.

Allan, R.J. and Roxon, J.J., 1974, Metabolism by intestinal bacteria; the effect of bile salts on tartrazine azo reduction, *Xenobiotica,* **4,** 637–43.

Bakke, O.M. and Midtvedt, T., 1970, Influence of germ-free status on the excretion of simple phenols of possible significance in tumour promotion, *Experientia,* **26,** 519.

Barker, H.A., 1981, Amino acid degradation by anaerobic bacteria, *Ann. Rev. Biochem.,* **50,** 23–40.

Berthelot, A., 1911, Récherches sur la flore intestinale: isolement des microbes qui attaquent spécialement les produits ultime de la digestion des protéiques, *Comptes Rend. Acad. Sci.,* **153,** 306–9.

Berthelot, A., 1918, Récherches sur la flore intestinale: contribution a l'étude des microbes producteurs du phenol, *Ann. Inst. Pasteur.,* **32,** 17–36.

Bone, E.S., Tamm, A. and Hill, M.J., 1976, The production of urinary phenols by gut bacteria and their possible role in the causation of large bowel cancer, *Am. J. Clin. Nutr.,* **29,** 1448–54.

Boutwell, R.K. (1974) The function and mechanism of promoters of carcinogenesis, *CRC Crit. Rev. Toxicol.,* **2,** 419–43.

Boutwell, R.K. and Bosch, D.K., 1959, The tumour-promoting action of phenol and related compounds on the mouse skin, *Cancer Res.,* **19,** 413–27.

Bryan, G.T., 1971, The role of urinary tryptophan metabolites in the etiology of bladder cancer, *Am. J. Clin. Nutr.,* **24,** 841–47.

Chung, K.-T., 1983, The significance of azo reduction in the mutagenesis and carcinogenesis of azo dyes, *Mutat. Res.,* **114,** 269–81.

Chung, K.-T., Stevens, S.E. and Cerniglia, C.E., 1992, The reduction of azo dyes by the intestinal microflora, *Crit. Rev. Microbiol.,* **18,** 175–90.

Crabtree, H.C., 1992, Carcinogenicity ranking of aromatic amines and nitro compounds, *Mutat. Res.,* **64,** 155–62.

Cummings, J.H., Hill, M.J., Bone, E.S., Branch, W.J. and Jenkins, D.J., 1976, The effect of meat protein with and without dietary fibre on colonic function and metabolism, *Am. J. Clin. Nutr.,* **32,** 2094–101.

Daniel, J.W., 1962, The excretion and metabolism of edible food colours, *Toxicol. Appl. Pharmacol.,* **4,** 572–94.

Doolittle, D.J., Sherrill, J.M. and Butterworth, B.E., 1983, Influences of intestinal bacteria, sex of the animal and position of the nitro group on the hepatic genotoxicity of nitrotoluene isomers *in vivo, Cancer Res.,* **43,** 2836–42.

Drasar, B.S. and Hill, M.J., 1974, *Human Intestinal Flora,* pp. 85–88, London: Academic Press.

Dunning, W.J., Curtis, M.R. and Malin, M.E., 1950, The effect of added dietary tryptophan on the occurrence of 2-acetylaminofluorene induced liver and bladder cancer in rats, *Cancer Res.,* **10,** 454–59.

El-Bahoumy, K., Sharma, C., Louis, Y.M., Reddy, B. and Hecht, S.S., 1983, The role of the intestinal microflora in the metabolic reduction of 1-nitropyrene to 1-aminopyrene in conventional and germ-free rats and humans, *Cancer Lett.,* **19,** 311–16.

Fouts, J.R., Kamm, J.J. and Brodie, B.B., 1957, Enzymatic reduction of prontosil and other azo dyes, *J. Pharm. Exp. Therap.,* **120,** 291–96.

Gale E.F., 1946, The bacterial amino acid decarboxylases, *Adv. Enzymol.,* **6,** 1.

Gingell, R. and Walker, R., 1971, Mechanism of azo reduction by *Streptococcus faecalis.* II. The role of soluble flavins, *Xenobiotica,* **1,** 231–39.

Goldin, B.R. and Gorbach, S.L., 1976, The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer, *J. Nat. Cancer Inst.,* **57,** 371–75.

Goldin, B.R. and Gorbach, S.L., 1977, Alterations in fecal microflora enzymes related to diet, age, *Lactobacillus* supplements and dimethylhydrazine, *Cancer,* **40,** 2421–26.

Goldin, B.R. and Gorbach, S.L., 1984, Alteration of the intestinal flora by diet, oral antibiotics and *Lactobacillus* decreased production of free amines from aromatic nitro compounds, azo dyes and glucuronides, *J. Nat. Cancer Inst.,* **73,** 689–95.

Guest, D., Schnell, S.R., Rickert, D.E. and Dent, J.G., 1982, Metabolism of 2, 4-dinitrotoluene by intestinal microorganisms from rat, mouse and man, *Toxicol. Appl. Pharmacol.,* **64,** 160–68.

Hanke, M.T. and Koessler, K.K., 1924, On the faculty of the normal intestinal bacteria to form toxic amines, *J. Biol. Chem.,* **59,** 835–53.

Hartman, C.P., Fulk, G.E. and Andrews, A.W., 1978, Azo reduction of trypan blue to a known carcinogen by a cell-free extract of a human intestinal anaerobe, *Mutat. Res.,* **58,** 125–32.

Hartman, C.P., Andrews, A.W. and Chung, K.-T., 1979, Production of mutagen from Ponceau 3R by a human intestinal anaerobe, *Infect. Immunol.,* **23,** 686–89.

Haughton B.G. and King H.K., 1961, Induced formation of leucine decarboxylase in *Proteus vulgaris, Biochem J.,* **80,** 268.

Hayes M.L. and Hyatt, A.T., 1974, The decarboxylation of amino acids by bacteria derived from dental plaque, *Arch. Oral Biol.,* **19,** 361–67.

Higson, F.K., 1992, Microbial degradation of nitroaromatic compounds, *Adv. Appl. Microbiol.,* **37,** 1–19.

Hill, M.J., 1986, *Microbes and Human Carcinogenesis,* London: Edward Arnold.

Howard, P.C., Heflich, R.H., Evans, F.E. and Beland, F.A., 1983a, DNA adducts formed *in vitro* and in *Salmonella typhimurium* upon metabolic reduction of the environmental mutagen 1-nitropyrene, *Cancer Res.,* **43,** 2052–58.

Howard, P.C., Beland, F.A. and Cerniglia, C.E., 1983b, Reduction of the carcinogen 1-nitropyrene to 1-aminopyrene by rat intestinal bacteria, *Carcinogenesis,* **4,** 985–90.

Johnson, K.A., 1975, The Ecology Of The Gut Flora, *FIMLS Thesis,* London: Institute of Med. Lab. Sciences.

Johnson, K.A., 1977, The production of secondary amines by the human gut bacteria and its possible relevance to Carcinogenesis, *Med. Lab. Sci.,* **34,** 131–43.

Kiese, M., 1974, *Methaemoglobinemia: A Comprehensive Treatise,* Cleveland: CRC Press Inc.

Levin, A.A. and Dent, J.G., 1982, Comparison of the metabolism of nitrobenzene by hepatic microsomes and caecal microflora from Fischer 344 rats *in vitro* and the relative importance of each *in vivo, Drug Metabol. Dispos.,* **10,** 450–54.

Lin, C.S., 1992, Evaluating the safety of food and food additives with pharmacokinetic data, *Crit. Rev. Fd. Science Nutr.,* **32,** 191–95.

Mallett, A.K., Wise, A. and Rowland, I.R., 1983, Effect of dietary cellulose on the metabolic activity of the rat caecal microflora, *Arch. Toxicol.,* **52,** 311–17.

Mirsalis, J.C., Hamm, T.E., Sherrill, J.M. and Butterworth, B.E., 1982, Role of gut flora in genotoxicity of dinitrotoluene, *Nature,* **295,** 322–23.

Morris D.R. and Fillingame, R.H., 1974, Regulation of amino acid decarboxylation, *Ann. Rev. Biochem.,* **43,** 303.

Rickert, D., 1987, Metabolism of nitro aromatic drugs, *Drug Metab. Rev.,* **18,** 23–53.

Roxon, J.J., Ryan, A.J. and Wright, S.E., 1967, Reduction of water-soluble azo dyes by intestinal bacteria, *Food Cosmetic Toxicology,* **5,** 367–69.

Saz, A.K. and Slie, R.B., 1954, Reversal of aureomycin inhibition of bacterial cell-free nitroreductase by manganese, *J. Biol. Chem.,* **210,** 407–12.

Scheline, R.R. and Longberg, B., 1965, The absorption, metabolism and excretion of the sulfonated dye acid yellow by rats, *Acta Pharm. Toxicol,* **23,** 1–8.

Scheline, R.R., Nygaard, R.T. and Longberg, B., 1970, Enzymatic reduction of the azo dye acid yellow by extracts of *Streptococcus faecalis* isolated from rat intestine, *Food Cosmetic Toxicology,* **8,** 55–58.

Vince, A., 1986, Metabolism of ammonia, urea and amino acids and their significance in liver disease, in Hill, M.J. (Ed.), *Microbial Metabolism in the Digestive Tract,* pp. 83–105, Boca Raton: CRC Press.

Walker, R., 1970, The metabolism of azo compounds: a review of the literature, *Food Cosmetic Toxicology,* **8,** 659–76.

Wang, C.Y., Lee, M.S., King, C.M. and Warner, P.O., 1980, Evidence for nitroaromatics as direct-acting mutagens of airborne particulates, *Chemosphere,* **9,** 83–7.

Wise, A., Mallett, A.K. and Rowland, I.R., 1983, Dietary protein and caecal microbial metabolism in the rat, *Nutr. Cancer,* **4,** 267–72.

Section 3

Carbohydrate metabolism

Chapter 8
Carbohydrate metabolism

M.J.Hill

8.1
Introduction

Saccharolytic fermentation is the main source of energy for the vast majority of bacteria in the gastrointestinal tract, but there is a wide range of abilities to ferment individual mono- or disaccharides (Table 8.1). These fermentation patterns have formed a vital part of the basis of bacterial taxonomy from the early part of this century (when the ability to ferment lactose was thought to distinguish between commensal and pathogenic bacteria) to the current position (where they are the basis of speciation of many bacterial genera).

There is considerable detailed information on the metabolism of monosaccharides, particularly glucose and fructose, but this will not be discussed in detail in this chapter. All that will be considered here is the qualitative ability to ferment individual monosaccharides and the acid end-products of fermentation produced from them. In the human gut mono- and disaccharides represent only a small fraction of the carbohydrate available to bacteria but the range of glycosides and polysaccharides in the diet must first be hydrolyzed to release the fermentable monosaccharides.

In this chapter there will first be a brief summary of the relevant aspects of mono-saccharide metabolism, then a review of the role of bacterial glycosidases and finally a section on polysaccharide metabolism in the colon.

8.2
Monosaccharide metabolism

A wide range of monosaccharides is available in the diet or can be released from dietary glycosides or polysaccharides. Not all are utilizable by all bacterial species or genera as is illustrated in Table 8.1. Almost all bacterial species are able to utilize glucose and ferment it to pyruvate via the Embden-Meyerhof (EM) pathway—this pathway is present in all of the major gut organisms except the bifidobacteria. The pyruvate is then fermented to a range of fatty acid end-products of fermentation, the pattern of which can be used to identify the producer organism (Table 8.2).

Many monosaccharides are metabolized to glucose-6-phosphate or fructose-6-phosphate and then are metabolized by the EM pathway; consequently the end-products of fermentation of most monosaccharides are similar to those produced from glucose.

Table 8.1 Utilization of monosaccharides by some common species of gut bacteria.

	Ara.	*Fruct.*	*Mann.*	*Rham.*	*Rib.*	*Xyl.*	*Gala.*
Peptococcus spp.	–	–	–	–	–	–	–
Peptostreptocccus spp.	–	+	–	–	–	–	+
B. fragilis	–	+	+	–	–	+	+
B. ovatus	+	+	+	+	+	+	+
Eub. rectale	+	+	+	–	+	+	+
Eub. lentum	–	–	–	–	–	–	–
Prop. acnes	–	+	+	–	–	–	+
L. plantarum	+	+	+	–	+	+	+
Bif. bifidum	–	+	–	–	–		+
Bif. adolescentis	+	+	+	–	+	+	+

Key: Ara.=arabinose; Fruct.=fructose; Mann.=mannose; Rham.=rhamnose; Rib.=ribose; Xyl.=xylose; Gala.=galactose.

Table 8.2 Presence of the Embden-Meyerhof pathway in various genera of gut bacteria (in order of numerical importance in the colon).

Organism	*Count of total bacteria per gram faeces*	*E–M pathway*
Bact. fragilis	10^{11}	Present
Other *Bacteroides* spp.	10^{11}	Present
Bifidobacterium spp.	10^{11}	Absent
Propionibacteria	10^{11}	Present
Eubacteria	10^{10}	Present
Fusobacteria	10^{9}	Present
Lactobacilli	10^{8}	Present
Enterobacteria	10^{7}	Present
Clostridia	10^{7}	Present
Enterococci	10^{6}	Present

8.3 Glycosidases

Monosaccharides are released from conjugated glycosides and from di- and oligosaccharides by the action of specific glycosidases, the principal of which are β-glucosidase, β-galactosidase and β-glucuronidase together with the corresponding α-enzymes of the first two. Unlike the intestinal mucosal enzymes (which are substrate-specific) the bacterial glycosidases are able to hydrolyze a wide range of substrates including a range of artificial substrates useful in enzyme assays. Using such artificial substrates Hawksworth *et al.* (1971) assayed all five enzymes in 50 strains of each of the 6 principal bacteria generally found in the human gut (Table 8.3). Whereas coliforms were the best producers of β-glucuronidase, enterococci were the most active producers of β-glucosidase, while lactobacilli were the best producers of α-glucosidase, α-galactosidase and β-galactosidase.

Table 8.3 Major short-chain fatty acid end-products of glucose fermentation.

Genus	*Formic*	*Acetic*	*Propionic*	*Butyric*	*Lactic*	*Succinic*
Bacteroides	•	+	+	•	+	+
Bifidobacteria	•	+	•	•	+	•
Fusobacteria	•	+	•	+	•	•
Eubacteria	+	•	•	•	•	•
Propionibacteria	•	+	+	•	•	•
Clostridia	•	+	+	+	+	+
Coliforms	+	+	•	•	+	•
Streptococci	•	•	•	•	+	•
Lactobacilli	•	•	•	•	+	•

•=small amounts or absent.

8.3.1 *β*-galactosidase

β-galactosidase, or lactase, is produced by strains of most genera, but is not produced by *Salmonella* spp. or *Shigella* spp. of the major coliform species. The bacterial enzyme has a broad substrate specificity; in particular it will readily hydrolyze lactulose as well as lactose whereas the human intestinal mucosal enzyme is specific for lactose. It is an inducible enzyme in most bacterial genera and so is not produced *in vivo* in the mouth or upper gut where glucose is present. However, in the lower gut there is no such suppression of enzyme induction and the full potential for enzyme production will be expressed. The enzyme is constitutive in strains *of Enterococci*.

The enzyme is highly relevant because in most human populations (and all other animal species) the intestinal mucosal lactase is only produced during infancy and childhood. In consequence when milk or milk products are consumed by such persons, the lactose is not digested in the small bowel but reaches the large bowel where it is metabolized to acid and gas (CO_2 and hydrogen from formate and as by-products of production of other fatty acids). When sufficient lactose is consumed then diarrhoea results, and this proved to be a major problem in populations receiving dried milk as part of food-aid programmes (McCracken, 1970).

In contrast the lack of substrate-specificity in the bacterial enzyme has been utilized in the design of lactulose (fructose-galactose) which is used to generate caecal acid as part of the treatment of hepatic cirrhosis (Bircher *et al.,* 1966; Vince *et al.,* 1974; Weber, 1979).

8.3.2 *β*-glucosidase

This enzyme is widely distributed, but in the study by Hawksworth *et al.* (1971) the *Enterococci* were the most active producers of the enzyme. The human gut mucosal enzyme is an isomaltase which has a narrow substrate range; in contrast the bacterial enzyme will act on a very wide range of substrates. The diet is a rich source of *β*-glucosides (Table 8.4) and release of aglycones from glucosides is responsible for the toxicity of a number of plants (Table 8.5).

Cycad flour, produced from ground cycad nuts, is a major starch source in much of south-east Asia and the Pacific Basin, and historically was even more so. However, before it can be used there is an exhaustive aqueous extraction procedure to remove the cycasin, methylazomethanol-*β*-D-

Table 8.4 Production of glycosidases by the principal genera of gut bacteria. Mean enzyme activity is expressed as μMoles substrate degraded per hour per 10^8 cells.

	α–galase	*β–galase*	*α–glucase*	*β–glucase*	*β–glucuro-nidase*
Escherichia	12.7± 2.1*	42.4± 3.2	5.9± 0.5	5.8± 2.5	24.7±2.1
Enterococci	20.8± 4.3	53.8± 6.0	14.0± 1.1	192.7±19.5	2.9±0.6
Lactobacilli	97.7±12.1	90.6±10.7	26.6±35	26.0± 7.4	1.6±0.2
Bifidobacteria	28.2± 3.8	39.1± 4.7	20.7± 3.0	29.3± 6.0	1.9±0.8
Bacteroides	24.0± 4.8	50.7± 4.9	9.8± 2.0	35.1± 4.8	6.0±3.5
Clostridia	53.1± 8.1	13.7± 2.7	30.1± 6.4	22.1± 5.0	11.3±2.3

*± Standard deviation galase=galactosidase glucase=glucosidase

Table 8.5 Some plant glucosides.

Trivial name	*Composition*
Iso-maltose	6–0–α–glucosyl–β–D–glucoside
Gentiobiose	6–0–β–glucosyl–β–D–glucoside
Melibiose	6–0–α–D–galactosyl–α–D–glucoside
Trehalose	α–glucosyl–α–glucoside
Aesculin	dihydroxycoumarin–β–D–glucoside
Salicin	hydroxymethylphenyl–β–D–glucoside

glucoside. Cycasin is nontoxic to germ-free rodents or when given intravenously to conventional animals; when fed to conventional animals the glucoside (which is resistant to mucosal β-glucosidase) is hydrolyzed by the gut bacterial enzyme to release the aglycone methylazoxymethanol. This last is a potent hepatotoxin in man and rodents and is a potent colon carcinogen in rodents (Laqueur and Spatz, 1968).

Cassava is a widely-used carbohydrate source in many tropical countries, but again it can only be used after exhaustive aqueous extraction to remove the amygdalin which is normally present. Amygdalin is a cyanogenetic glucoside which is not hydrolyzed by mammalian mucosal β-glucosidase but is readily metabolized by the bacterial enzyme to release the benzaldehyde cyanhydrin aglycone. The latter spontaneously hydrolyzes to release cyanide. Similarly, linamarus grasses contain a cyanogenetic glycoside, linamarin, which can be fatal to animals grazing on the grasses.

Cascara sagrada used to be a widely-used laxative; its active components are the β-glucosides of a family of emodin-related compounds. The glucoside has no effect on the mucosa of germ-free animals but the aglycone stimulates colonic motility, giving rise to the cathartic or laxative action. When fed to rodents the aglycone was inactive because it was degraded to an inactive form while in the acid environment of the stomach. The glucoside conjugation is therefore needed to protect the aglycone until it can be released at its site of action in the colon by bacterial action.

Plants of the digitalis family produce a family of cardioactive glycosides related to digoxin, and to the lanatosides. These do not depend for their cardioactive action on release of the aglycone—it is the glycoside which is active—but many have gastrointestinal side-effects which are thought to be caused by the aglycones released by bacterial action.

8.3.3 glucuronidase

A wide range of ingested carcinogens and toxic compounds are detoxified in the liver by hydroxylation and then excreted in bile as the *β*-glucuronide. Such hydrophilic derivatives can then pass through the small intestine and into the colon for faecal excretion. However, the glucuronides may be hydrolyzed by bacterial *β*-glucuronidase and, if this occurs in the ileum, results in release of a lipophilic aglycone which is absorbed and returned via the portal blood system to the liver for reconjugation and resecretion in the bile. This enterohepatic circulation (EHC) of a xenobiote results in its retention in the body for longer than would otherwise occur. The extent of EHC depends on the density of the bacterial flora in the small intestine and on the ease of hydrolysis of the glucuronide by the bacterial enzyme. For example, the small bowel of the rat is heavily colonized whilst that of the rabbit is not; stilboestrol has a half-life within the rat of 5 days compared with less than 24 hours in the rabbit due to differences in EHC. EHC is utilized clinically in the treatment of typhoid with the antibiotic chloramphenicol. The antibiotic is highly effective against *Salmonella typhi* but is absorbed from the small bowel and returned to the liver where it is conjugated as the mono- or diglucuronide. The conjugates have no antibiotic activity and are not absorbed from the small bowel. The infecting *S. typhi,* however, is a good source of *β*-glucuronidase and releases the active antibiotic from its conjugate, thereby allowing the chloramphenicol to have a further bactericidal action on the infecting organism. Thus, EHC allows,chloramphenicol to have a multiple action against gut infections. The conjugate is ultimately excreted in the urine where, again, the most common organisms in urinary tract infection *(E. coli, Klebsiella)* are potent producers of *β*-glucuronidase and so release the antibiotic to act against them.

EHC, if unmonitored, can cause problems in therapy, however. Morphine undergoes EHC and its half-life in the body can vary markedly from the average. In consequence, a standard dosing regime may lead to a build-up of serum levels (in good circulators) or atypically low levels (in rapid excretors); drug levels need therefore to be monitored carefully.

8.4 Polysaccharide breakdown

Although most gut bacteria produce a range of mono- and disaccharidases, such substrates are rarely available directly from the diet in significant amounts. In populations which have no small intestinal lactase but which nevertheless consume milk or milk products (e.g. India) significant amount of lactose may reach the colon. Similarly, in persons consuming lactulose as a stool bulking agent or in diabetics consuming mannitol as a sugar replacement, fermentable disaccharide or sugar alcohol will be available to the colonic bacteria.

Nevertheless, even in such persons the main source of carbohydrate reaching the colon will be undigested starch and indigestible 'dietary fibre'. These can be utilized if they are first broken down by extracellular enzymes to oligosaccharides that are small enough to be transported across the plasma membrane, where they can be digested by intracellular enzymes. Only at that final stage does the organism derive sole benefit from its metabolic activity, whilst the extracellular enzymes benefit the total flora.

Whilst there is a considerable literature on starch digestion there is relatively little on the fraction of starch available to the colonic flora. The situation is reversed with dietary fibre, where although

we have good data on the characteristics of dietary fibre there is little known about the bacterial metabolism of such compounds.

8.4.1
Starch hydrolysis

Starch contains two major polyglucose components, namely amylose and amylopectin. Amylose consists of simple linear chains of glucose linked by α(1–4) bonds; it is readily digested by α-amylase, which hydrolyzes randomly the α(1–4) linkages in the middle part of the chain to yield maltose and maltotriose, both of which can be degraded to glucose by a-glucosidase.

Amylopectin consists of amylose chains linked via branches with α(1–6) linkages, α-amylase is able to digest those parts of the chains between the α(1–6) linkages yielding maltose and maltotriose plus oligosaccharides of 4–6 glucose units and containing the bonds with α(1–6) linkages. There is an iso-amylase which hydrolyses the α(1–6) linkages, but this is much less widely distributed than the α-amylase.

Starch usually consists of about 25% amylose and 75% amylopectin. Starch metabolism must be widespread amongst gut bacteria although there have been few reports of such activity. In a study by Scheppach *et al.* (1988) in volunteers treated with acarbose to induce starch malabsorption, the stool wet weight increased by 68%, the faecal excretion per day of n-butyrate increased by 182% and of total short chain fatty acids by 95%, indicating that starch is a good substrate for gut bacterial metabolism. Many organisms (e.g. some *Streptococci*) use starch granules as reserve energy sources and produce both α- and β-amylases. The products of amylase action are readily taken up and digested by starch-metabolizing bacteria.

Starch is potentially well digested by pancreatic enzymes and until relatively recently it was assumed that all starch was, by definition, digested in the small bowel. However, Stephen *et al.* (1983) demonstrated the passage of some dietary starch into the colon. Presumably in many food sources some amylase-sensitive bonds are physically protected (e.g. by lignified layers) from enzyme attach. In addition they may be sterically protected by, for example, *o*-esterification. In addition, there are fractions of starch, termed ‘resistant starch’, which are much more slowly hydrolyzed by amylases. Carbohydrate analysts have now been able to define specific ‘resistant starches’ (Englyst and Cummings 1987, Englyst *et al.*, 1990), but it is important to stress that these definitions are based on chemical structure and not on physiological behaviour, and it has still to be established that they give a good measure of the starch reaching the colon.

8.4.2
Dietary fibre

‘Dietary fibre’ is defined as the cell wall macromolecules which are not digested by small bowel enzymes and so reach the large bowel. Early attempts to study fibre were based on the assumption that starch and soluble fractions were readily digested. The fraction assayed was termed ‘crude fibre’ and was the fraction remaining after digestion with weak acid and alkali and consisted essentially of cellulose and lignin. However, early studies by nutritionists included a further fraction in fibre termed hemicellulose— essentially an insoluble polysaccharide which is slowly dissolved in hot water. Early studies (e.g. Williams and Olmstedt, 1936) showed that the hemicelluloses were well digested during colonic transit whilst cellulose and lignin are usually only very poorly digested.

Table 8.6 Some examples of plant glucosides with pharmacological activity.

Trivial name	*Aglycone*	*Action*
Amygdalin	Benzaldehyde cyanhyrin	Cyanide toxicity
Linamarin		Cyanide toxicity
Cycasin	Methylazoxymethanol	Hepatotoxin and carcinogen
Cascarasagrada	Emodin analogues	Cathartic

Table 8.7 Starch metabolism by gut bacteria.

Genera/species in which amylase has been reported
Bacteroides vulgatus
Bifidobacterium spp.
Fusobacterium spp.
Butyrivibrio spp.
Bacteroides spp. (unspecified)

Table 8.8 Digestibility of various fibre fractions and sources (data from various sources).

Cellulose	10–50%
Non-cellulose polysaccharide	50–80%
Pectins/gums	85–100%
Ispaghula	85–100%
Lignins	<5%
Agar	60%
Wheat bran	30–40%
Peas	80–90%
Carrot	80–90%

With the revival of interest in fibre in more recent decades soluble polysaccharides have been included within the definition of dietary fibre and more studies have been carried out on pure polysaccharides such as pectin, guar gum, cellulose etc., as well as on mixed fibres from different sources (e.g. sugar cane fibre, rich in lignin; wheat bran; oat bran; potato and pea fibre, etc.). In general, pure polysaccharides are more readily degraded by gut bacteria than are the same polysaccharides in crude material (e.g. cellulose as a pure fraction compared with cellulose in wheat bran). Table 8.8 gives data on the digestibility of various fibre fractions.

In parallel with the progress in the studies of fibre digestibility in the colon there has been considerable progress in the analytical methodology and this has permitted a better definition of the relationship between the components of fibre. Thus, Van Soest (1967) developed a fractionation method using detergents; his acid detergent fibre (ADF) was similar to crude fibre whilst his neutral detergent fibre (NDF) is essentially similar to the insoluble fibre fraction. Southgate (1969) had developed assay methods that could be used in the construction of food tables, based on colorimetric assays. Later Englyst *et al.* (1982) developed assays based on the gas chromatographic separation of monosaccharides. This has allowed a better understanding of the composition of the individual polysaccharides in dietary fibre.

It had long been assumed that dietary fibre, since it was resistant to gut mucosal enzymes, was not metabolized by gut bacteria, and passed through the gut undegraded. This was despite the very good work of Williams and Olmstedt (1936) showing that in fact the hemicellulose fraction is highly degraded. In fact, dietary fibre is a major source of carbon and energy for the (dominant) saccharolytic component of the colonic flora (Vercallotti *et al.,* 1977; Salyers, 1979). However, there has been no parallel increase in our understanding of the detailed microbial fermentation of dietary fibre components. Hoskins and Boulding (1981) and Hoskins *et al.* (1985) have studied the role of bacterial glycosidases in mucin degradation and have identified a function for β-galacto sidase, N-acetylglucosaminidase, α-fucosidase and neuraminidase. Salyers *et al.* (1977a) have studied the degradation of a polyglucan, laminarin, by endo- and exoglucanase of *Bacteroides* spp.; the exoglucanase releases glucose monomers from the non-reducing end of the laminarin chain whilst the endoglucanase hydrolyzes bonds in the middle of the chain to release di-, tri- or oligoglucosides. In the three strains of *Bacteroides* spp. studied by Salyers *et al.* (1977b) the glucanases were intracellular; this contrasted with the glucanases of other species. Salyers *et al.* (1977a,b) investigated the breakdown of a range of polysaccharides by species of *Bacteroides, Bifidobacterium, Eubacterium, Fusobacterium* and *Peptostreptococcus.* Strains of all except the last genus degraded starch, *Bacteroides* spp. and *Eubacterium* spp. metabolized pectin, whilst a hemicellulose containing a mixture of xylan, galactomannan and arabinomannan was degraded by most species of *Bacteroides* and *Bifidobacterium.* None of the organisms tested was able to degrade cellulose *in vitro* even though the purified cellulose used is extensively degraded in the gut. A wide range of colonic bacterial species can utilize cellobiose, which would be expected to be a major product of cellulose degradation. Perhaps consortia of organisms are needed to produce endocellulase or perhaps researchers have simply failed to reproduce the necessary gut conditions for cellulolysis. Betian *et al.* (1977) reported a strain of *Bacteroides* spp. able to degrade cellulose but this ability must be widely occurring.

8.4.3
Products of polysaccharide degradation

The major end-products of fermentation of polysaccharides are ethanol, short-chain fatty acids (SCFA), CO_2, H_2 and methane. The principal SCFAs are formate, acetate, propionate, butyrate, lactate and fumarate. In mixed bacterial populations such as those found in the gut formate is readily degraded by formate lyase to CO_2 and H_2. *Veillonella* spp. are end-chain fermenters using lactate as a major energy source yielding CO_2 and water. In addition, during bacterial synthesis in the colon, considerable amounts of lactate would be utilized in amino acid synthesis.

8.5
Toxicological importance of polysaccharide degradation

The SCFAs are important for healthy bowel function in humans (Hoverstad and Bjorneklett, 1984), being metabolic regulators of ion absorption in the colon (Roediger, 1989). Butyrate is also a major fuel source for colonocytes (Roediger, 1980), and inhibition of fatty acid oxidation causes experimental ulcerative colitis (Roediger and Nance, 1986). This is consistent with an observation by Roediger (1980) that there is impaired butyrate metabolism in the colonic mucosa of colitics, recently confirmed by Chapman *et al.* (1994). Senagore *et al.* (1992) have extended these

observations by demonstrating that enemas of butyrate ameliorate the symptoms of proctosigmoiditis. Interestingly, faecal stream diversion causes an amelioration of the symptoms of inflammatory bowel disease (Winslet *et al.,* 1994) which would suggest that the situation is very much more complex than a simple butyrate effect. Presumably the faecal stream contains irritant factors that are more active than the protective agents.

There have been indications from *in vitro* studies that butyrate may have anti-neoplastic properties. For example, n-butyrate alters chromatin accessibility to DNA repair enzymes (Smith, 1986). This has been discussed at length in the chapter by Csordas. If butyrate had an important effect in the human colon in preventing carcinogenesis it would be expected that patients with colon cancer or colon adenomas would have lower colonic butyrate concentrations than controls, but this is not so (Clausen *et al.,* 1991). Further, as with inflammatory bowel disease, diversion of the faecal stream causes a regression of colorectal polyps suggesting that the causal agents in the gut lumen are more potent than the protective agents.

An intriguing role for starch digestion is via its effect on cell proliferation in the colon and the consequent synthesis of vitamins. The importance of these to the balance of vitamins such as biotin, riboflavin and vitamin K is discussed in detail in Chapter 15.

References

Betian, H.G., Linehan, B.A., Bryant, M.P. and Holdeman, L.V., 1977, Isolation of a cellulolytic *Bacteroides* from human faeces, *Appl. Environ. Microbiol.,* **331,** 1009–10.

Bircher, J., Muller, J., Guggenheim, P. and Haemmerli, U.P., 1966, Treatment of chronic portal-systemic encephalopathy with lactulose, *Lancet,* **i,** 890–93.

Chapman, M.A.S., Grahn, M.F., Boyle, M.A., Hulton, M., Rogers, J. and Williams, N.S., 1994, Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis, *Gut,* **35,** 73–76.

Clausen, M.R., Bonnen, H. and Mortensen, P.B., 1991, Colonic fermentation of dietary fibre to short chain fatty acids in patients with adenomatous polyps and colonic cancer, *Gut,* **32,** 923–28.

Englyst, H.N. and Cummings, J.H., 1987, Digestion of polysaccharide of potato in the small intestine of man, *Am. J. Clin. Nutr.,* **45,** 423–31.

Englyst, H.N. and Kingman, S.M., 1990, Dietary fiber and resistant starch: a nutritional classification of plant polysaccharides, in Kritchevsky, D., Bonfield, C. and Anderson, J.W. (Eds) *Dietary Fiber,* pp. 49–66, New York: Plenum.

Englyst, H.N., Wiggins, H.S. and Cummings, J.H., 1982, Determination of non-starch polysaccharide in plant foods by gas-liquid chromatography of constituent sugars as alditol acetates, *Analyst,* **107,** 307–18.

Hawksworth, G.M., Drasar, B.S. and Hill, M.J., 1971, Intestinal bacteria and the hydrolysis of glycosidic bonds, *J. Med. Microbiol.,* **4,** 451–59.

Hoskins, L. and Boulding, E.T., 1981, Mucin degradation in human colonic ecosystems, *J. Clin. Invest.,* **67,** 163–72.

Hoskins, L.C., Augustines, M., Mckee, W.B., Boulding, E.T., Kriaris, M. and Niedenmeyer, G., 1985, Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins, *J. Clin. Invest.,* **75,** 944–53.

Hoverstad, T. and Bjorneklett, A., 1984, Short chain fatty acids and bowel function in man, *Scand. J. Gastro.,* **19,** 1059–65.

Laqueur, G. and Spatz, M., 1968, Toxicology of cycasin, *Cancer Res.,* **28,** 2262–67.

McCracken, R.D., 1970, Adult lactose tolerance, *J. Am. Med. Ass.,* **213,** 2257–60.

Roediger, W.E.W., 1980, The colonic epithelium in ulcerative colitis: an energy deficient disease? *Lancet,* **ii,** 712–15.

Roediger, W.E.W., 1989, Short chain fatty acids as metabolic regulators of ion absorption in the colon, *Acta Vet. Scand.,* **86 (Suppl.)**, 116–25.

Roediger, W.E.W. and Nance, S., 1986, Metabolic induction of experimental ulcerative colitis by inhibition of fatty acid oxidation, *Br. J. Exp. Path.,* **67,** 773–82.

Salyers, A.A., 1979, Energy sources of major fermentative anaerobes, *Am. J. Clin. Nutr.,* **32,** 158–63.

Salyers, A.A., Vercelletti, J.R., West, S.E.H. and Wilkins, T.D., 1977a, Fermentation of mucin and plant polysaccharide by strains of *Bacteroides* from the human colon, *Appl. Environ. Microbiol.,* **33,** 319–22.

Salyers, A.A., West, S.E.H., Vercelletti, J.R. and Wilkins, T.D., 1977b, Fermentation of mucin and plant polysaccharides by anaerobic bacteria from the human colon, *Appl. Environ. Microbiol.,* **34,** 529–33.

Scheppach, W., Pomare, E.W., Elia, M. and Cummings, J.H., 1988, The effect of starch malabsorption on faecal short chain fatty acid excretion in man, *Scand. J. Gastro.,* **23,** 755–59.

Senagore, A.J., McKigan, J.M., Scheider, M. and Ebron, J.S., 1992, Short chain fatty acid enemas: a cost-effective alternative in the treatment of nonspecific proctosigmoiditis, *Dis. Colon Rect.,* **35,** 923–27.

Smith, P.J., 1986, n-Butyrate alters chromatin accessibility to DNA repair enzymes, *Carcinogenesis,* **7,** 423–29.

Southgate, D.A.T., 1969, Determination of carbohydrate in foods. II. Unavailable carbohydrate, *J. Set. Food Agric.,* **20,** 332–35.

Stephen, A.M., Haddad, A.C. and Phillips, S.F., 1983, Passage of carbohydrate into the colon. Direct measurements in humans, *Gastroenterol.,* **85,** 589–95.

Van Soest, P.J., 1967, Development of a comprehensive system of food analysis and its application to forages, *J. Anim. Sci,* **26,** 119–35.

Vercallotti, J.R., Salyers, A.A., Bullard, W.S. and Wilkins, T.D., 1977, Breakdown of mucin and plant polysaccharide in the human colon, *Can. J. Biochem.,* **55,** 1190–96.

Vince, A., Zeegan, R., Drinkwater, J.E., *et al.,* 1974, The effect of lactulose on the faecal flora of patients with portal encephalopathy, *J. Med. Microbiol.,* **7,** 163–68.

Weber, F.L., 1979, The effect of lactulose on urea metabolism and nitrogen excretion in cirrhotic patients, *Gastroenterol.,* **77,** 518–26.

Williams, R.D. and Olmstedt, W.H., 1936, The effect of cellulose, hemi-cellulose and lignin on the weight of stool: a contribution to the study of taxation in man , *J. Nutr.,* **11,** 433–49.

Winslet, M.C., Allan, A., Poxon, V., Youngs, D. and Keighley, M.R., 1994, Faecal diversion for Crohn's colitis: a model to study the role of the faecal stream in the inflammatory process, *Gut,* **35,** 236–42.

Chapter 9
Toxicology of butyrate and short-chain fatty acids

A.Csordas

9.1
Butyrate as an inhibitor of histone deacetylases

The discovery that butyrate at millimolar concentrations causes hyperacetylation of eukaryotic chromatin (Riggs *et al.,* 1977) by inhibiting histone deacetylases (Cousens *et al.,* 1979) triggered a large number of investigations on the biological role of histone acetylation, in which butyrate was used as a tool for achieving a higher acetylated state of core histones. The previous observations, that butyrate treatment leads to the induction of the globin gene in Friend erythroleukemia cells (Leder and Leder, 1975) and increases the activity of various enzymes in other cell lines (Prasad and Sinha, 1976), were consistent with a positive regulatory role of histone acetylation in eukaryotic gene expression (Allfrey *et al.,* 1964). In addition it was noted that butyrate is an inhibitor of cell growth (Wright, 1973) and an inducer of differentiation (Prasad, 1980; Kruh, 1982). Moreover, butyrate-treated cells exhibited drastic changes in cell morphology and a large number of other pleiotropic effects. Generally, the effects of butyrate appeared to be reversible. The manifold effects of butyrate observed in studies with cell lines derived from a great variety of species and tissues were the subject of previous reviews (Prasad, 1980; Kruh, 1982; Kruh *et al.,* 1992).

This chapter focuses on the special situation of colonocytes which, due to the metabolism of gut bacteria, are constantly exposed to butyrate, propionate and acetate. The effects of butyrate—as an inhibitor of growth, inducer of differentiation and regulator of transcription—on colonic epithelial cells, colon cancer cells and transformed cell lines of other origins will be more closely examined. The potential of butyrate in cancer prevention, cancer therapy and other medical applications will be discussed. As hyperacetylation of histones is the main mechanism of action of butyrate, special attention will be paid to the role of butyrate as a signal for modulating chromatin structure and gene expression.

9.2
Butyrate as both metabolic fuel and inducer of differentiation

Butyrate affects the growth of colonocytes and colon cancer cells in a concentration-dependent biphasic manner. Being the main and preferential source of metabolic energy for these cells, butyrate acts as a stimulator of growth at a lower concentration range; however, as it was noted above, at certain concentrations *in vitro* it inhibits growth of animal cells and in several cell types induces differentiation. The preferential use of butyrate as fuel for metabolic energy is a special feature of

colonic epithelial cells. The growth-inhibiting effect of butyrate with arrest of the cell cycle in the G1 and G2 phases is a general one, observed with cell lines from all animal species and tissues which have been investigated *in vitro*. As far as induction of differentiation is concerned, some cell types are readily induced—as for instance erythroleukemia cells—others not at all, but in most instances at least some increase of differentiation markers can be observed. The cells of the large bowel divide and differentiate in an environment where they are exposed to butyrate produced by gut bacteria. Therefore the question arises whether in the course of evolution intestinal epithelial cells have developed a higher resistance to butyrate, with regard to inhibition of growth, induction of differentiation and interference with gene expression. Since most of the effects of butyrate are due to hyperacetylation of core histones, the immediate question is whether histone deacetylase(s) of intestinal epithelial cells (and colon cancer cells) are less sensitive to inhibition by butyrate than those of other tissues.

The butyrate concentrations used for inhibiting histone deacetylases for achieving efficient hyperacetylation in various animal cell types, as reported in a great many papers, were in the range of 0.5–6mM. However, with regard to the butyrate-sensitivity of animal deacetylases, one can say that there are no drastic differences either when comparing non-transformed cells with transformed ones of any tissue, or when comparing colon cancer cell lines with cancer cell lines derived from other tissues.

In view of a lack of long-term cultures, data with reference to non-transformed intestinal epithelial cells are scarce. However, contrasting effects of butyrate on the expression of phenotypic markers of differentiation in neoplastic and non-neoplastic colonic epithelial cells have been reported. In response to a 24-hour exposure to 1 mM or 4 mM butyrate, differentiation markers increased in LIM1215 colon cancer cells but decreased in epithelial cells isolated from a surgically resected, histologically normal colon. Proliferation of LIM1215 cells was suppressed, but there was no growth inhibition or toxic effect on the normal epithelial cells up to 4 mM/l butyrate during the 24 hour exposure (Gibson *et al.*, 1992).

On the other hand, compared with the colorectal carcinoma cell lines HT29 and PC/JW/FI, the pre-malignant PC/AA cells exhibited a higher sensitivity to butyrate. Concentrations of sodium butyrate (2 mM) that killed early-passage PC/AA cells allowed the late-passage PC/AA cells and the carcinoma lines to proliferate (Berry and Paraskeva, 1988).

Although colonic epithelial cells have a short life span of less than seven days, in this time they rapidly differentiate into mature mucin-secreting cells or colonocytes. Therefore in the long-term *in vivo* situation, even minor differences in the sensitivity to butyrate of colonic epithelial cells in different stages of differentiation and/or carcinogenesis could mean that butyrate has a crucial function in control of growth and differentiation. Alternatively, cells may be able to adapt to higher concentrations of butyrate, or there may be other regulatory mechanisms keeping the steady-state concentration of butyrate below a growth-inhibiting level within the cells. Although at present we do not know whether *in vivo* colonic epithelial cells can adapt their growth behaviour to different levels of butyrate, there are reports of cell lines which became butyrate-resistant *in vitro*. For instance, variant HTC cells were isolated which can replicate in the presence of 6 mM sodium butyrate and exhibit at the same time substantially decreased nucleosomal acetylation. Cells of the original HTC line, upon exposure to 6 mM butyrate, stop DNA replication after one day and die in several days. However, resistant lines arise when exposure is started at lower butyrate concentrations and, after exposure for several weeks, shifted by 0.5 mM to a higher concentration. It was indicated that these cells have more of a deacetylase activity which is less sensitive to sodium butyrate

(Chalkley and Shires, 1985). Butyrate-resistant HeLa S3 cell lines were isolated by treatment of cultures every two weeks with successive 0.2 mM increases in butyrate concentration, until cultures resistant to 5 mM butyrate were obtained. Since these HeLa variant cell lines, capable of multiplying in the presence of 5 mM sodium butyrate, produced both subunits of human chorionic gonadotropin, cell-cycle arrest does not seem to be a requirement for the previously observed butyrate-induced ectopic expression of this glycoprotein hormone (Milsted *et al.,* 1987). Furthermore, a tumourigenic subline of the T14 human bladder cancer cell line was adapted to grow in 5 mM butyrate. The adapted cells showed altered growth characteristics and were morphologically quite different, being large and flattened. Some of the distinctive growth characteristics proved to be irreversible as they persisted when grown in medium without butyrate. The butyrate-adapted T14 cell line showed decreased ouabain sensitivity but no changes in *ras* oncogene expression. Although this adapted cell line was still tumourigenic in nude mice, the tumours differed grossly and microscopically from those produced by the sensitive cell line (Flatow *et al.,* 1989). Sublines of HL-60 human leukemia cells resistant to induction of differentiation by butyric acid were isolated after mutagenesis, or after gradual increase of the butyrate concentration, until normal growth rate was achieved in medium containing greater than 1 mM butyric acid (Fischkoff *et al.,* 1990). Thus, according to these *in vitro* experiments, cells possess the ability to adapt to a higher concentration range of butyrate. However, as the example of the T14 bladder cancer cell line shows, this is associated with irreversible changes in growth characteristics *in vitro* as well as *in vivo*. Thus, even if cells are able to develop resistance to butyrate, they may not remain unaffected in certain other parameters of their growth behaviour.

Inhibition of growth by butyrate is not necessarily linked to loss of viability. Although 1.25 mM butyrate or 10 mM propionate caused significant reductions in the proliferation of endothelial cells, there were no significant effects on the viability of these cells with either of the agents up to 25 mM, the highest concentration tested. The inhibition of endothelial cell proliferation by short-chain fatty acids may contribute to the pathogenic effects of dental plaque in periodontal disease (Tse and Williams, 1992). A study of primary cultures of adult rat hepatocytes claims that butyrate (5 mM) represents a non-toxic compound capable of improving the maintenance of cell culture characteristics (Engelmann *et al.,* 1987). In primary cultures of hepatocytes it was found that at a lower concentration range (0.5 or 1 mM) butyrate stimulated DNA synthesis but at higher concentrations (5 mM) became an inhibitor of growth, 1 mM propionate being almost as stimulatory as 1 mM butyrate (Staecker *et al.,* 1987). Thus, in addition to growth promotion by providing metabolic energy, there could be a specific stimulatory effect of butyrate and propionate for replicative DNA synthesis at a lower concentration range. In numerous other studies, however, with a great many different cell types, the highest butyrate concentrations which cells can take were used in order to achieve maximum yield of hyperacetylation. For this reason the effects of short-chain fatty acids at lower concentration ranges were not extensively investigated.

Since for colonocytes butyrate is the preferential source of energy, it can be expected that their biphasic response to butyrate is especially pronounced. Thus, apparently paradoxically, a lack of butyrate as well as a high butyrate concentration would both act against proliferation, while an intermediate concentration would most efficiently support growth of these cells. Whether *in vivo* butyrate plays an essential role in growth control of colonocytes, whether and within what time frame they are able to adapt to varying concentrations of short-chain fatty acids, and whether and to what extent the steady-state concentrations of short-chain fatty acids are regulated by other signals, remains to be seen.

Although the manifold character of butyrate effects may suggest that there is more than one mechanism by which butyrate acts, it should be emphasized that histone acetylation is certainly the major mechanism of butyrate action responsible for growth arrest, interference with gene expression and induction of differentiation in the animal cell. Therefore, in what follows, the role of histone acetylation (Csordas, 1990; Turner, 1991; Bradbury, 1992) will be considered more closely.

9.3
Comparison of butyrate with other short-chain fatty acids

Inhibition of histone deacetylases is not exclusively a property of butyrate. To a lesser extent, acetate, propionate, isobutyrate, valerate, hexanoate, as well as isomers and derivatives of short-chain fatty acids are also inhibitors of histone deacetylases, propionate and valerate being, next to n-butyrate, the most effective. The mechanism of inhibition is a non-competitive one and it is generally assumed that butyrate (or another short-chain fatty acid) binds as a lipophilic detergent to a site present on histone deacetylases, for which apparently a chain of four carbon atoms, with a methyl group at one end and a carboxyl group at the other end, has the highest binding affinity. This site appears to be a conserved one in histone deacetylases of the animal kingdom, since in all animal cell lines investigated, histone deacetylases have been found to be sensitive, within the same order of magnitude, to inhibition by butyrate. However, histone deacetylases of lower eukaryotic organisms are generally less sensitive to butyrate and those of *Saccharomyces cerevisiae* and some plants seem to be completely non-responsive. Notably, for the butyrate-non-responsive plants, acetate is an efficient inhibitor of histone deacetylases.

Since, in early studies with animal cell lines, butyrate was found to be most efficient for achieving hyperacetylation, data as to the effects of other short-chain fatty acids are rather scarce. In most of the studies in which the effects of butyrate were compared with those of other short-chain fatty acids, a gradual decrease of these effects was observed according to the differing chain length. Since not only butyrate but also large amounts of propionate and acetate are produced by gut bacteria, the concentrations of these fatty acids must also be considered in evaluating the impact of gut bacteria on growth and differentiation of colonic epithelial cells. Thus it can be assumed that the effects described in this chapter for butyrate also hold true for the other short-chain fatty acids, even though with lower efficiency. Therefore other short-chain fatty acids will be specially mentioned only if they have been found equally efficient, drastically different or in some respect superior in their performance compared to butyrate. The binding site on animal histone deacetylases might well be optimal for a C4-chain, but it is conceivable that other enzymes may have binding sites which are optimal for another chain length. In this way various short-chain fatty acids may exhibit specific effects, for instance by interacting with proteins of signal transduction. Considering that the tranquillizing effect of baldrian tea is common knowledge, one can say in the historical perspective that a high degree of specificity associated with the actions of at least one short-chain fatty acid, namely valerate, has been well known for a long time.

9.4
Acetylation neutralizes positive charges at the N-terminal domains of core histones

Hyperacetylation of core histones can be achieved by treatment of cell cultures with millimolar concentrations of butyrate. Many studies using such butyrate-treated cell cultures focused on the

role of histone acetylation in transcription. In the attempt to unravel the mechanism of eukaryotic gene expression, it was expected that butyrate treatment of cells would lead via hyperacetylation to activation of genes, and the goal of many experiments was to prove that there is a positive correlation between acetylation of core histones and transcriptional activation of genes.

Reversible acetylation of core histones is the major post-translational modification of nucleosomes and thus the major mechanism for the dynamic fine-tuning of electrostatic forces at the N-terminal domains of core histones located at the surface of the nucleosome core particle. The acetylation status of chromatin results from the equilibrium between histone transacetylases and deacetylases. Histone transacetylases transfer an acetyl group from acetyl CoA to ε-amino groups of specific internal lysine residues of core histones, thereby neutralizing a positive charge, by converting the amino group into an acetamide. The lysine residues which are targets of acetylation are located in highly conserved positions at the N-terminal tails of core histones. There are two positions of acetylation in histone H2A, four each in histones H2B and H4, and five in H3. While histone deacetylases are inhibited by butyrate (and by other short-chain fatty acids), histone transacetylases remain unaffected, and consequently upon incubation of eukaryotic cells with butyrate, the so-called hyperacetylated state of chromatin is achieved. As histone acetylation neutralizes positive charges of internal lysine residues of core histones, this may decrease their affinity to the negatively-charged DNA and thus be a mechanism of derepression of eukaryotic genes. The basic idea—along the lines of prokaryotic gene regulation and the Jacob-Monod concept—in the context of eukaryotic gene expression was, that histones, the repressers of eukaryotic chromatin which are bound to DNA by electrostatic interactions, could be removed more easily from inactive, repressed genes when their net positive charge is reduced by acetylation. A positive correlation between histone acetylation and gene expression was postulated by Vincent Allfrey and co-workers, who discovered this reversible post-translational modification, and who also reported on physiological situations consistent with their hypothesis (Ruiz-Carrillo *et al.,* 1974).

The recognition that butyrate induces the globin gene (Leder and Leder, 1975) and various enzymes (Prasad and Sinha, 1976) can be considered as the starting point for a large number of investigations examining the activity of genes in relation to histone hyperacetylation, as achieved by butyrate treatment of cell cultures. The results of these studies revealed that, for animal cells, butyrate is an inhibitor of growth, in many cases an inducer of differentiation markers and for certain genes an activator of transcription. The main expectation of this era of research, however, that butyrate treatment of cells would generally cause activation of transcription, or at least provide a necessary if not a sufficient requirement for transcriptional activation of genes, was not fulfilled. A rather complex picture emerged, as consequent to butyrate treatment some genes became activated, others repressed, yet others remained unaffected.

9.5
The impact of the butyrate signal on chromatin structure

It has to be pointed out that the N-terminal domains of core histones, where the positions of acetylation are located, are not the sites of major electrostatic DNA-protein interactions responsible for organization and stability of the nucleosome core particle. They can even be cut off by proteases without altering the gross organization and affinity of DNA-protein binding within the nucleosomal fragment. These highly basic N-terminal domains at the surface of the nucleosome core particle interact with the negatively charged phosphodiester backbone of DNA within and adjacent to the

core particle, thus shielding the negative charge in inter-nucleosomal interactions. Moreover, due to the specificity of the acetylation pattern and the location at the surface of the core particle, the N-terminal tails are ready for specific protein-protein interactions. Thus, histone acetylation affects not so much the binding of core histones to DNA as their interactions with other proteins. Core histones interact with regulatory non-histone proteins as well as histone H1, and are involved in inter-nucleosomal interactions. However, it would be wrong to say that the N-terminals have nothing to do with nucleosomal stability, as their hyperacetylation introduces a minor conformational change into the nucleosome core particle (Bode *et al.,* 1983; Ausio and van Holde, 1986) which may facilitate nucleosome disruption and histone displacement by transcription factors. Hyperacetylation of histones H3 and H4 reduces the linking number change per nucleosome core particle, i.e. it alters the superhelical topology of nucleosomal DNA (Norton *et al.,* 1990). Thus acetylation of the N-terminal tails may alter the path of DNA within the nucleosome core particle or change the shape of the histone octamer around which the DNA is coiled in the nucleosome. These acetylation-induced conformational changes may be essential for the binding specificity of regulatory proteins and they may, at the same time, facilitate nucleosome disruption. The non-random pattern of acetylation generated at a specific chromatin site provides for a high degree of specificity with regard to protein structure of the *trans-acting* factor as well as the location of the gene.

Even after most extensive butyrate treatment of cell cultures, whereby the so-called hyperacetylated state of chromatin is established, not all the nucleosomes with all their acetylation positions become fully acetylated. Even at the highest degree of acetylation achieved, only less than half of the possible acetylation positions are in fact acetylated. There are chromatin domains which respond to butyrate more rapidly and to a larger extent than do the bulk histones, i.e. there are chromatin regions which become fully acetylated, while others reach only an intermediate degree of acetylation, and yet others remain unaffected. Newly-synthesized nucleosomal histones in chromatin are more accessible to transacetylases than older, pre-existing histones (Cousens and Alberts, 1982). This differential distribution of hyperacetylation is mostly due to steric hindrance of the differentially compacted chromatin, but location and specificity of transacetylases/deacetylases are also contributing factors. There are populations of acetylated histones which differ in their turnover. In the embryonic chicken erythrocyte, for instance, 30% of core histones are stably acetylated, while the acetylation status of 2% changes rapidly. Moreover, the positions of acetylation are used in a sequential and non-random manner, resulting in specific patterns of acetylation. Distinct patterns of acetylation have been found to be associated with different physiological situations and distributed specifically with regard to chromosomes and chromosomal domains (Turner, 1991). The specific patterns of acetylation are distinct for each type of core histone and can be introduced in a site-specific manner. Thus, with the non-random pattern of acetylation at their N-terminals, individual core histones are equipped for protein-specific and site-specific interactions with other proteins, such as regulatory trans-acting factors. In that way, core histones, as the most conserved proteins representing the highest degree of structural monotony in the cell, can appear with many faces, providing as subunits for higher assembled structures the highest degree of structural variety. An example of highly-specific interaction of histone H4 with a regulatory protein was found in yeast. An intact N-terminal H4 histone tail is required for repression of the silent mating type loci and also for the efficient activation of a number of inducible promoters in the yeast *S. cerevisiae* (Grunstein *et al.,* 1992).

Based on many observations there is overwhelming evidence that active chromatin is enriched in higher acetylated nucleosomes. A15- to 30-fold enrichment in sequences of active genes has been found in association with hyperacetylated chromatin (Hebbes *et al.,* 1988). Furthermore, active chromatin with hypomethylated DNA contains maximally hyperacetylated core histones (Tazi and Bird, 1990). In spite of the overwhelming evidence for its close local and temporal association with transcriptionally-active genes and with a less condensed, nuclease-sensitive chromatin structure, the exact biological role of histone acetylation still remains to be clarified.

A higher degree of histone acetylation was found to be correlated not only with transcription but also with other physiological situations such as histone deposition on newly replicated DNA, and histone displacement by protamines during spermatogenesis. Newly-synthesized histones are acetylated before their deposition on to DNA. Deacetylation of the newly-formed higher-acetylated nucleosomes follows after a lag as chromatin matures and becomes more compacted. Histones are highly acetylated before their displacement by protamines. Therefore, it can be assumed as certain that histone acetylation has more than one function and is involved in different processes in the nucleus. The concept of multiple functions is also underlined by the distinct acetylation patterns associated with different physiological situations such as transcription, replication and spermatogenesis, and by the specific distribution of certain patterns with regard to chromosomes and chromosomal regions (Turner, 1991).

Butyrate-mediated histone acetylation, and derepression of genes consequent to it, primarily involve the more extended regions of chromatin, i.e. euchromatin (‘potentially active’ genes) while heterochromatin appears to be affected by acetylation to a much lesser extent. Constitutive heterochromatin is not converted to euchromatin in butyrate-treated cell cultures. When ear fibroblast cultures of three different *Peromyscus* strains, which differ considerably in their genomic content of heterochromatin but have essentially the same euchromatin content, were treated for 24 hours with 15 mM butyrate, the percentages of unacetylated histones H3 and H4 remaining after butyrate treatment were proportional to the amount of constitutive heterochromatin (Halleck and Gurley, 1981). In untreated cultures, too, the percentage of unacetylated histones was correlated to the heterochromatin content. These observations suggest that a fraction of histones in constitutive heterochromatin is inaccessible to acetylation. A considerable fraction of core histones in heterochromatin becomes monoacetylated after butyrate treatment. The fact that acetylation *per se* is possible but does not proceed above the level of monoacetylation suggests that accessibility by transacetylases is not the only regulatory factor of histone acetylation.

There is recent evidence that the positioning of genes within chromatin loops strongly affects their transcriptional response to butyrate. Butyrate increases the production of human lymphoblastoid interferon and this principle has been used for producing interferon on a large scale. In the course of investigations of the underlying mechanism it was found that the stimulatory effect is due to the vicinity of sequences representing the so-called scaffold/matrix-attached regions (SAR/MAR elements). It was demonstrated that the stimulatory effect of butyrate is greatest if one or especially two SAR/MAR elements are adjacent to a gene (Klehr *et al.,* 1992). Activation of the human interferon-β gene causes the disruption of a nucleosome ahead of the upstream SAR element; and the same nucleosome is disrupted consequent to the action of butyrate. Based on this knowledge, cell lines can be constructed in which a transfected gene is expressed more efficiently following the addition of butyrate to the medium.

There is genetic and physical evidence for the specific interactions of the N-terminal tails of core histones with regulatory proteins. The exact pattern of acetylation certainly plays an essential role

for the specificity of these interactions. There are examples of nucleosomes which, by being positioned on regulatory sequences, prevent the access of transcription factors (Straka and Hörz, 1991; Bresnick *et al.,* 1992; Lee *et al.,* 1993; Tsukiyama *et al.,* 1994). There are also other examples of nucleosomes such that their positioning on regulatory sequences is the prerequisite for specific factor binding. This is the case, for instance, for an enhancer sequence of the albumin gene, which is, in a tissue-specific manner in liver cells, precisely positioned in an array of nucleosomes (McPherson *et al.,* 1993). Thus the binding specificity of transcription factors can be determined by the chromatin structure at the level of nucleosomal organization of a gene. There are transcription factors which are excluded from their binding site by a nucleosome and there are others with a binding specificity for distorted (nucleosomal) DNA. Such contrasting binding specificities of transcription factors with regard to the nucleosomal organization would explain why butyrate-induced hyperacetylation, as a prelude for nucleosome disruption, results in activation of genes in one case, and has the opposite effect in another. No effect at all, i.e. neither induction nor stimulation nor down-regulation of transcription after butyrate treatment, would be the case for transcription of those genes which are located in chromatin regions beyond the reach of transacetylases. Likewise, butyrate treatment can be expected to have hardly any effect on the initiation of transcription of those genes, the regulatory sequences of which are not associated with histones (i.e. regulatory sequences positioned on linker DNA or in nucleosome-free regions). Moreover, even after removal of a repressing chromatin structure, genes can remain silent in the absence of specific *trans-acting* factors. Experiments made possible by the genetics of yeast suggest that one component required for activation of the transcription process is the disruption of repressive chromatin structures. A second component, however, lies in the interaction of the gene-specific transcription factors with the basal transcriptional machinery (Grunstein *et al.,* 1992; Wolffe, 1994).

Turning now from the initiation to the elongation event of transcription, hyperacetylated, i.e. conformationally more ‘open’, ‘split’, nucleosomes may facilitate the passing-by of RNA polymerase at the nucleosome cores during the elongation event of eukaryotic transcription, and it is conceivable that *in vivo,* butyrate-induced hyperacetylation increases the rate of elongation. *In vitro* it was shown that elongation of transcription with a DNA template organized in nucleosome cores is possible, even when core histones are covalently cross-linked and not hyperacetylated (O’Neill *et al.,* 1993). If DNA with a covalently cross-linked histone octamer can be transcribed, then obviously disintegration of the histone octamer is not a prerequisite for the elongation event of transcription. Although the exact mechanism by which the transcribing RNA polymerase overcomes the impediment of DNA-bound histone octamers is not known, O’Neill *et al.* (1993) have shown that the histone octamer can be removed by RNA polymerase as a compact unit.

9.6
Hyperacetylation and arrest of the cell cycle

In situations in which a specific acetylation pattern is required, complete hyperacetylation of all the acetylation positions due to butyrate treatment creates a non-physiological acetylation pattern. In this case, butyrate-induced hyperacetylation wipes out the specificity of electrostatic interactions and prevents their regulated changes. Inhibition of turnover accomplished by the inhibition of histone deacetylase(s) prevents essential events in remodelling of chromatin structure, and this appears to be the main molecular event leading to growth inhibition with arrest in the G1 and G2 phases. This

effect of butyrate is an interesting aspect of cancer therapy. Prerequisite for growth inhibition is a stable high level of fully-acetylated histones, the most important aspect thus being the efficient inhibition of deacetylases. Butyrate (administered intravenously) proved to be ineffective in the therapy of cancer because of the low actual serum concentrations resulting from its fast metabolic use. It should also be mentioned that there are new drugs which are more efficient inhibitors of histone deacetylases. Analogues of butyrate are being developed and tested (Planchon *et al.,* 1992; Carstea *et al.,* 1993; Rabizadeh *et al.,* 1993). Moreover, drugs with completely different structures were discovered which are most efficient inhibitors of histone deacetylases. Trichostatin A, a *Streptomyces* product, was shown to cause hyperacetylation, at doses as low as nanomolar concentrations, in various cell lines and also to be a non-competitive inhibitor of a partially purified histone deacetylase from mouse mammary gland tumour cells. Trichostatin A was further shown to cause induction of differentiation in Friend cells, and inhibition of the cell cycle of normal rat fibroblasts specifically in the G1 and G2 phases (Yoshida *et al.,* 1990). The fact that this inhibitor of histone deacetylases with a chemical structure entirely different from that of butyrate is, like butyrate, an inhibitor of cell growth and inducer of differentiation, is a further argument for histone hyperacetylation being the underlying mechanism of these effects. Moreover, it was previously observed that HTC cells continued to grow even when 70% of the H3 and H4 histones were acetylated in the presence of 6 mM n-propionate (Rubenstein *et al.,* 1979); in the presence of Trichostatin A, too, growth continues at this degree of hyperacetylation and stops only when tetra-acetylated (fully-acetylated) H4 appears. Thus, in agreement with the arguments mentioned above, efficient inhibition of histone deacetylases leading to a stable high level of hyperacetylation is necessary for an arrest of the cell cycle.

For the elucidation of the mechanism of action of butyrate, the comparison with Trichostatin A, a chemically unrelated inhibitor of histone deacetylases, is extremely helpful (Yoshida *et al.,* 1990). It was not only shown that Trichostatin A is an inhibitor of growth and inducer of differentiation, but also that Trichostatin A activates those genes for which butyrate is an activator and inhibits glucocorticoid-mediated transcription, as butyrate does. This clearly shows that for inhibition of growth, induction of differentiation and interference with gene expression, hyperacetylation due to inhibition of deacetylases is the mechanism of butyrate action. While for inhibition of growth a persisting high level of hyperacetylation is the requirement, for induction of differentiation and certain types of gene activations a transient wave of acetylation appears to be sufficient.

Butyrate may also interfere with signal transduction pathways by altering the precisely-regulated expression of proteins through the mechanism of histone acetylation (Lewis and Levine, 1992). It would, however, certainly be wrong to try to link all the pleiotropic effects of butyrate to histone acetylation. Partitioning into membranes and, last but not least, fatty acid metabolism, are further scenarios for short-chain fatty acids, which should not be ignored. Butyrate increased, whereas propionate decreased gluconeogenesis in isolated rat hepatocytes, and glycolysis was decreased by butyrate but increased by propionate (Anderson and Bridges, 1984). Thus, propionate, which inhibits hepatic acetate metabolism, acts to increase glucose use and decrease glucose production. That way plant fibres—via gut bacteria and their short-chain fatty acid metabolites—may influence hepatic glucose metabolism.

9.7
Butyrate and activation of gene expression

The fact that for certain types of gene regulation and for differentiation only a transient, site-specific increase of acetylation is required, could have implications for the toxicology of butyrate and other short-chain fatty acids. A site-specific wave of hyperacetylation could be efficiently delivered even by a 'weak' inhibitor of deacetylation, and therefore the amounts of short-chain fatty acids as produced by gut bacteria may strongly affect gene expression and differentiation in colonic epithelial cells. That way, there may be a close link between diet and events of gene expression and differentiation in colonic epithelial cells.

Recently it was shown that a stable, full acetylation of core histones favours the binding of transcription factor TFIIIA to its binding site on the *Xenopus borealis* 5S RNA gene organized within a nucleosome (Lee *et al.,* 1993), whereby a ternary complex is formed. This was shown with nucleosomes which have been reconstituted with hyperacetylated core histones isolated from butyrate-treated HeLa cells.

Most efficient induction of transcription as a result of persisting efficient inhibition of deacetylases can be expected when a conformational change needs to be induced into the nucleosome as a prelude for its subsequent disruption. Likewise, a high level of hyperacetylation will lead to activation of genes when simply a decrease of the net positive charge at the N-terminal tails of the core histones is required for overcoming repression, without the involvement of specific acetylation patterns and their dynamic changes. Maximum inhibition of deacetylases will also be necessary for induction of genes located in chromatin regions, where the required level of acetylation is only delivered with low efficiency. In this case, even an efficient deacetylase inhibitor will lead only to that intermediate level of acetylation which is required for transcriptional activation. If efficient induction of transcription is achieved by persistent maximal inhibition of deacetylases, this strongly suggests that in this type of gene regulation dynamic changes of specific acetylation patterns are not involved.

It was shown by two-dimensional electrophoresis that in butyrate-treated (5 mM) Friend erythroleukaemic cells many proteins which are not detectable in the control cells are synthesized *de novo* and the synthesis of several other proteins is inhibited. Such alterations of the expressed protein pattern do not occur when dimethyl sulphoxide—which does not cause histone hyperacetylation—is used as inducer of differentiation (Reeves and Cserjesi, 1979). However, with a similar analysis it was shown that only a small number of proteins can be induced by butyrate (6 mM) in HTC cells. Thus, there are clear cell type-specific differences in the response of genes to butyrate (Rubenstein *et al.,* 1979).

Furthermore, in studies with the F9 embryonic carcinoma cells of the mouse it was found that the expression of metallothionein (MT) genes is enhanced by butyrate treatment in undifferentiated stem cell lines (F9 and OC15) as well as in differentiated cells (PSA5E and OC15 END). MT genes in cells pre-treated with butyrate were hyper-sensitive to metal induction. Butyrate enhanced the rate of accumulation of MT mRNA in response to metals, increased the sensitivity of the MT gene to metals, and protected the cells from the toxic effects of high concentrations of metals. The responses to butyrate and metal ion were selective in that no accumulation of *c-myc, c-fms,* HSP-70, or AFP mRNA was detected. However, *c-fos* mRNA accumulated in cells exposed to toxic concentrations of metals (50 μM and higher) and this was also potentiated by butyrate treatment. Based on these

observations it was suggested that butyrate alters the chromatin structure of the MT-I and MT-II genes leading to an increased transcriptional response to metals (Andrews and Adamson, 1987).

Butyrate is a promising new drug in the treatment of diseases with defective α-globin gene, such as sickle cell anaemia and α-thalassaemia (Perrine *et al.,* 1989; Perrine and Faller, 1993). It was observed that infants having high plasma levels of α-amino-*n*-butyric acid in the presence of maternal diabetes did not undergo the normal developmental gene switch from the production of predominantly γ-globin to that of α-globin before birth (Perrine *et al.,* 1985), whereas other developmental processes were not delayed. Perrine *et al.* (1985) identified n-butyric acid as being responsible for the activation of the fetal globin gene. It was also observed that butyrate induces a specific embryonic gene in adult chickens by actions at 5• flanking sequences and, furthermore, selectively stimulates the globin gene in fetal sheep, cultured human erythroid cells, and adult non-human primates. In the case of the γ-globin gene, as of several other genes, it was found that regulatory sequences upstream of the promoter are the targets of butyrate-induced actions.

Butyrate was found to have a low order of toxicity, as children and adults with cancer who had been treated with sodium butyrate, and healthy adults and various animals which had received sodium or arginine butyrate, did not show any major side effects (Blau *et al.,* 1993). These observations suggested that n-butyric acid may maintain fetal-globin expression in a quite safe and specific manner. A Phase I trial in which patients with sickle cell anaemia and with a-thalassaemia syndromes received arginine butyrate infusions, showed that in all patients there was a substantial increase of fetal-globin synthesis (Perrine *et al.,* 1993). This medical application of butyrate, demonstrating its ability to reverse ontogeny, opens a new perspective in the treatment of genetic diseases. To awaken dormant genes is an elegant alternative of gene therapy (Bunn, 1993). In the majority of thalassaemia patients the upstream pair of γ-globin genes is intact and fully functional. In the case of sickle cell disease, both in the test-tube and in the patient, fetal haemoglobin is highly effective in inhibiting the polymerization of haemoglobin S.

For long-term induction of fetal genes, however, oral application would be desirable, and recently, by oral application of sodium phenylbutyrate a substantial increase in the number of F-cells (erythrocytes with fetal haemoglobin) was achieved (Dover *et al.,* 1992).

Further examples of activation of dormant genes, observed in many different cell types, are the inductions of placental and intestinal isoenzymes of alkaline phosphatase, in cells where synthesis of these enzymes is ectopic. Butyrate induces peptidic hormones, for instance, human chorionic gonadotropin, in HeLa cells. In several different cell types butyrate induces the synthesis of receptors of thyroid hormone and insulin. In human colon fibroblasts butyrate increases the expression of c-erbA oncogene (Bahn *et al.,* 1988). On the other hand, in cells which express high amounts of these hormone receptors, it leads to their down-regulation. It has been noted in different cell types that butyrate, in all phases of the cell cycle, increases the expression of the *c-fos* and *c-jun* oncogenes and inhibits the expression of *c-myc.*

9.8
Butyrate and inhibition of gene expression

Quite surprisingly, it was observed that butyrate prevents steroid hormone-mediated gene expression. This was shown for the induction of tyrosine aminotransferase by glucocorticoids in hepatoma cells, and for the induction of ovalbumin and transferrin by oestradiol in the chick oviduct (Kruh, 1982). The effect is reversible; after removal of butyrate, steroid hormone-mediated gene

expression can be induced again. Since protein synthesis, glucocorticoid receptor level and translocation of glucocorticoid-receptor complex were found to be normal under the conditions of butyrate treatment, it was assumed that a step which requires some alteration of chromatin structure is involved in the activation of the tyrosine aminotransferase gene. In the following investigations of the mechanism of glucocorticoid-mediated gene regulation it was shown that nucleosome disruption is required for glucocorticoid-mediated activation of the mouse mammary tumour virus promoter, and this is prevented by butyrate (Bresnick *et al.,* 1990). For transcriptional activation, two out of the six positioned nucleosomes on the regulatory region of MMTV have to be disrupted by the glucocorticoid receptor. The positioned nucleosomes prevent the binding of the transcription complex in absence of a hormonal signal. The mechanism of MMTV nucleosome disruption is not known but it was shown that removal of H1 from the linker DNA is part of the 'disruption' process. After displacement of histone H1, nuclear factor 1 (NF1) and other factors can bind to their recognition sequences which were blocked by H1. One would expect that hyperacetylation favours glucocorticoid-mediated gene expression if, for that process, nucleosome disruption is required, since acetylation has been shown in other systems to facilitate nucleosome disruption (Lee *et al.,* 1993). Exactly the opposite is the case. How can this apparent paradox be explained? The answer could be that specific interactions of the N-terminal tails with transcription factors are not possible when their specific acetylation pattern is wiped out by hyperacetylation. For the same reasons chromatin-remodelling steps are prevented by stable hyperacetylation of the involved nucleosomal histones.

Thus the apparently contradictory effects of butyrate on gene expression might be explained by a differing impact of histone acetylation on the transcriptional regulation of genes in a given cell type. When a stable initiation complex within a disrupted nucleosome (conformationally altered nucleosome) has to be formed, it can be expected that butyrate would enable the activation of such genes, since hyperacetylation would maintain the activated nucleosome in a stable 'open' conformation. The opposite effect, that is, inhibition of gene transcription, would be the result of hyperacetylation (i.e. of butyrate treatment) for genes with regulatory processes involving a dynamic on-and-off of transcription factors and/or histones. In the case of such genes, butyrate can be expected to inhibit chromatin-remodelling processes by preventing turnover of acetyl groups (i.e. the interplay of transacetylases and deacetylases) and thereby freezing nucleosomally-organized histones as well as free histones in the hyperacetylated state.

The third situation, namely, no effect of butyrate, neither stimulatory nor inhibitory, can be expected when the chromatin structure plays no role in repression and activation of a gene. This would be the case when regulatory sequences are not under the control of core histones or when they are repressed by core histones but require for their activation protein factors which, however, remain absent in spite of butyrate treatment. Butyrate would fail to have any effects also, as discussed earlier, when the gene is located in a chromatin region which is out of reach of the enzymes of histone acetylation. Thus, the efficiency of the deacetylase inhibitor, the positioning of a gene in a chromatin loop, the positioning of its regulatory sequences with regard to nucleosomes and the specificity of interactions of core histones with *trans-acting* factors involved in the activation process, will together determine the impact of butyrate on the transcriptional activation of a gene.

9.9
Butyrate and human colon cancer cell lines

The effect of butyrate on the expression of cellular oncogenes, and on growth and differentiation in various colon cancer cell lines, was the subject of several investigations. Analysis of the *c-myc* levels in human colon tumour cell lines of defined biological phenotype has shown that there is an inverse correlation between the *c-myc* level and the differentiation phenotype. Fast-growing, poorly differentiated cell lines which are more tumourigenic have the highest *c-myc* levels. It has been reported that in the cell line SW837, derived from an adenocarcinoma of the rectum, butyrate activates the synthesis of a cellular protein which causes a rapid decline in the level of *myc* mRNA. This effect was dose-dependent and was maximal at 1 mM. Among different short-chain fatty acids tested, butyrate was found to be the most potent. Valeric acid was less effective and acetic, propionic, isobutyric and capric acids did not cause a significant change in the level of *myc* mRNA. The reduction of the *myc* mRNA level caused by butyrate was blocked by inhibitors of protein synthesis, and was rapidly reversed by removing butyrate (Herold and Rothberg, 1988). Both propionate and butyrate but not acetate were found to inhibit growth of the human adenocarcinoma cell line HT29 and to cause an increase in alkaline phosphatase activity, which reflects a more differentiated phenotype. These results suggest that propionate, like butyrate, may play an important role in the physiology of the colon and could partially account for the protective effect of dietary fibres with respect to colon carcinogenesis (Gamet *et al.,* 1992). Butyrate-induced differentiation of HT-29 colon carcinoma cells is potentiated by 1, 25-dihydroxyvitamin D_3 (Tanaka *et al.,* 1990) and also by deoxycholate (Desai *et al.,* 1993).

In the colon carcinoma cell line Caco-2 which spontaneously undergoes enterocytic differentiation in culture, butyrate was found to induce *c-fos* very rapidly at a post-transcriptional level and after a time-lag to stimulate *c-fos* transcriptionally. It was shown that the ATF-CRE binding site located upstream of the *c-fos* transcriptional start site is a target for butyrate-induced *fos* transcription. Furthermore, increased binding activity of the CRE transcription factor was found in butyrate-treated cells (Souleimani and Asselin, 1993a). In the same cell line it was shown that degradation of normal *c-myc* mRNA is not coupled to translation, and down-regulation of *c-myc* expression takes place not at the level of transcription but at a post-transcriptional level. Furthermore, it was suggested that butyrate induces a factor involved in *c-myc* mRNA degradation (Souleimani and Asselin, 1993b). In another study *c-myc* was detected constitutively expressed in all of the six human colon tumour cell lines analysed. The poorly differentiated cell lines HCT116, RKO and C showed *c-myc* levels that averaged twofold greater than their well-differentiated counterparts, i.e. GEO, CBS and FET When *c-myc* levels and responses to serum induction were analyzed in the presence of various inducers of differentiation, distinct patterns of sensitivity and resistance emerged. As a result of butyrate treatment, nuclear *c-myc* levels were reduced in all the six colon cancer cell lines examined. However, only the well-differentiated human colon tumour cell lines were responsive to transforming growth factor-β (TGF-β), and only one of the colon tumour lines (GEO) responded to retinoic acid. Increased levels of *c-myc* protein were found to correlate well with greater growth rates and with lower differentiation class (Taylor *et al.,* 1992).

Colo 320 cells are small-cell neuroendocrine colonic carcinoma cells known to actively express the *myc* proto-oncogene. In haematopoietic cells *myc* expression was found necessary for maintaining the undifferentiated phenotype. When Colo 320 cells were exposed to 5 mM sodium butyrate for 7 days, *c-myc* expression decreased three-fold and this was accompanied by a decrease

of self-replicative potential. In an effort to demonstrate a direct cause-and-effect relationship between *myc* expression and the colony-forming capacity of the Colo 320 cells, they were exposed to an antisense *c-myc* oligonucleotide; this resulted in a concentration-dependent decrease in colony-forming capacity, suggesting that both butyrate and the antisense DNA acted by inhibiting *myc* expression. Thus, it can be concluded that in Colo 320, and possibly also in other colon cancer cell lines, the growth-inhibiting effect of butyrate is not only due to hyperacetylation but also to suppression of *myc*. However, it remains to be seen whether there is a direct link between hyperacetylation and suppression of *myc*. Furthermore, the specific inhibition of colony-forming capacity by antisense DNA suggests that the role of *myc* expression in Colo 320 cells is similar to that in haematopoiesis, where *myc* expression maintains colony-forming capacity and represents a primitive cell phenotype (Collins *et al.*, 1992). Thus, the elucidation of the molecular determinants of *c-myc* repression in normal colonic epithelial cells may yield the key information about one of the fundamental defects in colorectal carcinoma cells.

Treatment of the undifferentiated colon carcinoma cell line MIP-101 with butyrate resulted in a more normal phenotype, including diminished growth rate, elimination of anchorage-independent growth, and decreased tumourigenicity. Moreover, in this cell line butyrate suppressed the transforming activity of an activated N-rax oncogene in the NIH-3T3 transfection assay (Stoddart *et al.,* 1989). In another study the effects of butyrate on the response of the RCA colon carcinoma cell line to TGF-β1 were examined. In the RCA cells, induction of differentiation by butyrate was demonstrated, as judged by an increase in cellular alkaline phosphatase, and this was accompanied by a decreased growth rate. TGF-β1 applied alone did not significantly alter the growth or state of differentiation of the RCA cells. Addition of TGF-β1 to cells pre-grown in the presence of butyrate resulted in a stimulation of growth. Cells pretreated with TGF-β1 remained sensitive to the growth-inhibitory and differentiation-inducing effects of butyrate (Lewis and Levine, 1992). These results suggest that butyrate may alter the expression of proteins responsible for a stimulatory signal response to TGF-β1 in RCA cells, and the mechanism of action of butyrate may be linked in some way with signal transduction.

9.10
Butyrate and various other transformed cell lines

Butyrate was found to be an efficient inducer of differentiation in various erythroleukaemia cell lines, such as Friend leukaemia cells of the mouse, human K562 cells and HL60 leukaemia cells. In the chemotherapy of leukaemia, however, the problem is the short half-life of butyrate due to metabolism and the reversible nature of the butyrate effects. In order to deal with this problem, several analogues with longer half-lives have been synthesized and some of them appear to be promising. As mentioned earlier, other more efficient inhibitors of deacetylases with structures entirely different from that of butyrate such as, for instance, Trichostatin A, also show great promise as new drugs.

In comparison to other differentiation-inducing agents, distinct features of the butyrate-induced differentiation pathway have been often observed. F9 teratocarcinoma cells of the mouse can be induced to differentiate by both retinoic acid and butyrate; however, in the latter case, both plasminogen activators, namely, tissue-type (tPA) and urokinase-type plasminogen activator (uPA) are induced, whereas with retinoic acid alone only tPA is induced (Takeda *et al.,* 1992).

In mammary tumour cells butyrate causes a decrease in the number of oestrogen receptors. Butyrate treatment of MCF-7 cells led to a decrease of both the cytosolic and the nuclear (KCl extractable) oestrogen receptor levels. Confluent cells did not reach as high a level of histone hyperacetylation as rapidly-growing cells (Stevens *et al.,* 1984). In those cell lines that expressed the oestrogen receptor, growth inhibition by butyrate was accompanied by a more differentiated phenotype. The ZR-75–1 cell line showing the most pronounced potential for differentiation was suggested as a model for analysis of gene expression during differentiation of the mammary secretory epithelium (Graham and Buick, 1988). 13-cis-retinal acts as a negative modulator of differentiation and protects MCF-7 cells from the growth-inhibitory and differentiation-inducing action of butyrate (Resnicoff and Medrano, 1989).

Addition of butyrate to cultured breast carcinoma cell lines caused a concentration-dependent growth inhibition in monolayers and markedly reduced colony-forming efficiency in soft agar. In the cell line MCF-7, cell replication ceased after addition of 1 mM butyrate. Growth arrest and induction of a more differentiated phenotype was preceded by a block in G1 phase of the cell cycle (Guilbaud *et al.,* 1990).

Normal proliferating and neoplastic mammary cells in culture have cryptic prolactin receptors. These cryptic sites represent 80–95% of the total receptors and can be unmasked by energy depletion. Since lactating mammary tissue and other prolactin targets do not contain cryptic receptors, they may be important in the growth response to prolactin. Therefore, the effects of butyrate on the cryptic sites were investigated. Since butyrate decreased the density of cryptic sites, inhibited cell growth and evoked the expression of some morphological features of differentiation, it was suggested that the loss of cryptic prolactin receptors might be involved in the acquisition of a differentiated phenotype in mammary cells (Costlow, 1984).

Treatment of mammary carcinoma cells with butyrate reduces the prolactin receptor binding-affinity and receptor gene expression. It was shown that butyrate inhibits prolactin receptor gene expression by a transcriptional mechanism which does not require continuing protein synthesis (Ormandy *et al.,* 1992a). Prolactin receptor and oestrogen receptor are co-ordinately expressed in human breast cancer cell lines and in human breast tumour biopsies. In MCF-7 and T-47D breast cancer cell lines it was shown that treatment with 0.3–10 mM butyrate resulted in a parallel decrease of prolactin and oestrogen mRNA levels, thus supporting the hypothesis that the expression of these two receptors is coupled (Ormandy *et al.,* 1992b).

Addition of 5 mM butyrate to HeLa cells is reported both to stimulate gonadotropin α-subunit biosynthesis and to block cell cycling in G1. Investigation of these two actions of butyrate on HeLa cells showed that they may be associated with two independent mechanisms. The induction of a-subunit synthesis was due to an increase in the rate of transcription of the α gene and, using synchronized populations of the HeLa cells, it was shown that butyrate stimulates α-transcription throughout the cell cycle. In contrast, treated cells arrest in G1 only if exposed to butyrate for a discrete period during the previous S phase (Darnell, 1984). This study apparently suggests that butyrate leads to arrest in G1 through a cell cycle-specific action which is distinct from its direct effect on transcription. However, both effects can be explained by the mechanism of hyperacetylation. Only in the sterically more accessible chromatin structure of the S-phase is the high degree of hyperacetylation achieved which is necessary for arrest in G1, whereas the α-HCG gene is sterically accessible for transacetylases throughout the cell cycle.

9.11
Butyrate and multi-drug resistance

Sodium butyrate, but not dimethylsulphoxide induced the synthesis of villin, a protein of the brush border microvillar cytoskeleton, in a rat colon cancer cell line. Neither butyrate nor dimethylsulphoxide altered the *mdr*-1 mRNA expression or multi-drug resistance-associated cellular transport of doxorubicin. These results show that *mdr*-1 gene expression and activity are independent of other brush border proteins induced by differentiating agents at the apical pole of the epithelial cell (Petit *et al.,* 1993).

In an established colon differentiation model, introduction of the c-H-ras-1 oncogene into a poorly differentiated human colon carcinoma cell line results in the acquisition of a more differentiated phenotype. Down-regulation of *mdr*-1 mRNA was shown to accompany ras-related differentiation events resulting in decreased phosphoglycoprotein (Pgp) synthesis and a significant reduction in membrane Pgp; consistent with that a reduction in Pgp-mediated drug resistance was observed in *ras* transfectants. Alternatively, when differentiation was induced by butyrate, there was an up-regulation of *mdr*-1 mRNA and Pgp synthesis but no alteration in drug sensitivity, and the levels of membrane-associated Pgp remained unchanged during exposure to sodium butyrate. Thus, modulation of Pgp expression in colon differentiation depends upon the agent used in inducing differentiation (Kramer *et al.,* 1993).

In another study it was reported that although the level of Pgp increased 25-fold after sodium butyrate treatment in SW620 human colon carcinoma cells, the intracellular accumulation of vinblastin, adriamycin, and actinomycin D increased rather than decreased, while colchicine showed the expected decrease in accumulation, as a result of increased efflux. In a Pgp-expressing multi-drug-resistant SW620 subline, butyrate treatment resulted in increased Pgp levels with decreased phosphorylation of Pgp. Time-course studies after butyrate treatment revealed a tight relationship between decreased Pgp phosphorylation and increased vinblastin accumulation, suggesting that the specificity of Pgp transport can be modulated by Pgp-phosphorylation in such a way that the transport of vinblastin, adriamycin or actinomycin D, but not that of colchicine, is diminished after dephosphorylation (Bates *et al.,* 1992).

Drug resistance can be associated with an altered specificity of reversible acetylation. Histone acetylation is specific for core histones, and the linker histone H1 is not a substrate of transacetylases. However, in the human colon cancer cell line LoVo/DX which was selected for resistance to the drug doxorubicin, it was shown that doxorubicin induces the acetylation of histone H1 (Mannironi and D'Incalci, 1988).

9.12
Butyrate and cancerogenesis in vivo

The effects of butyrate on mammary tumourigenesis by 7, 12-dimethylbenz(a)anthracene were investigated. As reported previously, a high incidence of mammary tumours was observed in rats fed a basal diet containing 20% margarine. However, the tumour-enhancing effect of margarine was inhibited when sodium butyrate was added to the high margarine diet, whereas butyrate did not cause any effect when added as a supplement to the basal diet. Thus, an inhibitory effect of butter on mammary tumourigenesis, previously reported by the same authors, may be caused by the butyrate content of milk lipids (Yanagi *et al.,* 1993).

To determine the *in vivo* relevance of fermentative production of butyrate, colonic butyrate concentrations were manipulated by feeding different dietary fibres and related to tumour development using the dimethylhydrazine model of large bowel cancer in the rat. It has been shown that guar gum and oat bran, while highly fermentable, are associated with low butyrate levels in the distal colon, while wheat bran causes significantly higher butyrate concentrations. Diets containing 10% of these fibres were administered for 3 weeks before, for 10 weeks during, and for 20 weeks after dimethylhydrazine administration. Thereafter, the animals were sacrificed and examined for tumours. Significantly fewer tumours were seen in the rats fed with wheat bran, when compared with those fed guar or oat bran, and the total tumour mass was also lower in rats fed wheat bran. Interestingly, rats on a diet without fibre had an intermediate tumour mass. There was a negative correlation between the butyrate concentration in stool and tumour mass. However, no such correlation could be observed with regard to acetate in stool, and stool volume. These findings indicate that fibre associated with high butyrate concentrations in the distal large bowel is protective against large bowel cancer, while soluble fibres that do not raise distal butyrate concentrations, are not protective (McIntyre *et al.,* 1993).

9.13
Butyrate and intestinal cells in vivo

Butyrate and other short-chain fatty acids are the products of anaerobic fermentation of carbohydrate (residual fibre which escaped complete digestion in the small intestine) by gut bacteria. Another source of butyrate is milk where it represents about 10% of fatty acids in lipids. For a further evaluation of a role of butyrate as tumour-suppressing compound, the *in vivo* concentrations of butyrate and other short-chain fatty acids should be related to those of *in vitro* experiments. The term '*in vivo*' in the following is used also for operative specimens, perfused colon, or freshly isolated colonocytes, in contrast to long-term cell cultures.

The colon contains an active microflora which ferment 30 g or more carbohydrate daily, derived from diet or intestinal secretions, with the production of at least 300 mM of short-chain fatty acids (acetic, propionic and butyric acids). In addition, about 6 g of urea is degraded to NH_3. These metabolic processes result in the generation of solutes which are then transported across the mucosa and which alter the pattern of water and electrolyte transport. Short-chain fatty acids are rapidly absorbed by passive diffusion as undissociated acids, although anion transport, possibly through a paracellular route, is also feasible (Cummings, 1984).

When concentrations of short-chain fatty acids were measured in venous blood of healthy subjects after a fast followed by various oral doses of the fermentable carbohydrate lactulose, or pectin, significant levels of propionate or butyrate were not detected in any blood samples. Blood acetate, however, rose in both cases, though with different kinetics. Thus, fermentation in the large intestine makes an important contribution to blood acetate levels in man and may influence metabolic processes in other organs, too (Pomare *et al.,* 1985).

Suspensions of isolated epithelial cells (colonocytes) from the human colon were used to assess utilization of respiratory fuels which are normally available to the colonic mucosa *in vivo* (Roediger, 1980a). Cells were prepared from operative specimens of the ascending and descending colon of several patients. The fuels used were, butyrate, on the one hand, which is produced only by anaerobic bacteria in the colonic lumen, and on the other, glucose and glutamine, which are normally present in the circulation. Oxygen consumption attributable to butyrate, when this was the

only substrate, was 73% in the ascending colon and 75% in the descending colon. In the presence of 10 mM glucose these proportions changed to 59% and 72%, respectively. Aerobic glycolysis was observed in both the ascending and descending colon. Glucose oxidation accounted for 85% of the oxygen consumption in the ascending and 30% in the descending colon in the absence of butyrate; in the presence of 10 mM butyrate these proportions decreased to 41% in the cells of the ascending colon and to 16% in those of the descending colon. Based on the assumption that events in the isolated colonocytes reflect utilization of fuels *in vivo,* the hypothesis was put forward that fatty acids of anaerobic bacteria are a major source of energy for the colonic mucosa, particularly in the distal colon (Roediger, 1980a).

Fermentable dietary fibre components are known to stimulate colonic crypt proliferation. As these compounds are rapidly degraded to short-chain fatty acids by the anaerobic microflora, the hypothesis was tested that this trophic effect of fibre may be mediated by short-chain fatty acids. Biopsies were taken from normal caecal mucosa of individuals during routine colonoscopy. They were incubated for three hours with sodium salts of short-chain fatty acids at physiological concentrations (acetate 60 mmol/L+ propionate 25 mmol/l+butyrate 10 mmol/l) or equimolar NaCl as a control. Caecal crypt proliferation was raised significantly in all incubation experiments with short-chain fatty acids. Butyrate (10 mmol/l, increase+89%) and propionate (25 mmol/1+70%) were about as effective in stimulating proliferation as the combination of three (103%), the effect of acetate being minor. Increasing the butyrate concentration to 25 mmol/l or 60 mmol/l did not result in a further increase of cell labelling. The short-chain fatty acids stimulated proliferation in the basal three crypt compartments only. An expansion of the proliferative zone to compartments 4 and 5 was not observed. Thus, it can be concluded that butyrate and propionate, especially, are luminal trophic factors for the caecal epithelium (Scheppach *et al.,* 1992a).

However, a growth-inhibiting effect of butyrate for normal colonocytes was observed in the rat model. The effects of increasing amounts of wheat bran (in AIN-76 semisynthetic diet) on the concentration of colonic luminal short-chain fatty acids, epithelial cell histone acetylation and cytokinetics, were studied in groups of Sprague-Dawley rats. Luminal contents were removed from the colon at sacrifice, frozen quickly, and analyzed for short-chain fatty acids by gas-liquid chromatography. Histone acetylation was assessed in cells isolated from the same animals. Cell proliferation was measured after a short pulse *in vivo* with (^{3}H)-thymidine. A significant inverse correlation between luminal butyrate levels and colonic cell proliferation was demonstrated. In addition, there was a positive linear correlation between luminal butyric acid levels and colon epithelial cell histone acetylation. From these data it was concluded that colonic butyrate levels can modulate DNA synthesis in the proliferative compartments of colonic crypts. The localization of dividing cells was unchanged and no induction of terminal differentiation was detectable, which is contrary to what has been observed for transformed cells in culture (Boffa *et al.,* 1992).

9.14
Short-chain fatty acids and human diseases other than cancer

Suspensions of colonocytes (isolated colonic epithelial cells) were prepared from mucosa of the descending colon from patients with quiescent ulcerative colitis (UC), with acute UC, and control subjects. In each group metabolic performance was investigated by assessing utilization of *n*-butyrate, the main respiratory fuel of colonic mucosa, as well as utilization of glucose and glutamine. In both acute and quiescent UC, oxidation of butyrate to CO_2 and ketones was

significantly lower than in control tissues, and the decrease correlated with the state of the disease. Enhanced glucose and glutamine oxidation compensated for decreased butyrate oxidation in UC, indicating that colonocytes in colitis were not metabolically degenerate cells. Thus, failure of butyrate oxidation reflects a variable yet definite metabolic defect in the mucosa in UC. Diminished oxidation of butyrate can explain the characteristic distribution of colitis along the colon, especially the frequency of UC in the distal colon. It is suggested that failure of butyrate oxidation in UC is the expression of an energy deficiency disease of the colonic mucosa (Roediger, 1980b).

No available test objectively measures impairment of function of the inflamed colonic mucosa in ulcerative colitis. To assess functional viability of the mucosa, rectal bicarbonate output in patients with different forms of colitis was measured by rectal dialysis in the presence of water and butyrate. In acute ulcerative colitis, compared with controls, bicarbonate output and pH were reduced; stimulated bicarbonate output with butyrate (incremental bicarbonate output) was reduced by 80% in acute ulcerative colitis. The results indicate that bicarbonate output is a useful and selective test of mucosal function in acute ulcerative colitis. A diminished incremental bicarbonate output with butyrate supports the above expressed view that in UC, oxidation of bacterial fatty acids in the mucosa is inadequate (Roediger *et al.,* 1984).

Short-chain fatty acid irrigation has been shown to ameliorate inflammation in diversion colitis. The effect of butyrate was tested in patients with distal ulcerative colitis who had been unresponsive to or intolerant of standard therapy. After butyrate irrigation, stool frequency (number/day) decreased and discharge of blood ceased in 9 out of 10 patients. Endoscopic score and histological degree of inflammation became more favourable. These data suggest that butyrate deficiency may play a role in the pathogenesis of distal ulcerative colitis (Scheppach *et al.,* 1992b).

Other factors, acting in combination with butyrate, add to the complexity of the *in vivo* situation in the colon. A recent investigation of the effects of butyrate, Ca^{2+}, and deoxycholate has shown that calcium and sodium ions and deoxycholate have complex influences in combination with butyrate on mucosal proliferation (Bartram *et al.,* 1993).

As butyrate acts in the nucleus by altering chromatin structure, the effects of this simple compound on the animal cell are profound and manifold. Since histone deacetylases are the main target of butyrate, most of the effects can be linked to the molecular event of hyperacetylation. Histone acetylation, however, has manifold functions. Thanks to the progress in the elucidation of the biological role of histone acetylation the chances are improving for the understanding of the molecular mechanism responsible for a given butyrate effect. Investigations with non-transformed intestinal epithelial cells were hampered by the lack of long-term cultures. Here the availability of conditionally-immortalized intestinal cell lines from transgenic mice (Whitehead *et al.,* 1993) should be helpful. However, there is still a long way to go, with further *in vitro* and *in vivo* studies, before a complete picture emerges of the contribution of gut bacteria, the constant companions of man, to human health.

References

Allfrey, V.G., Faulkner, R. and Mirsky, A.E., 1964, Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis , *Proc. Natl. Acad. Sci.,* **51,** 786–94.

Anderson, J.W. and Bridges, S.R., 1984, Short-chain fatty acid fermentation products of plant fiber affect glucose metabolism of isolated rat hepatocytes, *Proc. Soc. Exp. Biol. Med.,* **177,** 372–76.

Andrews, G.K. and Adamson, E.D., 1987, Butyrate selectively activates the metallothionein gene in teratocarcinoma cells and induces hypersensitivity to metal induction, *Nucl. Acids Res.,* **15,** 5461–75.

Ausio, J. and van Holde, K.E., 1986, Histone hyperacetylation: its effects on nucleosome conformation and stability, *Biochem.,* **25,** 1421–28.

Bahn, R.S., Zeller, J.C. and Smith, T.J., 1988, n-Butyrate increases *c-erb* A oncogene expression in human colon fibroblasts, *Biochem. Biophys. Res. Commun.,* **150,** 259–62.

Bartram, H.P., Scheppach, W., Schmid, H., Hofmann, A., Dusel, G., Richter, F., Richter, A. and Kasper, H., 1993, Proliferation of human colonic mucosa as an intermediate biomarker of carcinogenesis: effects of butyrate, deoxycholate, calcium, ammonia, and pH, *Cancer Res.,* **53,** 3283–88.

Bates, S.E., Currier, S.J., Alvarez, M. and Fojo, A.T., 1992, Modulation of P-glycoprotein phosphorylation and drug transport by sodium butyrate, *Biochem.,* **31,** 6366–72.

Berry, R.D. and Paraskeva, C., 1988, Expression of carcinoembryonic antigen by adenoma and carcinoma derived epithelial cell lines: possible marker of tumour progression and modulation of expression by sodium butyrate, *Carcinogenesis,* **9,** 447–50.

Blau, C.A., Constantoulakis, P., Shaw, C.M. and Stamatoyannopoulos, G., 1993, Fetal hemoglobin induction with butyric acid: efficacy and toxicity, *Blood,* **81,** 529–37.

Bode, J., Gomez-Lira, M.M. and Schröter H., 1983, Nucleosomal particles open as the histone core becomes hyperacetylated, *Eur. J. Biochem.,* **130,** 437–45.

Boffa, L.C., Lupton, J.R., Mariani, M.R., Ceppi, M., Newmark, H.L., Scalmati, A. and Lipkin, M., 1992, Modulation of colonic epithelial cell proliferation, histone acetylation, and luminal short chain fatty acids by variation of dietary fiber (wheat bran) in rats, *Cancer Res.,* **52,** 5906–12.

Bradbury, E.M., 1992, Reversible histone modifications and the chromosome cell cycle, *BioEssays,* **14,** 9–16.

Bresnick, E.H., John, S., Berard, D.S., LeFebvre, P. and Hager, G.L., 1990, Glucocorticoid receptor-dependent disruption of a specific nucleosome on the mouse mammary tumor virus promoter is prevented by sodium butyrate, *Proc. Natl. Acad. Sci.,* **87,** 3977–81.

Bresnick, E.H., Bustin, M., Marsaud, V., Richard-Foy, H. and Hager, G.L., 1992, The transcriptionally-active MMTV promoter is depleted of histone H1, *Nucl. Acids Res.,* **20,** 273–78.

Bunn, H.F., 1993, Reversing ontogeny, *N. Engl J. Med.,***328,** 129–31.

Carstea, E.D., Miller, S.P.F., Christakis, H. and O'Neill, R.R., 1993, Analogues of butyric acid that increase the expression of transfected DNAs, *Biochem. Biophys. Res. Commun.,* **192,** 649–56.

Chalkley, R. and Shires, A., 1985, The isolation of HTC variant cells which can replicate in butyrate, *J. Biol. Chem.,* **260,** 7698–704.

Collins, J.F., Herman, P., Schuch, C. and Bagby Jr, G.C., 1992, *c-myc* antisense oligonucleotides inhibit the colony-forming capacity of Colo 320 colonic carcinoma cells, *J. Clin. Invest.,* **89,** 1523–27.

Costlow, M.E., 1984, Differentiation-inducing agents decrease cryptic prolactin receptors in cultured rat mammary tumor cells, *Exp. Cell Res.,* **155,** 17–23.

Cousens, L.S. and Alberts, B.M., 1982, Accessibility of newly synthesized chromatin to histone acetylase, *J. Biol. Chem.,* **257,** 3945–49.

Cousens, L.S., Gallwitz, D. and Alberts, B.M., 1979, Different accessibilities in chromatin to histone acetylase, *J. Biol. Chem.,* **254,** 1716–23.

Csordas, A., 1990, On the biological role of histone acetylation, *Biochem. J.,* **265,** 23–38.

Cummings, J.H., 1984, Colonic absorption: the importance of short chain fatty acids in man, *Scand. J. Gastroenterol. Suppl.,* **93,** 89–99.

Darnell, R.B., 1984, Independent regulation by sodium butyrate of gonadotropin alpha gene expression and cell cycle progression in HeLa cells, *Mol. Cell. Biol.,* **4,** 829–39.

Desai, T.K., Nathan, D.F. and Morin, M.J., 1993, Potentiation of butyrate-induced differentiation in human colon tumor cells by deoxycholate, *Cancer Lett.,* **69,** 181–86.

Dover, G.J., Brusilow, S. and Samid, D., 1992, Increased fetal hemoglobin in patients receiving sodium 4-phenylbutyrate, *New Engl. J. Med.,* **327,** 569–70.

Engelmann, G.L., Staecker, J.L. and Richardson, A.G., 1987, Effect of sodium butyrate on primary cultures of adult rat hepatocytes, *In Vitro Cell. Dev. Biol.,* **23,** 86–92.

Fischkoff, S.A., Hoessly, M.C. and Rossi, R.M., 1990, Characterization of sublines of HL-60 human leukemia cells resistant to induction of differentiation by butyric *acid, Leukemia,* **4,** 302–6.

Flatow, U., Rabson, A.B., Hand, P.H., Willingham, M.C. and Rabson, A.S., 1989, Characterization and tumorigenicity of a butyrate-adapted T24 bladder cancer cell line, *Cancer Invest.,* **7,** 423–35.

Gamet, L., Daviaud, D., Denis-Pouxviel, C., Remesy, C. and Murat, J.C., 1992, Effects of short-chain fatty acids on growth and differentiation of the human colon-cancer cell line HT29, *Int. J. Cancer,* **52,** 286–89.

Gibson, P.R., Moeller, I., Kagelari, O., Folino, M. and Young, G.P., 1992, Contrasting effects of butyrate on the expression of phenotypic markers of differentiation in neoplastic and non-neoplastic colonic epithelial cells *in vitro, J. Gastroenterol., Hepatol.,* **7,** 165–72.

Graham, K.A. and Buick, R.N., 1988, Sodium butyrate induces differentiation in breast cancer cell lines expressing the estrogen receptor, *J. Cell Physiol.,* **136,** 63–71.

Grunstein, M., Durrin, L.K., Mann, R.K., Fisher-Adams, G. and Johnson, L.M., 1992, in McKnight, S.L. and Yamamoto, K.R. (Eds) *Transcriptional Requlation,* pp. 1295–315, New York: Cold Spring Harbor, Laboratory Press.

Guilbaud, N.F., Gas, N., Dupont, M.A. and Valette, A., 1990, Effects of differentiation-inducing agents on maturation of human MCF-7 breast cancer cells, *J. Cell Phys.,* **145,** 162–72.

Halleck, M.S. and Gurley, L.R., 1981, Histone acetylation and heterochromatin content of cultured *Peromyscus* cells, *Exp. Cell Res.,* **132,** 201–13.

Hebbes, T.R., Thorne, A.W. and Crane-Robinson, C., 1988, A direct link between core histone acetylation and transcriptionally active chromatin, *EMBO J.,* **7,** 1395–402.

Herold, K.M. and Rothberg, P.G., 1988, Evidence for a labile intermediate in the butyrate induced reduction of the level of *c-myc* RNA in SW837 rectal carcinoma cells, *Oncogene,* **3,** 423–28.

Klehr, D., Schlake, T., Maass, K. and Bode, J., 1992, Scaffold-attached regions (SAR elements) mediate transcriptional effects due to butyrate, *Biochem.,* **31,** 3222–29.

Kramer, R., Weber, T.K., Arceci, R., Morse, B., Simpson, H., Steele Jr, G.D. and Summerhayes, I.C., 1993, Modulation of mdr-1 expression by a H-ras oncogene in a human colon carcinoma cell line, *Int. J. Cancer,* **54,** 275–81.

Kruh, J., 1982, Effects of sodium butyrate, a new pharmacological agent, on cells in culture, *Molec. Cell. Biochem.,* **42,** 65–82.

Kruh, J., Defer, N. and Tichonicky, L.L., 1992, Action moléculaire et cellulaire du butyrate, *C. R. Soc. Biol,* **186,** 12–25.

Leder, A. and Leder, P., 1975, Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells, *Cell,* **5,** 319–22.

Lee, D.Y., Hayes, J.J., Pruss, D. and Wolffe, A.P., 1993, A positive role of histone acetylation in transcription factor access to nucleosomal DNA, *Cell,* **72,** 73–84.

Lewis, L.R. and Levine, A.E., 1992, Sodium butyrate alters the response of a human colon carcinoma cell line to transforming growth factor-β1, *Cancer Lett.,* **63,** 33–40.

Mannironi, C. and D'Incalci, M., 1988, Doxorubicin induces the acetylation of histone H1 in a human colon cancer cell line (LoVo/DX) selected for resistance to the drug, but not in the sensitive parental line (LoVo), *Biochem. Biophys. Res. Commun.,* **155,** 1221–29.

McIntyre, A., Gibson, P.R. and Young, G.P., 1993, Butyrate production from dietary fibre and protection against large bowel cancer in a rat model, *Gut,* **34,** 386–91.

McPherson, C.E., Shim, E.Y., Friedman, D.S. and Zaret, K.S., 1993, An active tissue-specific enhancer and bound transcription factors existing in a precisely positioned nucleosomal array, *Cell,* **75,** 387–98.

Milsted, A., Genduso Day, D.L., Pensky, J. and Cox, R.P., 1987, Phenotypes of HeLa S3 variant cell lines resistant to growth inhibition by sodium butyrate, *In Vitro Cell. Dev. Biol.,* **23,** 395–402.

Norton, V.G., Marvin, K.W., Yau, P. and Bradbury, E.M., 1990, Nucleosome linking number change controlled by acetylation of histones H3 and H4, *J. Biol. Chem.,* **265,** 19848–52.

O'Neill, T.E., Smith, J.G. and Bradbury, E.M., 1993, Histone octamer dissociation is not required for transcript elongation through arrays of nucleosome cores by T7 RNA polymerase *in vitro, Proc. Natl. Acad. Sci.,* **90,** 6203–7.

Ormandy, C.J., de Fazio, A., Kelly, P.A. and Sutherland, R.L., 1992a, Coordinate regulation of oestrogen and prolactin receptor expression by sodium butyrate in human breast cancer cells, *Biochem. Biophys. Res. Commun.,* **182,** 740–45.

Ormandy, C.J., de Fazio, A., Kelly, P.A. and Sutherland, R.L., 1992b Transcriptional regulation of prolactin receptor gene expression by sodium butyrate in MCF-7 human breast cancer cells , *Endocrinology,* **131,** 982–84.

Perrine, S.P. and Faller, D.V., 1993, Butyrate-induced reactivation of the fetal globin genes: a molecular treatment for the *α*-hemoglobinopathies, *Experientia,* **49,** 133–37.

Perrine, S.P., Greene, M.F. and Faller, D.V., 1985, Delay in the fetal globin switch in infants of diabetic mothers, *New Engl. J. Med.,* **312,** 334–38.

Perrine, S.P., Miller, B.A., Faller, D.V., Cohen, R.A., Vichinsky, E.P., Hurst, D., Lubin, B.H. and Papayannopoulou, T., 1989, Sodium butyrate enhances fetal globin gene expression in erythroid progenitors of patients with Hb SS and a thalassemia, *Blood,* 74, 454–59.

Perrine, S.P., Ginder, G.D., Faller, D.V., Dover, G.H., Ikuta, T., Witkowska, H.E., Cai, S.P., Vichinsky, E.P. and Olivieri, N.F., 1993, A short-term trial of butyrate to stimulate fetal-globin-gene expression in the *β*-globin disorders, *New Engl. J. Med.,* **328,** 81–86.

Petit, J.M., Chauffert, B., Dimanche-Boitrel, M.T., Genne, P., Duchamp, O. and Martin, F., 1993, mdr 1 gene-expression and villin synthesis in a colon cancer cell line differentiated by sodium butyrate, *Anticancer Res.,* **13,** 487–90.

Planchon, P., Magnien, V., Beaupain, R., Mainguene, C., Ronco, G., Villa, P. and Brouty-Boye, D., 1992, Differential effects of butyrate derivatives on human breast cancer cells grown as organotypic nodules *in vitro* and as xenografts *in vivo, In Vivo,* **6,** 605–10.

Pomare, E.W., Branch, W.J. and Cummings, J.H., 1985, Carbohydrate fermentation in the human colon and its relation to acetate concentrations in venous blood, *J. Clin. Invest.,* **75,** 1448–54.

Prasad, K.N., 1980, Butyric acid: a small fatty acid with diverse biological functions, *Life Sci.,* **27,** 1351–58.

Prasad, K.N. and Sinha, P.K., 1976, Effect of sodium butyrate on mammalian cells in culture: a review, *In Vitro,* **12,** 125–32.

Rabizadeh, E., Shaklai, M., Nudelman, A., Eisenbach, L. and Rephaeli, A., 1993, Rapid alteration of *c-myc* and *c-jun* expression in leukemic cells induced to differentiate by a butyric acid prodrug, *FEBS Lett.,* **328,** 225–29.

Reeves, R. and Cserjesi, P., 1979, Sodium butyrate induces new gene expression in Friend erythroleukemic cells, *J. Biol. Chem.,* **254,** 4283–90.

Resnicoff, M. and Medrano, E.E., 1989, Growth factors and hormones which affect survival, growth, and differentiation of the MCF-7 stem cells and their descendants, Exp. *Cell Res.,* **181,** 116–25.

Riggs, M.G., Whittaker, R.G., Neumann, J.R. and Ingram, V.M., 1977, n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells, *Nature,* **268,** 462–64.

Roediger, W.E.W., 1980a, Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man, *Gut,* **21,** 793–98.

Roediger, W.E.W., 1980b, The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet,* **2,** 712–15.

Roediger, W.E.W., Lawson, M.J., Kwok, V., Grant, A.K. and Pannall, P.R., 1984, Colonic bicarbonate output as a test of disease activity in ulcerative colitis, *J. Clin. Pathol.,* **37,** 704–7.

Rubenstein, P., Sealy, L., Marshall, S. and Chalkley, R., 1979, Cellular protein synthesis and inhibition of cell division are independent of butyrate-induced histone hyperacetylation, *Nature,* **280,** 692–93.

Ruiz-Carrillo, A., Wangh, L.J., Littau, V.C. and Allfrey, V.G., 1974, Changes in histone acetyl content and in nuclear non-histone protein composition of avian erythroid cells at different stages of maturation, *J. Biol. Chem.,* **249,** 7358–68.

Scheppach, W., Bartram, P., Richter, A., Richter, F., Liepold, H., Dusel, G., Hofstetter, G., Ruthlein, J. and Kasper, H., 1992a, Effect of short-chain fatty acids on the human colonic mucosa *in vitro, J. Parenter. Enter. Nutr.,* **16,** 43–48.

Scheppach, W., Sommer, H., Kirchner, T., Paganelli, G.M., Bartram, P., Christl, S., Richter, F., Dusel, G. and Kasper, H., 1992b, Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis, *Gastroenterol.,* **103,** 51–56.

Souleimani, A. and Asselin, C., 1993a, Regulation *of c-fos* expression by sodium butyrate in the human colon carcinoma cell line Caco-2, *Biochem. Biophys. Res. Commun.,* **193,** 330–36.

Souleimani, A. and Asselin, C., 1993b, Regulation of *c-myc* expression by sodium butyrate in the colon carcinoma cell line Caco-2, *FEBS Lett.,* **326,** 45–50.

Staecker, J.L., Sawada, N. and Pilot, H.C., 1987, Stimulation of DNA synthesis in primary cultures of adult rat hepatocytes by sodium butyrate, *Biochem. Biophys. Res. Commun.,* **147,** 78–85.

Stevens, M.S., Aliabadi, Z. and Moore, M.R., 1984, Associated effects of sodium butyrate on histone acetylation and estrogen receptor in the human breast cancer cell line MCF-7, *Biochem. Biophys. Res. Commun.,* **119,** 132–38

Stoddart, J.H., Lane, M.A. and Niles, R.M., 1989, Sodium butyrate suppresses the transforming activity of an activated N-ras oncogene in human colon carcinoma cells, *Exp. Cell Res.,* **184,** 16–27.

Straka, C. and Hörz, W., 1991, A functional role for nucleosomes in the repression of a yeast promoter, *EMBO J.,* **10,** 361–68.

Takeda, M., Kosaka, M., Nishina, Y., Sawada, K., Matsumoto, K. and Nishimune, Y., 1992, Teratocarcinoma F9 cells induced to differentiate with sodium butyrate produce both tissue-type and urokinase-type plasminogen activators, *J. Cell. Biochem.,* **49,** 284–89.

Tanaka, Y., Bush, K.K., Eguchi, T., Ikekawa, N., Taguchi, T., Kobayashi, Y. and Higgins, P.J., 1990, Effects of 1,25-dihydroxyvitamin D_3 and its analogs on butyrate-induced differentiation of HT-29 human colonic carcinoma cells and on the reversal of the differentiated phenotype, *Arch. Biochem. Biophys.,* **276,** 415–23.

Taylor, C.W., Kim, Y.S., Childress-Fields, K.E. and Yeoman, L.C., 1992, Sensitivity of nuclear *c-myc* levels and induction to differentiation-inducing agents in human colon tumor cell lines, *Cancer Lett.,* **62,** 95–105.

Tazi J. and Bird, A., 1990, Alternative chromatin structure at CpG islands, *Cell,* 60, 909–20.

Tse, C.S. and Williams, D.M., 1992, Inhibition of human endothelial cell proliferation *in vitro* in response to n-butyrate and propionate, *J. Periodont. Res.,* **27,** 506–10.

Tsukiyama, T., Becker, P.B. and Wu, C., 1994, ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor, *Nature,* **367,** 525–32.

Turner, B.M., 1991, Histone acetylation and control of gene expression, *J. Cell Sci.,* **99,** 13–20.

Whitehead, R.H., VanEeden, P.E., Noble, M.D., Ataliotis, P. and Jat, P.S., 1993, Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2K^h-tsA58 transgenic mice, *Proc. Natl. Acad. Sci.,* **90,** 587–91.

Wolffe, A.P., 1994, Transcription: in tune with the histones, *Cell,* **77,** 13–16.

Wright, J.A., 1973, Morphology and growth rate changes in Chinese hamster cells cultured in presence of sodium butyrate, *Exp. Cell Res.,* **78,** 456–60.

Yanagi, S., Yamashita, M. and Imai, S., 1993, Sodium butyrate inhibits the enhancing effect of high fat diet on mammary tumorigenesis, *Oncology,* **50,** 201–4.

Yoshida, M., Kijima, M., Akita, M. and Beppu, T., 1990, Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by Trichostatin A, *J. Biol. Chem.,* **265,** 17174–79.

Section 4

Fat metabolism

Chapter 10
Bacteria and fat digestion

M.J.Hill

10.1
Introduction

Fat, by virtue of its very high energy density, is an important component of human nutrition and fat digestion in the healthy person is an extremely efficient process. Even on the high fat diets consumed in many western countries only 1–4% of dietary fat is lost in faeces per day. Fats are important nutrients not only because of their high energy density but also because, as hydrophobic molecules, they are essential components of cell membranes.

Early studies suggested that dietary fat was digested in a micellar form; for example in the seventeenth century Rudbeck noted the milky appearance of material from the thoracic duct after a fat meal. Claude Bernard in the nineteenth century reported the opacification of mesenteric lacteal during the course of digestion of fat; he also suggested a role for pancreatic juice in fat digestion. Knowledge slowly accumulated over the next century and the basic elements of fat digestion have been known for many decades (although details are still being worked out).

In principle, after homogenization of the food in the stomach the fat is emulsified by biliary action in the duodenum; this increases the surface area available for lipolytic activity and provides a particle size ideal for absorption in the micellar phase from the small bowel. This process allows the ready and efficient absorption of large amounts of fat together with the associated fat-soluble vitamins.

This process occurs in a part of the gut that in health is not normally colonized by bacteria and so there would appear to be no major role, *a priori,* for bacteria in fat digestion. However, although fat digestion is 96–99% efficient in the healthy gut, there is a residual amount that escapes digestion and enters the large bowel, where it is readily available for bacterial metabolism, the products of which may be absorbed by passive diffusion and utilized by the host. In addition there are various disease states in which the small bowel is colonized by bacteria. In this chapter I will review the aspects of fat digestion, particularly those that are affected by bacteria in small bowel infection; this will be followed by the effects of bacterial infection on fat digestion, and finally a review of the bacterial digestion of the residual fat which reaches the colon.

10.2
Fat digestion

Fat is emulsified in the duodenum by bile salts to give a ternary complex of bile salt, fatty acid and triglyceride; bile secretion is triggered by the gut hormone cholecystokinin which regulates the amount as well as the time of bile secretion. The ternary mixture gives micelles of a size which optimizes digestion of the fat by pancreatic enzymes. The hydrolysis of the fat is not complete but yields a mixture of fatty acids, glycerol, monoglyceride and diglyceride together with small amounts of triglyceride. The extent of digestion depends on the type of fat consumed and on the intestinal milieu. As the amount of free fatty acid in the micelles increases so the pancreatic lipase is displaced;

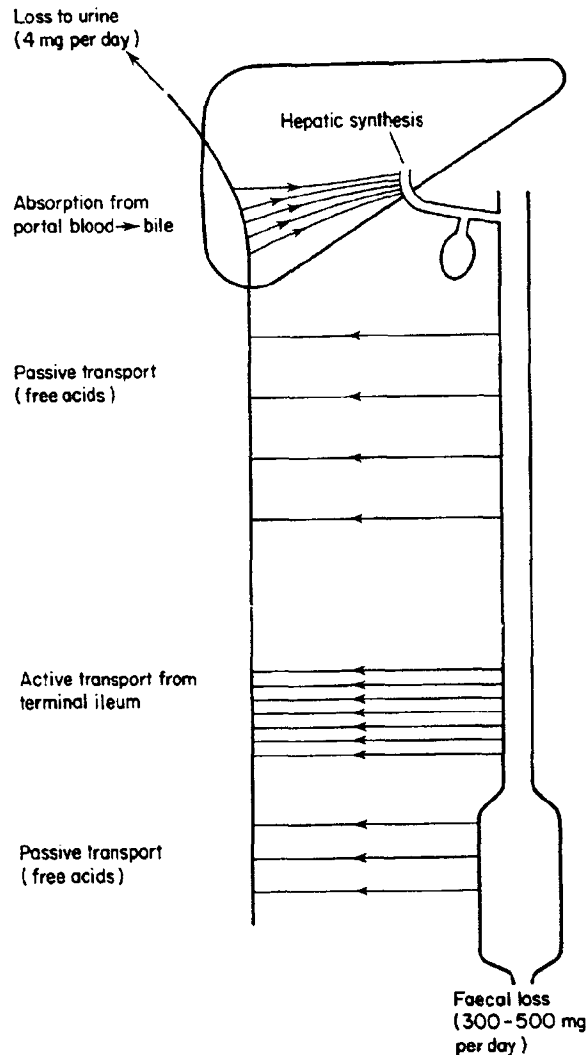


Figure 10.1 The enterohepatic circulation of bile

this releases the enzyme for absorption to and hydrolysis of other droplets of fat but also means that the fat digestion in any individual droplet will never be completed.

Fat is absorbed from the colon as droplets; those less than 0.5 *µm* in diameter pass through 'pores' in the brush border and into the mucosal cells, where they are admixed with phospholipid and transferred to the lymph as chylomicrons. Fatty acids released from fat by hydrolysis and separated from the micelles are absorbed by a separate mechanism and are transferred to the liver via the portal blood system.

The micellar size is of great importance both to lipolysis and to absorption from the gut. Micelles of the optimal size are formed by conjugated bile acids; free bile acids have poorer detergent properties and so form larger micelles; these are relatively poorly absorbed from the gut and their smaller specific surface area makes them less readily available to pancreatic lipase.

Conjugated bile acids are not absorbed with the fat particles but return to the gut lumen to participate in further micelle formation. In consequence, although there is slow removal of bile conjugates by passive diffusion, a high luminal concentration of bile acid conjugates is maintained throughout the length of the small bowel and ensures that the critical micellar concentration (CMC, needed to permit micelles of optimal size to be formed) is exceeded throughout the duodenum, jejunum and upper ileum. The conjugated bile acids are recovered by an active transport mechanism from the terminal ileum and returned via the portal blood system to the liver for resecretion in bile, thus completing the enterohepatic circulation (EHC) of the bile salts. Figure 10.1 illustrates the EHC of bile salts.

The bile pool undergoes 6–10 enterohepatic cycles per day, depending on the amount of dietary fat, and 2–3 cycles per meal. The EHC of bile salts is highly efficient with only 1–4% lost in each cycle. The total bile acid pool is 2–5 g and this is sufficient to enable the 100–150 g fat per day in the western diet to be digested readily and efficiently. Normally 2–4 g per day of fat is lost in faeces, almost entirely in the form of fatty acid and bacterial lipid; a faecal fat level of more than 5 g per day is diagnostic of steatorrhoea—fat malabsorption.

10.2.1
Metabolism of fats and phospholipids

Although fat digestion is highly efficient the dietary intake is such that gram quantities enter the caecum, principally in the form of glycerides, phospholipids and fatty acids together with smaller amounts of more complex lipid. In addition large quantities of short-chain fatty acid (SCFA) are generated in the colon during the digestion of complex carbohydrate (as described in Chapters 8 and 9).

Lipases able to hydrolyze the ester linkages between glycerol and long-chain fatty acids (LCFA) in glycerides have been detected in a wide range of species and genera of gut bacteria (Table 10.1). The released LCFA is not utilized by most bacterial species and in fact has considerable antimicrobial activity (with unsaturated being more bactericidal than saturated fatty acids). It is likely, therefore, to have an influence on the composition of the gut bacterial flora. The observations that persons consuming a high fat diet have a distinctive caecal flora rich in non-sporing anaerobic bacteria is consistent with such a selective antibacterial effect. The released glycerol, in contrast, is rapidly utilized by most gut bacterial species and faecal glycerol levels are very low. The lipases have been most widely studied in the clostridia, where they are a useful taxonomic indicator in the rapid identification of some species.

Table 10.1 Production of lipase anaerobic non-sporing bacterial species from the human large intestine.

Species producing lipase	*Non-producers of lipase*
Bacteroides melaninogenicus *Bacteroides intermedius*	Other *Bacteroides* spp.
Fusobacterium necrophorum	Other *Fusobacterium* spp.
Eubacterium combesii *Propionibacterium avidum* *Propionibacterium granulosum*	Other *Eubacterium* spp. Other *Propionibacterium* spp.
–	*Lactobacillus* spp.
–	*Bifidobacterium* spp.
–	*Peptococcus* spp.

Table 10.2 Production of lipase and lecithinase by some species of *Clostridium*.

	Lipase	*Lecithinase*
Cl. sordelii	–	+
Cl. bifermentans	–	+
Cl. sporogenes	+	–
Cl. histolyticum	–	–
Cl. perfringens	–	+
Cl. novyi	+	+
Cl. haemolyticum	–	+
Cl. difficile	–	–
Cl. septicum	–	–
Cl. butyricum	–	–

Although the fatty acids are not widely utilized in the gut the unsaturated acids are hydrogenated to their saturated analogues or hydrated to their hydroxy-fatty acid analogues (Pearson, 1971). In consequence, although more than 30% of dietary fatty acids are unsaturated, the faecal lipids contain only small amounts of such LCFA.

The study of the metabolism of phospholipids by bacteria was initially prompted by their taxonomic value in clostridial classification. Phospholipase A yields lysolecithin from lecithin; phospholipase A, removes the fatty acid from position 1 of glycerol whilst the A_2 enzyme removes the unsaturated fatty acid from the C-2 position. Phospholipase B hydrolyzes the acyl bond in lysolecithin to give glycerophosphocholine. Phospholipase C hydrolyzes the glycerophosphatidyl linkage to give glycerol and choline phosphate. Phospholipase D hydrolyzes the phosphocholine linkage to release choline and glycerophosphate. The phospholipases of *Clostridium* spp. have been extensively studied but the enzymes from the other gut bacteria have not received the same attention. Table 10.2 describes lipase and lecithinase production by the most commonly isolated species of clostridia (Smith, 1977). Choline, released by phospholipase D, undergoes N-dealky-lation to yield first trimethylamine, then dimethylamine, then methylamine and finally ammonia. The amines are absorbed and ultimately excreted in urine, but are also secreted in gastric juice and vaginal secretion, where they may provide a substrate for N-nitrosation (see Chapter 6).

10.3 *Bile acid conservation*

Two bile acids are synthesized in the liver, namely cholic (3α7α12α-trihydroxy-cholan-24-oic) and chenodeoxycholic (3α7α-dihydroxyclolan-24-oic) acids and these primary bile acids are secreted in bile as their glycine or taurine conjugates—the primary bile salts. There is normally a higher proportion of glycine (60%) than taurine (40%) conjugates, and more cholic (60%) than chenodeoxycholic (40%) acid synthesized in the liver. They are potent detergents and efficient emulsifiers of fat in the small bowel. When emulsified fat droplets are taken up from the gut, the bile salts are returned to the bowel lumen where they are able to form mixed micelles with remaining fat droplets and so aid their absorption. Bile salts can be absorbed slowly from the gut by passive diffusion and are removed efficiently from the terminal ileum by an active transport mechanism. This is an effective process and is 96–99% efficient, with only 1–4% entering the colon during each enterohepatic cycle. The bile pool is circulated 2–3 times per meal and 6–10 times per day; the rate of circulation increases with the amount of dietary fat.

Bile secretion is controlled by cholecystokinin to ensure that the bile salt concentration exceeds the CMC—the level needed to emulsify fat sufficiently to yield droplets small enough to be optimally absorbed from the small bowel. The importance of this controlled delivery of bile is demonstrated by the effect of cholecystectomy on fat digestion. If the luminal concentration of bile salts fails to achieve the CMC then fat digestion is impaired; such a failure can occur if:

(a) the bile pool size is insufficient—congenital lack of bile salts is associated with severe steatorrhoea;
(b) the bile salts are deconjugated to free bile acids and further degraded to the secondary bile acids —bile acids are less efficient detergent molecules than the bile salts; secondary bile acids are less efficient than the primary bile acids synthesized by the liver;
(c) the bile salts (which are only slowly absorbed from the small bowel and so maintain the CMC) are deconjugated to free bile acids which can then be readily absorbed from the small bowel leaving the residual bile below the CMC.

The bile pool size is determined by the rate of synthesis of new bile acids and the rate of their loss from the EHC; these two are normally in balance ensuring maintenance of the pool size. Excess losses can occur as a result of either impaired reabsorption from the terminal ileum or impaired hepatic transfer from the portal blood to the bile. Ileal reabsorption requires a healthy terminal ileum, but this can be impaired in regional ileitis (Crohn's disease) or ileal resection (usually for the treatment of ileitis or other severe inflammatory disease of the ileum). It is likely that there is a role for bacteria in the causation or maintenance of ileitis but it has still to be elucidated. Impaired hepatic transfer results from hepatitis; this can have a number of possible causes including viral hepatitis.

10.4 *Bacterial metabolism of bile salts*

The main role for bacteria in bile acid conservation and fat digestion is through bile salt degradation. In general, the small bowel is largely free of bacteria but bacteria can be present in

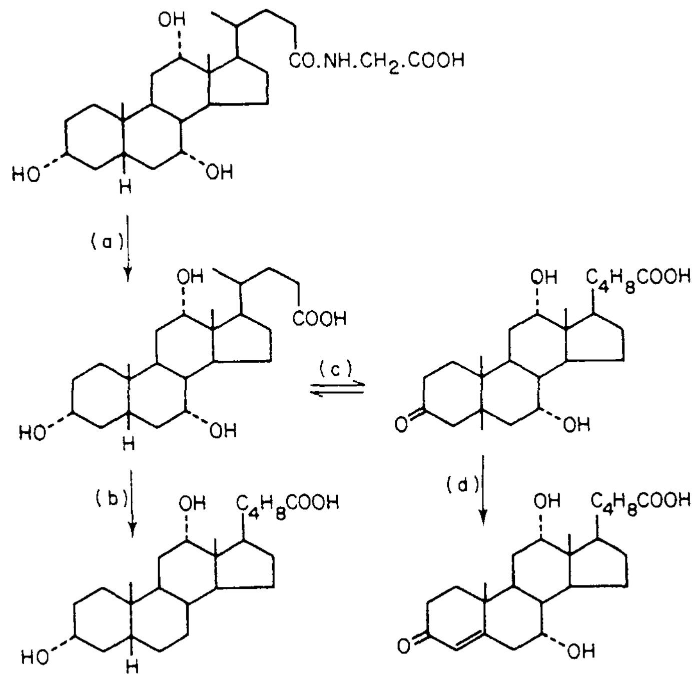


Figure 10.2 Pathways for bile salt metabolism

sufficient concentrations to cause significant bile salt degradation in blind loop syndrome, diverticulitis, mucosal infection in the duodenum or jejunum and severe gallbladder or biliary tract infection. Although these are the situations in which bile salt degradation affects fat absorption, bile salt metabolism occurs as a normal event in the colon with the bile salts lost to the EHC as the substrates.

Bile salts can be metabolised via a range of pathways which are illustrated in Figure 10.2 for cholylglycine.

10.4.1 Cholylglycine hydrolase

The first step in bile salt metabolism is the hydrolysis of the amide bond to release the free cholic acid and glycine by cholylglycine hydrolase. This enzyme is produced by a wide range of colon anaerobes (Table 10.3), particularly the dominant *Bacteroides* spp. and *Bifidobacterium* spp. The enzyme was first described in detail by Nair *et al.* (1967); they isolated the enzyme from *Clostridium perfringens* as a cell-free product which was equally active on glycine and taurine conjugates and had a pH-optimum of 5.6–5.8. Further cell-free enzyme preparations have been isolated by other groups (e.g. Hill and Drasar, 1968; Aries and Hill, 1970) and Table 10.4 illustrates some of the characteristics of hydrolase from different organisms. In the colon bile salts are almost completely deconjugated and faeces contain very small amounts, if any, of bile salts. Estimates of enzyme

activity suggest that they are deconjugated within 30 minutes of entering the caecum; this compares with a normal colonic transit time of 40–80 hours.

10.4.2
7-Dehydroxylase

7-Dehydroxylation is the second step in bile salt metabolism in the colon. It removes the 7a-hydroxyl group from cholic acid (CA) to yield deoxycholic acid (DC), and from chenodeoxycholic acid (CD) to yield lithocholic acid (LC). The products of microbial metabolism, DC and LC, are termed secondary bile acids. This reaction can only take place after initial deconjugation since the dehydroxylase has a requirement for a free 24-carboxyl group. In faeces the primary bile acids account for only 0–15% of the total; despite the rapid removal of the conjugating amino acid by the hydrolase, and the 40–80-hour gut transit time, this suggests that the 7-dehydroxylase is widely distributed amongst the colonic bacterial flora. This was confirmed in studies of fresh bacterial isolates by Hill and Drasar (1968), as illustrated in Table 10.5. A significant proportion of strains of the dominant genera *Bacteroides* spp. and *Bifidobacterium* spp. were capable of dehydroxylation of

Table 10.3 Production of cholyglycine hydrolase by gut bacteria.

Genes	*Number Tested*	*Percentage Active*
E. coli	342	0
Strep. salivarius	112	0
Strep. viridans	22	0
Enterococcus spp.	252	91
Lactobacillus spp.	60	0
Bacteroides spp.	54	76
Bifidobacterium spp.	194	80
Clostridium spp.	130	95
Veillonella spp.	92	50

Table 10.4 The characteristics of cholylglycine hydrolase from different species (data from Hill and Drasar, 1968).

	Enterococcus	*Clostridium*	*Bacteroides*	*Bifid.*
Location	Cytoplasm	Cytoplasm	Cytoplasm	Extra-cellular
Activity				
glycineconj.	+++	+++	+++	+++
taurineconj.	+	+++	+++	–
% inhibition by 8.5 mM free bile acid	0%	50%	10%	0%
pH–optimum	4.8	5.5	5.5	5.5
Constitutive	++	++	++	++
Inducibility	–	–	–	–
Redox requirements	anaerobic	anaerobic	anaerobic	anaerobic

Table 10.5 Production of 7-dehydroxylase by fresh isolates of human gut bacteria (data from Hill and Drasar, 1968).

Organisms	*Percentage producing 7α–dehydroxylase*	
	UK isolates	*Ugandan isolates*
E. coli	0	0
Strep. salivarius	0	0
Strep. viridans	0	0
Ent. faecalis	3	0
Lactobacillus spp.	0	0
Bacteroides spp.	44	3
Bifidobacterium spp.	40	4
Clostridium spp.	34	6
Veillonella spp.	4	3

Table 10.6 Characteristics of the cholvl 7a-dehydroxylase from four different genera.

Test	*Characteristics*
pH for:	
production	greater than 6
activity	7–8
Constitutive activity	absent
Inducibility	+++
Substrate inhibition	+++
Redox requirements	anaerobic

cholic acid.

The dehydroxylase was studied in detail by Midtvedt and Norman (1967) from lactobacilli and by Aries and Hill (1970) from *Bacteroides, Bifidobacterium* and *Clostridium* spp. It was found to be inducible but was only produced at pH values greater than 6. The enzyme characteristics are described in Table 10.6. The enzyme is inhibited by excess substrate and is plasmid-mediated; in many strains the plasmid is unstable and the dehydroxylase is lost on subculture *in vitro*.

The molecular biology of the dehydroxylase has been studied by the group led by Hylemon (e.g. Coleman *et al.*, 1987; White *et al.*, 1988; Malloney *et al.*, 1990). They have also determined the detailed mechanism of the reaction (Hylemon *et al.*, 1991). Early studies by Samuelsson (1960) suggested that the first step in dehydroxylation was the removal of the elements of water across the 6–7 bond to yield a substituted 6-cholenic acid, followed by hydrogenation of the double bond to give DC from CA. Hylemon showed that the reaction is much more complex and involved an initial 3-oxo formation followed by 4-en-3-one, which was then dehydrated across the 6–7 bond to give a 4,6-dien-3-one; following this there was hydrogenation first of the 6–7 bond, then of the 4-en-3-one to give deoxycholic acid. An interesting by-product of the reaction was allodeoxycholic acid, which has been found in human faeces but the origin of which has been debated.

Table 10.7 Production of hydroxysteroid oxidoreductases by gut bacteria.

Organisms	*Number Tested*	*Hydroxyl group*		
		C3	*C7*	*C12*
E. coli	162	39	78	10
Strep. salivarius	80	0	0	0
Strep. viridans	12	0	0	0
Enterococcus spp.	90	29	81	10
Lactobacillus spp.	40	0	0	0
Bacteroides spp.	37	30	79	10
Bifidobacterium spp.	57	21	56	10
Clostridium spp.	70	47	87	10
Veillonella	60	10	50	0

Table 10.8 Properties of the hydroxy-steroid dehydrogenases active on the C-3, C-7 and C-12 hydroxyl groups.

	Hydroxyl group		
	C3	*C7*	*C12*
Inducible	+++	+++	+++
Constitutive	±	+	±
Substrate specificity			
tri:dihydroxy	1:1.2	1:1.2	1:1.1
pH–optimum:			
oxidation	10–11	9–10	8–8.5
reduction	6–8	6.5–7.5	6.0
Km (mM)	0.1–0.3	0.1–0.5	0.2
Inhibition by substrate excess	low	0–16%	low
Molecular weight		50–100KD	

10.4.3 Hydroxysteroid dehydrogenases

A further route for metabolism of bile acids by gut bacteria is via the oxidoreductase enzymes active on the 3, 7 and 12-hydroxyl groups. These enzymes were reported by Hoehn *et al.* (1944) and by Norman and Bergman (1960) and by Midtvedt and Norman (1967), who screened 55 assorted strains and demonstrated that many of them could produce oxidoreductases active at the 7, 3 or 12 positions. In a survey of more than 1100 faecal strains of bacteria (Table 10.7) it was demonstrated that the 7-oxidoreductase was the most widely distributed, followed by the enzyme active at the 3 position and that acting on the 12-hydroxyl group. There has been great interest in isolated purified oxidoreductases because they offer a prospect of detailed analysis of the bile acid profile without the need for chromatography; they have been reviewed by MacDonald *et al.* (1983). A detailed description of a highly purified 7-oxidoreductase has been given by Franklund *et al.*, (1990), and the same group went on to clone and sequence the gene coding for the enzyme (Baron *et al.*, 1991). Table 10.8 summarizes some of the properties of the oxidoreductases.

An important feature of the oxidoreductases is that the oxidation step requires oxygen; this is sufficient to make the enzymes of little importance in the healthy gut and there are usually only small amounts of oxo-bile acids in human faeces.

Table 10.9 The profiles of biliary and faecal bile acids.

Bile acid	*% of total bile acids*	
	Bile	*Faeces*
Primary		
Cholic acid	30–40%	<3%
Chenodeoxycholic acid	30–40%	<3%
Secondary		
Deoxycholic acid	15–25%	40–50%
Lithocholic acid	0–5%	30–40%
Keto bile acids	trace	0–10%
β–hydroxylated acids	0–10%	0–10%

In the normal human gut the metabolism of bile acids only occurs in the large bowel, and perhaps the final few inches of ileum, where the density of the bacterial flora is sufficient to ensure an adequate enzyme activity. During colonic transit the bile acids are rapidly deconjugated then 7-dehydroxylated; in most persons both reactions have gone to completion before the gut contents have reached the transverse colon (Boyer *et al.,* 1984). Little further degradation occurs, probably because by that time the gut contents have been dehydrated to a turgid mass which allows little opportunity for mixing and exposure of substrate to enzyme.

At colonic pH values DC is somewhat soluble and available for absorption by passive diffusion; it then returns to the liver for conjugation and resecretion in bile. Lithocholic acid is very hydrophobic but is sulphated by the colonic mucosa; a small proportion then returns to the liver as the 3-sulphate where it is conjugated at the 24 position and secreted in bile as the 3-sulphated lithocholic acid conjugate. During childhood the amount of DC in bile slowly increases and in adulthood the bile acid composition stabilizes at approximately 40% CA/40% CD/20% DC and less than 1% LC. Table 10.9 compares biliary and faecal bile acid profiles.

10.4.4 Toxicological consequences of bile acid metabolism

Faecal bile acids, particularly the secondary bile acids DC and LC, have been implicated in the aetiology of colorectal cancer (for reviews see Hill, 1986,1992; Owen *et al.,* 1987; Rafter *et al.,* 1986) and pan-colitis (Hill *et al.,* 1987). The evidence for this association is summarized in Table 10.10. In addition to this chronic toxicity there are acute effects of high colonic bile acid concentrations. Bile acids stimulate water secretion, and when colonic concentrations are high, for example when ileal recovery is impaired, there is a consequent cholerrheic diarrhoea. It is possible that colonic bile acids may facilitate the absorption of fat soluble vitamins and this may be particularly relevant to the vitamin K balance in humans.

Bacterial metabolism of bile salts is important in impairing fat digestion. When surgically-constructed 'blind loops' or small bowel diverticulae, strictures or fistulae become infected, their isolation from the luminal stream allows them to be heavily colonized (Drasar and Hill, 1974) and this results in the spillage of mixed populations of faecal anaerobes into the gut lumen. These organisms are usually potent metabolizers of bile salts (Hill and Drasar, 1968). They are particularly active in bile salt deconjugation (to the point where this reaction is widely used in a breath test of small bowel colonization) but may also have significant 7-dehydroxylase activity. The deconjugation

Table 10.10 The evidence implicating bile acids in colorectal carclnogenesls.

Type of study	*Observation*
Comparison of populations	Faecal bile acid (FBA) concentration correlated with colorectal cancer (CRC) incidence in many studies from many labs.
Animal studies	Diet manipulations that increase the FBA concentration increase the CRC prevalence and vice versa.
Carcinogenicity studies	A number of secondary bile acids (including the two principal faecal bile acids) are tumour promoters in the rat colon.
Bile acid-binding sites	Binding sites for deoxycholic acid present in more than 30% of colon cancers but less than 3% of tissues from adjacent sites.
Cohort studies	Long-term follow-up of cohorts of patients with chronic pan-colitis show that the risk of CRC is related to the FBA concentration.

Table 10.11 The causes of clinical consequences of blind-loop syndrome.

Type of study	*Observation*
Comparison of populations	Faecal bile acid (FBA) concentration correlated with colorectal cancer (CRC) incidence in many studies from many labs.
Animal studies	Diet manipulations that increase the FBA concentration increase the CRC prevalence and vice versa.
Carcinogenicity studies	A number of secondary bile acids (including the two principal faecal bile acids) are tumour promoters in the rat colon.
Bile acid-binding sites	Binding sites for deoxycholic acid present in more than 30% of colon cancers but less than 3% of tissues from adjacent sites.
Cohort studies	Long-term follow-up of cohorts of patients with chronic pan-colitis show that the risk of CRC is related to the FBA concentration.

decreases the ability of bile to emulsify the luminal fat and this effect is compounded when 7-dehydroxylation occurs.

Blind loop syndrome has a number of clinical consequences (Table 10.11) and these include steatorrhoea, malabsorption of vitamin B_{12}, C and the fat-soluble vitamins D and K, all of which respond to antibiotic therapy. The bacteroides are probably the organisms most responsible for deconjugation of bile salt and dehydroxylation in the small bowel (Drasar *et al.*, 1966; Donaldson, 1970), and the treatment of choice remains metronidazole together with an agent active against the facultative flora such as gentamycin.

References

Aries, V.C. and Hill, M.J., 1970, Degradation of steroids by intestinal bacteria. I. Deconjugation of bile salts, *Biochim. Biophys. Acta,* **202,** 526–34.

Baron, S.F., Franklund, C.V. and Hylemon, P.B., 1991, Cloning, sequencing, and expression of the gene coding for bile acid 7α-hydroxysteroid dehydrogenase from *Eubacterium* sp. strain VPI 12708, *J. Bacterial.,* **173,** 4558–69.

Boyer, J., Day, D.W. and Hill, M.J., 1984, Site of cholesterol degradation in the human gut, *Trans. Biochem. Soc.,* **12,** 1104–5.

Coleman, J.P., White, W.B., Egestad, B. and Hylemon, P., 1987, Biosynthesis of a novel bile acid nucleotide and mechanism of 7-dehydroxylation by an intestinal *Eubacterium* species, *J. Biol. Chem.,* **262,** 4701–7.

Donaldson, R.M., 1970, Blind loop *syndrome, Adv. Intern. Med.,* **16,** 191–97.

Drasar, B.S. and Hill, M.J., 1974, *Human Intestinal Flora,* London: Academic Press.

Drasar, B.S., Hill, M.J. and Shiner, M., 1966, The deconjugation of bile salts by human intestinal bacteria, *Lancet,* **i,** 1237–38.

Franklund, C.V., de Prada, P. and Hylemon, P.B., 1990, Purification and characterisation of a microbial NADP-dependent bile acid 7α-hydroxysteroid dehydrogenase, *J. Biol. Chem.,* **265,** 9842–49.

Hill, M.J., 1986, *Microbes and Human Carcinogenesis,* London: Edward Arnold.

Hill, M.J., 1992, Bile acids and colorectal cancer: a hypothesis, *Eur. J. Cancer Prev.,* **1** (**Suppl. 2**), 69–74.

Hill, M.J. and Drasar, B.S., 1968, Degradation of bile salts by human intestinal bacteria, *Gut,* **9,** 22–27.

Hill, M.J., Melville, D., Lennard-Jones, J.E., Neale, K. and Ritchie, J.K., 1987, Faecal bile acids, dysplasia and carcinoma in ulcerative colitis, *Lancet,* **ii,** 185–86.

Hoehn, W.M., Schmidt, L.H. and Hughes, H.B., 1944, Studies in bile acid metabolism, *J. Biol. Chem.,* **152,** 59–63.

Hylemon, P.B., Melone, P.D. and Franklund, C.V., 1991, Mechanism of intestinal 7-dehydroxylation of cholic acid: evidence that allo-deoxycholic acid is an inducible side-product, *J. Lipid Res.,* **32,** 89–96.

MacDonald, I.A., Williams, C.N., Sutherland, J.D. and MacDonald, C.D., 1983, Metabolism of primary bile acids by *Clostridium perfringens, Anal. Biochem.,* **135,** 349–54.

Mallonee, D.H., White, W.B. and Hylemon, P.B., 1990, Cloning and sequencing of a bile acid-inducible operon from *Eubacterium* sp. strain VPI 12708, *J. Bacterial.,* **172,** 7011–19.

Midtvedt, T. and Norman, A., 1967, Bile acid transformation by microbial strains belonging to genera found in intestinal contents, *Acta Path.* Mic. *Scand.,* **71,** 629–38.

Nair, P.P., Gordon, M. and Reback, J., 1967, The enzymatic cleavage of the carbon-nitrogen bond in 3α, 7α, 12α-trihydroxy-5β-cholan-24-oylglycine, *J. Biol. Chem.,* **242,** 7–11.

Norman, A. and Bergman, S., 1960, The action of intestinal microorganisms on bile acids, *Acta Chem. Scand.,* **14,** 1781–85.

Owen, R.W., Thompson, M.H., Hill, M.J., Malnguet, P., Wilport, M. and Roberfroid, M., 1987, The importance of the ratio of lithocholic to deoxycholic acid in large bowel carcinogenesis, *Nutr. Cancer,* **9,** 67–71.

Pearson, J., 1971, The etiology of steatorrhoea, *PhD Thesis,* London University.

Rafter, J.J., Eng, V., Furrer, R., Medline, A. and Bruce, W.R., 1986, Effect of dietary calcium and pH on the mucosal damage produced by deoxycholic acid on the rat colon, *Gut,* **27,** 1320–29.

Samuelsson, B., 1960, Bile acids and steroids: on the mechanism of the biological formation of deoxycholic acid from cholic acid, *J. Biol. Chem.,* **235,** 361–66.

Smith, L.D.S., 1977, The clostridia, in Laskin, A. and Lechevalier, H. (Eds) *Handbook of Microbiology,* pp. 337–45, Boca Raton: CRC Press.

White, W.B., Coleman, J.P. and Hylemon, P.B., 1988, Molecular cloning of a gene encoding a 45000-Dalton polypeptide associated with bile acid 7-dehydroxylation in *Eubacterium* sp strain VPI 12708, *J. Bacterial.,* **170,** 611–16.

Chapter 11
Interactions between fat substitutes and gut bacteria

T.G.Schlagheck and T.W.Federle

11.1
Fat substitutes

With consumers becoming more aware of medical and government recommendations for reducing dietary fat, and industry continuing to make progress in developing new fat-replacement technologies, widespread introduction of fat substitutes into the diet is becoming a reality. Fat substitutes represent a heterogeneous group of materials consisting of carbohydrate-, protein- and lipid-like substances that have widely different sensory, nutritional and functional properties. Fat substitutes are designed to decrease the consumption of conventional fats and associated calories while maintaining the organoleptic properties of fat-containing foods.

A variety of different fat substitute products are shown in Table 11.1. Since the chemical nature of fat substitutes differs significantly, the potential for interaction with the colonic microflora also varies greatly. The primary focus of this chapter will be on fat substitutes that have the potential to interact with and/or alter microbial metabolism. For example, Simplesse^{R} is a microparticulated protein that is completely digested by pancreatic enzymes, absorbed in the small intestine, and therefore is unlikely to interact with the microflora in the large bowel. Products such as polydextrose and gums, which are partially fermented, are exogenous substrates for microbial fermentation and may be sources of metabolic by-products that could affect the microbial environment. Olestra, which is a mixture of hexa-, hepta- and octa-esters of sucrose that is not digested or absorbed, passes into the colon as part of the intestinal chyme and also has the opportunity to potentially interact with the colonic microflora.

Investigating the interactions of fat substitutes with the colonic microflora presents new challenges to food scientists, microbiologists and gastrointestinal physiologists. With the exception of gums and some other carbohydrate-based fat substitutes, very few data on microbial interactions with fat substitutes can be found in the scientific literature. The design of a programme to study potential interactions between fat substitutes and the gut microflora should be based on an understanding of the chemical nature of the fat substitute. With this basic understanding the directed approach should include experiments which measure end-points of microbial metabolism and monitor the colonic functions supported by the presence of a viable microflora population. Although no single experiment or single end-point parameter is enough to provide a definitive answer to the effect of a fat substitute on the gut microflora community, the cumulative weight of multiple experiments and relevant, metabolic end-points can be used to assess the presence or absence of significant interactions between fat substitutes and the gut microflora.

This chapter will present an overview of the variety of approaches that can be used to study these potential interactions. In addition, it will review some recent work with olestra that can form a basis for future studies.

11.2
Potential interactions of fat substitutes with the intestinal microflora

In evaluating the potential interactions of a fat substitute with the gastrointestinal (GI) microflora, several questions need to be addressed:

- To what extent does the fat substitute reach the large intestine?
- Is the fat substitute metabolized in any way by the microflora?
- If metabolized, does the fat substitute serve as a nutrient for microbial growth and activity?
- Does the fat substitute or its biotransformation products affect the normal activity and function of the microflora, either directly or indirectly?

11.2.1
Transport into the large intestine

Since the GI microflora in healthy humans is largely confined to the large intestine, only fat substitutes that are not completely digested and absorbed in the stomach and small intestine will enter the colon to a significant degree and thus interact with the colonic microflora. Some of the fat substitutes that are digested and absorbed, and so contribute calories to the diet (e.g. carbohydrate and protein-based fat replacers) do not reach the colon to any significant extent. Some carbohydrate-based fat substitutes, such as gums, may undergo incomplete digestion in the upper gastrointestinal (GI) tract and hence reach the colon. Such materials are essentially fibres, whose fate and effect in the colon have been intensively investigated in recent years. Newer lipid-based fat substitutes, such as olestra, are not digested in any way by mammalian enzymes (Mattson and Volpenhein, 1972) and reach the colon intact. Generally, levels of exogenous lipids and lipidlike materials entering the large intestine are relatively low. On a normal diet, most humans excrete less than 5 or 6 g of fat per day, 90% of which consists of unsaturated C16 and C18 fatty acids and a significant fraction of which is of non-dietary origin (Wrong *et al.*, 1981).

11.2.2
Metabolism of fat substitutes

Metabolism of a fat substitute by the intestinal microflora can have important ramifications relative to the caloric value of the substitute, its interactions with the gut microflora and host compatibility. Microbial metabolism of fat substitutes may involve simple biotransformation, partial degradation or complete biodegradation. Potential biotransformations include reductive reactions (e.g. reduction of double bonds) and hydrolytic reactions as well as reactions which remove or modify functional groups (e.g. dehydroxylation and acetylation). Such biotransformation products may interact further with the microflora and need to be considered in assessing the overall effect of a fat substitute on intestinal microflora. When partially or completely degraded, the fat substitute could serve as a new exogenous nutrient for the microflora and consequently stimulate fermentation,

Table 11.1 Examples of fat substitutes

Fat substitute	*Chemical basis*	*Fate in gut*	*Interaction with gut microflora*
Polydextrose (Pfizer)	Modified glucose polymer (carbohydrate-based)	Limited digestion and absorption; Partial colonic fermentation; Remainder excreted in faeces	Possible
Stellar® (AE Staley)	Modified starch (carbohydrate-based)	Limited digestion and absorption; Colonic fermentation	Possible
Slendid® (Hercules); Carageenan; Guar gum	Gums, emulsifiers (carbohydrate-based)	Not digested in small intestine; Range from highly fermentable to unfermentable	Possible
Simplesse®, (Nutra-Sweet Co) Trailblazer®, (Kraft Foods)	Microparticulated protein (protein-based)	Digested to amino acids and absorbed in small intestine	Minimal to none
Olestra (Procter & Gamble); Sucrose polyesters (Unilever)	Fatty acid esters of sugar (lipid-based)	Higher esters (>5) are undigested, unabsorbed, unfermented	Possible

resulting in increased gas and short-chain fatty acid (SCFA) production in the colon. Since SCFA can be absorbed from the colon and utilized by the host, the calorie-lowering benefits of using a fat substitute could be lessened. In this regard, the fat substitute would be analogous to many of the fermentable fibres.

11.2.3 Effects on normal microflora function and activity

Fat substitutes that reach the colon, or their biotransformation products, could directly stimulate or inhibit bacterial activity in the colon. Such direct effects could impact overall microbial activity or be limited to specific metabolic activities or specific populations of bacterial species. In addition, indirect effects could result from physical/chemical changes in the growth environment of the GI bacteria, produced by the presence of a non-metabolized substance in the colon. Hypothetically, such a substance could alter the bioavailability to the bacteria of certain bacterial growth factors as well as reduce the bioavailability of endogenous inhibitors such as long-chain fatty acids and unconjugated bile acids.

11.3
Metabolism of fat substitutes by the gut microflora

11.3.1
Approaches for assessing the microbial metabolism of fat substitutes

Approaches for assessing the metabolism of fat substitutes can be divided into two general categories: *in vivo* and *in vitro*. Each approach has its own limitations and advantages.

In vivo *approaches*

In vivo studies can be conducted in animals and humans. These studies include material balance studies in which the difference in amount between the fat substitute that is egested is compared with total consumption over a period of time. Such studies are difficult to perform due to the necessity of recovering all faecal matter, quantitatively extracting the fat substitute from the faecal matrix, and obtaining a quantitative and accurate analysis of the extract. Although material balance studies can readily show if significant metabolism occurs, demonstrating a low level or a complete absence of metabolism is very difficult. The sensitivity of this method can be improved with the use of radioactive tracers, although this approach is generally not used in human studies due to the concern with radioactivity exposure.

An additional complication of material balance studies is in differentiating whether the host or the microflora is responsible when partial or full metabolism of a fat substitute is evident. To distinguish between mammalian and bacterial metabolism, comparisons can be made between germ-free and conventional laboratory animals. If metabolism occurring in conventional animals is absent in gnotobiotic animals, it suggests that the intestinal microflora is the causative agent. However, many morphological and physiological differences exist between conventional and germ-free animals, e.g. redox potential, intestinal pH, caecum size, intestinal wall morphology, immune responsiveness and epithelial cell turnover rate, which can complicate interpretation of the study's results (Coates and Gustafsson, 1984).

In vitro *approaches*

In vitro studies typically involve incubation of the substance of interest with human faeces or cultures of intestinal bacteria and monitoring the formation of products and/or disappearance of the substance. Monocultures, as well as defined and undefined mixed bacterial cultures, may be employed in these studies. *In vitro* approaches are more sensitive than *in vivo* studies, particularly if radiolabelled substances are utilized, and make it easier to calculate the mass balance as well as determining if the microflora is responsible for metabolism. It is possible with *in vitro* studies to test in replicate several human-derived inocula. Such incubations, however, are limited temporally and do not provide an opportunity for the organisms to adapt, which is often a very important factor affecting the metabolism of xenobiotic chemicals by microbial communities.

Adaptation is defined as a process whereby the rate or extent of metabolism is significantly changed as a result of prior exposure to the chemical (Spain *et al.,* 1980). Adaptation can result from derepression or induction of degradative enzymes not synthesized by the community prior to exposure, shifts in community structure in which populations with degradative abilities increase in

dominance, or selection of organisms with new metabolic activities acquired through gene transfer or mutation. Adaptation by the intestinal microflora is important in the metabolism of cyclamates (Scheline, 1973), 5-fluoruracil (Harris *et al.,* 1986) and 1-nitropyrene (Manning *et al.,* 1986).

In addition to adaptation, two other important factors that can affect the microbial metabolism of a fat substitute are its bioavailability and the nutritional environment of the microbes in the test systems. To ensure that negative findings are not the result of the fat substitute being unavailable for metabolism, the material should be presented to the bacterial cultures in a highly dispersed form, incorporating emulsifiers for lipid-based substances to maximize bioavailability. Furthermore, testing should be conducted under minimal and enriched nutritional conditions. Biotransformation occurs when a material is utilized as a carbon and energy source or when it is gratuitously co-metabolized during the catabolism of other substrates. The presence of other carbon sources in the media could provide the additional nutrients required to support growth of biotransforming populations as well as fuel co-metabolism. Alternatively, additional carbon sources could prevent metabolism through catabolite repression and inhibition.

Semi-continuous and continuous culture systems can be utilized to extend the length of *in vitro* studies (Manning *et al.,* 1987). Such systems provide an opportunity for adaptation to occur but require a great deal of maintenance. As a consequence, only one or two inocula can typically be tested, and one needs to be concerned whether the microbial community established *in vitro* remains comparable to that *in vivo*.

11.3.2 Definitive studies on the metabolism of olestra

Some of the most comprehensive work in determining the metabolism of a fat substitute by the colonic microflora has been focused on olestra, a lipid-based material. Since olestra is not digested or absorbed in the small intestine (Mattson and Volpenhein, 1972), it is present in the large intestine where it can potentially be metabolized by the colonic microflora.

In vivo *studies*

One *in vivo* approach which proved successful for determining that olestra was not metabolized by the microflora was a series of absorption, distribution, metabolism and elimination (ADME) studies conducted in the rat (Miller *et al.,* 1992). Groups of rats were sacrificed 1, 3, 7 and 21 days after being given a single gavage dose of ^{14}C-(sucrose) olestra. Radiolabel levels in urine, CO_2, faeces, blood and all organs were measured. The mean recovery of radiolabel was 98±14% of the administered dose. More than 99.8% of the radiolabel was recovered in the faeces, gut contents and gastrointestinal tract. A trace amount of the administered dose (0.14%) was recovered in urine, blood, tissues, carcass and CO_2. If radiolabelled olestra were metabolized to normal fermentation by-products (CO_2 and SCFA), ^{14}C from absorbed SCFA would be incorporated into the carbon pool of the host tissues and radiolabelled CO_2, absorbed from the gut lumen or derived from further metabolism of SCFA by the host, would be exhaled. The low level of radioactivity found uniformly in all tissues indicated metabolism of low levels of penta- and lower esters, which are minor constituents in olestra (<1%) and have been shown to be metabolized by pancreatic enzymes (Mattson and Volpenhein, 1972). Sucrose and free fatty acids are the products of hydrolysis of the

lower esters and are metabolized in the same manner that the body handles sucrose and fatty acids from other foods. The *in vivo* studies provided convincing evidence that there was no significant metabolism of olestra by gut microflora.

In vitro *studies*

An *in vitro* approach, which has been used to evaluate whether olestra is metabolized by the human faecal microflora, involved directly measuring if radiolabelled fermentation products were formed when radiolabelled olestra was incubated with faecal material (Nuck *et al.,* 1994). Under the anaerobic conditions in the colon, olestra hypothetically could be metabolized in a variety of ways. Ester linkages could be hydrolyzed yielding free fatty acids, olestra with fewer esters, and ultimately sucrose. The liberated sucrose and fatty acids could be metabolized to SCFA, methane, hydrogen and carbon dioxide. Alternatively, liberated unsaturated fatty acids could be hydroxylated or hydrogenated (Eyssen and Parmentier, 1974). In a like manner, it is possible that unsaturated fatty acids on intact olestra could be hydroxylated or reduced resulting in a more polar or saturated olestra.

To determine if any of these reactions were catalyzed by the colonic microflora, ^{14}C-olestra was incubated with faecal samples from seven subjects who had consumed olestra for up to 30 days, and the presence or absence of radiolabelled metabolites was determined. The pre-exposure period provided an opportunity for the microflora to adapt to the presence of olestra. Thus, an attempt was made to exploit the advantages of short-term *in vitro* incubations, while providing an opportunity for adaptation to occur *in vivo*.

Results of this study showed that no significant levels ($<0.1\%$) of ^{14}C-gases, SCFA or long-chain fatty acids were generated from the metabolism of radiolabelled olestra. In addition, the fatty acid composition of recovered olestra was the same as the starting material.

In summary, repeated testing under low and highly-enriched nutrient conditions with acclimated intestinal bacteria from multiple subjects indicated that olestra was not biotransformed in any way by the microflora of the human intestine. Therefore, olestra did not serve as a nutrient for intestinal bacteria or as a source of metabolic byproducts, and any effects on the microflora would be related to a direct or indirect action of olestra on the microflora.

11.4
Effect of fat substitutes on microbial metabolism

Microflora function in the gastrointestinal tract involves complex interactions between the bacteria and the host. An interdependence is established between the mammalian host and the intestinal bacteria based on a continual exchange of metabolic substrates and products between luminal digesta, the intestinal epithelium and the bacteria. Key factors which can affect this equilibrium are: (a) new substrates which can alter the microbial environment, (b) changes in the microbial metabolism of compounds in the gut, and (c) changes in the microbial metabolic processes which are important in maintaining colonic morphology and function.

The close interrelationship between the microflora and the host presents a challenge to the investigator attempting to understand the effects of a food, drug, or novel food ingredient, such as a fat substitute, on microbial metabolism. Three approaches for studying the effects of fat substitutes on microbial metabolism include (a) determination of end-points of microbial metabolism, (b)

measurement of indicators of colonic function, and (c) assessment of changes in microbial composition. While microbial metabolism and composition can be investigated by both *in vitro* and *in vivo* studies, the effects of changes in microbial metabolism on colonic function are best studied using *in vivo* experiments.

11.5
End-points of microbial metabolism

The colonic microflora carry out a variety of reductive, degradative and hydrolytic processes on the contents of intestinal chyme entering the large bowel. Changes in metabolic substrates, such as fibre or dietary lipid, or disruption of the colonic environment, such as antibiotic administration, have been shown to affect the metabolic processes carried out by the microflora, as measured by several end-points of microbial metabolism. These end-points include measures of (a) microbial fermentation, (b) bile acid, neutral sterol and bilirubin bioconversion, (c) degradation of mucin, trypsin activity and β-aspartyglycine, (d) metabolism of enterohepatically circulating steroids, (e) microbial nitrogen metabolism, and (f) xenobiotic metabolism. Several of these end-points used to assess microbial metabolism have been described as microflora-associated characteristics (MACs) (Midtvedt *et al.,* 1990). These same parameters can be used to assess the effects of fat substitute on microbial processes.

11.5.1
Microbial fermentation

The microflora obtain the bulk of their energy requirements by fermenting undigested carbohydrate including a variety of polysaccharides, unabsorbed sugars, oligosaccharides and resistant starches, and endogenous substances such as mucin and sloughed cells. Increasing the amount of undigested polysaccharide entering the colon, such as dietary fibre, has been shown to increase bacterial biomass, stool weight and the production of microbial fermentation by-products (Stephen and Cummings, 1980) including short-chain fatty acids, hydrogen, and in some individuals, methane (Bond *et al.*, 1971).

Short-chain fatty acids (SCFA), primarily acetic, propionic and butyric, are involved in the maintenance of colonic morphology and water and electrolyte balance in the large bowel. The relative distribution of the different SCFA reflects differences in the anaerobic intestinal microflora (Siigur *et al.,* 1990). Furthermore, disruption of microbial metabolism could affect total production of SCFA. This is evident from studies which showed as much as an eight-fold reduction in the faecal excretion of SCFA due to antibiotic administration (Hoverstad *et al.*, 1986).

A batch *in vitro* method was used to assess the effect of olestra on the production of SCFA and gaseous hydrogen and methane by human faecal microflora. Homogenates of methanogenic and non-methanogenic faecal samples, used as sources of the diverse microbial populations present in man, were inoculated separately into enriched anaerobic media containing olestra at concentrations of 0 or 20 mg/ml. Samples were incubated in sealed 60 ml serum bottles for 18 hours at 37°C. The olestra concentration in the medium was approximately two to three times higher than concentrations that might be expected to occur in the colon, assuming a predicted chronic consumption level of 7.0 g/day of olestra and 500–1000 ml of colonic contents. The production of

fermentation end-products in the cultures incubated in the presence of 20 mg/ml olestra did not differ from controls (Figure 11.1).

Appropriate *in vivo* studies would include measurement of breath gas production and faecal SCFA excretion, with several considerations necessary for the experimental design. Key aspects of a study designed to investigate the effects of a fat substitute on microbial metabolism should include: (a) controlled diets balanced for individual subject caloric needs, (b) an acclimation period of at least 21 days of daily consumption of the fat substitute, (c) breath gas and faecal sample collection regimens during a baseline period and the treatment period, (d) sampling regimens that adequately cover periods of time within which changes would be expected to occur, for example breath gas collection for at least 12 hours after consumption and collection of two or three repeated faecal samples during the sampling period, and (e) study populations large enough to give enough statistical power to measure treatment group differences.

11.5.2 Microbial bioconversions

Bile acids

The microflora of the distal small intestine and the colon play an important role in establishing the profile of primary and secondary bile acids in bile and faeces. Fifty per cent of the bile acid pool in the enterohepatic cycle is deconjugated by bacteria in the ileum and colon. Half of this pool of deconjugated bile acids is reabsorbed. The remainder are converted by bacteria to secondary bile acids which are then reabsorbed to varying degrees (Hofmann, 1989; Erlinger, 1987).

Changes in biliary and faecal bile acid profiles can occur when changes in the intestinal environment, such as diet, pH, antibiotics and disease, affect gut microflora (Kay, 1981; Reddy, 1981; Salvioli *et al.,* 1982; Rolfe, 1984; Midtvedt, 1985; Andrieux *et al.,* 1989). Thus, if a fat substitute altered the microbial processes responsible for biotransformation of bile acids, changes in the relative amounts of primary and secondary bile acids in faeces and in bile would be expected.

Biliary and faecal bile acid data from a rat study showed that olestra did not have a meaningful effect on relative amounts of primary and secondary bile acids in bile and faeces. Rats were fed olestra as 0 and 5% of the diet, and bile and faeces were collected at one and two months from animals in each group (Tables 11.2 and 11.3). The only statistically significant difference in biliary and faecal bile acids was a lower lithocholic acid concentration in bile at one month in the olestra-fed rats that was not present at two months. There was a statistically non-significant tendency for faecal primary bile acids (cholic and chenodeoxycholic) to be higher and faecal secondary bile acids (deoxycholic and lithocholic) to be lower in olestra-fed rats at both one and two months. Importantly, there was no consistent effect in the proportion of primary and secondary bile acids in bile, since this represents the majority of the bile acid pool biotransformed by the intestinal microflora.

Neutral sterols

Microflora in two-thirds of the human population bioconvert more than 60% of unabsorbed cholesterol to coprostanone and coprostanol; the remaining one-third of the population bioconvert

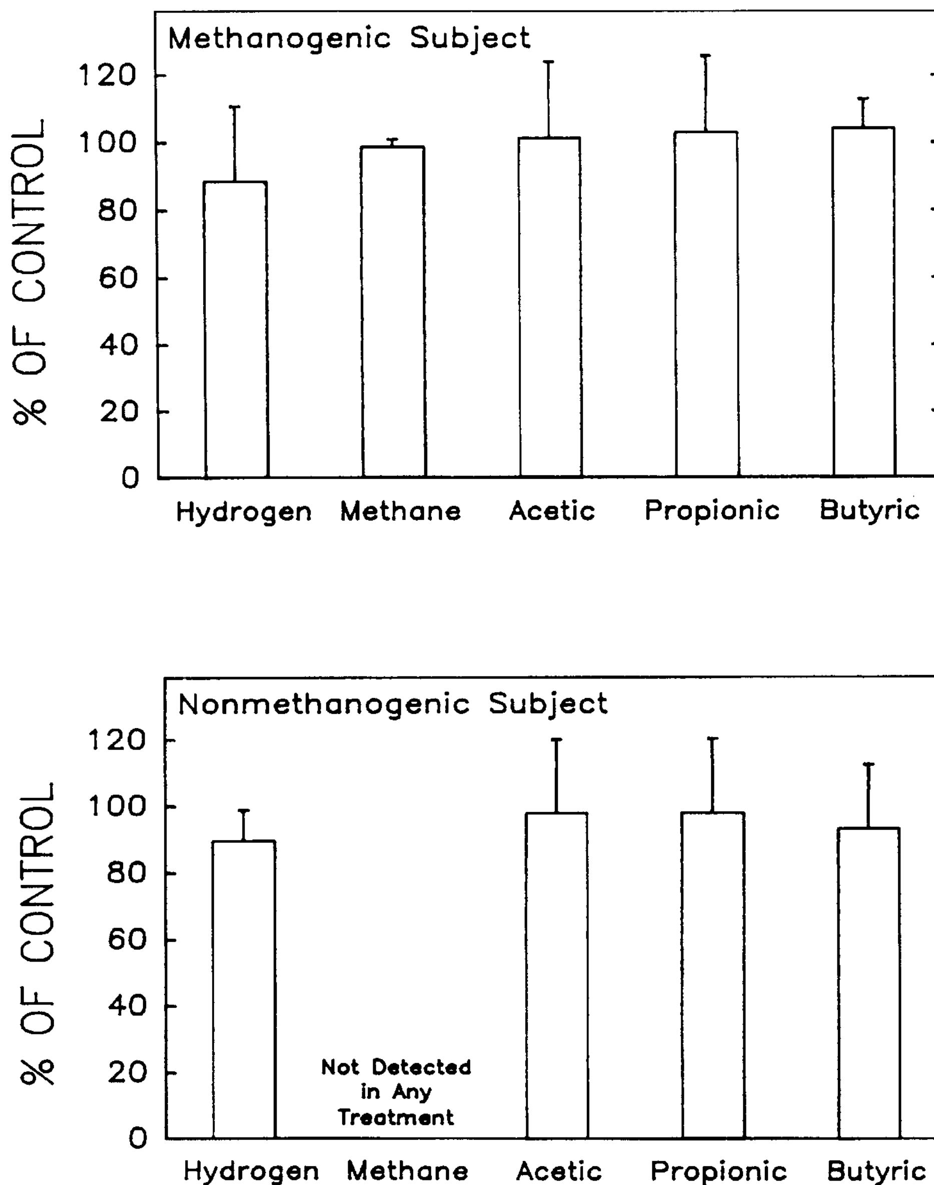


Figure 11.1 Production of fermentation by-products by faecal cultures in media containing olestra presented as a percentage of concentrations in control media.

less than 30% (Wilkins and Hackman, 1974; Jandacek *et al.,* 1980). The presence of a lipophilic fat substitute in the colon could potentially affect this bioconversion process.

The results of a study investigating this process show that olestra reduced neutral sterol (cholesterol and β-sitosterol) bioconversion (Jandacek *et al.,* 1980). Microbial bioconversion of

Table 11.2 Biliary bile acid composition (µg/ml) of rats fed olestra at 0 or 5% (w/w) of the diet for 2 months

	1 Month		*2 Months*	
Bile acid	*Control*	*Olestra*	*Control*	*Olestra*
	(n=5)	(n=5)	(n=5)	(n=5)
Cholic acid	176.3	199.1	253.9	283.8
	(37.7)†	(55.2)	(85.1)	(30.9)
Deoxycholic acid	5.6	10.3	9.6	5.5
	(1.6)	(3.8)	(2.1)	(1.2)
Chenodeoxycholic acid	29.8	28.9	28.7	40.7
	(4.9)	(7.0)	(9.2)	(6.1)
Lithocholic acid	21.6	11.8*	18.2	20.1
	(2.8)	(1.3)	(5.5)	(2.7)

*Significant difference ($p<0.05$) between control and olestra groups.
†Numbers in brackets indicate standard error of the mean.

Table 11.3 Faecal bile acid excretion (mg/day) of rats fed olestra at 0 or 5% (w/w) of the diet for 2 months

	1 Month		*2 Months*	
Bile acid	*Control*	*Olestra*	*Control*	*Olestra*
	(n=5)	(n=5)	(n=5)	(n=5)
Cholic acid	0.4	0.6	0.4	0.6
	(0.06)*	(0.09)	(0.06)	(0.09)
Deoxycholic acid	3.5	2.7	3.2	2.5
	(0.6)	(0.5)	(0.6)	(0.3)
Chenodeoxycholic acid	10.7	13.6	10.4	11.9
	(1.0)	(1.5)	(1.2)	(1.5)
Lithocholic acid	0.8	0.5	0.4	0.4
	(0.3)	(0.9)	(0.08)	(0.07)

No statistical differences between control and olestra values within a sampling period ($p>0.05$).
*Numbers in brackets indicate standard error of the mean.

neutral sterols decreased dose-responsively, by 1% for each gram of olestra consumed, in subjects who converted more than 60% of unabsorbed cholesterol during the baseline period. The evidence suggested that olestra had this effect by reducing the availability of neutral sterols to the microflora because of lipophilic interactions, rather than by affecting the microbial metabolic pathway, since greater than 80% of the sterols were still bioconverted in the presence of olestra.

Bilirubin

Urobilinogen is formed when the intestinal microflora deconjugates and dehydroxylate bilirubin, a bile pigment derived from heme catabolism and conjugated in the liver. Urobilinogen is present in faeces of healthy individuals and is absent when the microflora is absent (Saxerholt *et al.,* 1986). Antibiotics effective against Gram-positive anaerobes produced a tenfold or greater reduction in the amount of urobilinogen formed (Saxerholt *et al.,* 1986), while treatment of ulcerative colitis with anti-inflammatory sulphasalazone significantly reduced urobilinogen (Leijonmarck *et al.,* 1990).

11.6
Microbial degradation

In addition to fermentation of non-digested or partially-digested polysaccharide, the colonic microflora plays a significant role in degrading endogenous moieties secreted by the host. Monitoring of microbial degradation products in the faeces can be used to assess the effects of fat substitutes on this microbial function.

11.6.1
Mucin

Mucin secretion by the intestinal goblet cells is important in preserving the integrity of the intestinal mucosa and is a source of carbon and energy for the intestinal microflora. The intestinal microflora of healthy individuals degrades mucin so that only small amounts, if any, are detected in faeces. Agents which disturb the intestinal microflora or germ-free conditions result in large amounts of mucin in faeces (Carlstedt-Duke *et al.,* 1986).

11.6.2
Beta-aspartylglycine

β-aspartylglycine is not normally present in faeces since this dietary dipeptide is metabolized by the colonic microflora. When the microflora is lacking or seriously disturbed, as in patients treated with broad spectrum antibiotics, β-aspartylglycine can be detected in the faeces (Midtvedt *et al.,* 1986).

11.6.3
Trypsin-inactivation

The gut microflora is involved in the inactivation or degradation processes of intestinal tryptic activity (Norin *et al.,* 1988). Elevated faecal tryptic activity is a consistent characteristic of rats which are devoid of intestinal microflora (Norin, 1985). However, agents which disturb intestinal microbial activity do not always produce changes in faecal tryptic activity, and considerable intra- and inter-individual variation has been reported (Norin, 1985). Therefore, this measurable end-point may not be an optimal parameter for assessing gut microflora function.

11.7
Metabolism of enterohepatically circulating steroid hormones

Steroid hormones, including approximately 60% of circulating oestrogens, enter the enterohepatic circulation following conjugation in the liver. A similar proportion of the body pool of the synthetic steroid, ethinyl oestradiol, is conjugated in the liver and excreted in the bile (Simon and Gorbach, 1986). Most of the steroid hormones, both natural and synthetic, are excreted in the bile, deconjugated in the gut by the microflora and reabsorbed. Some investigators have attributed the failure of oral contraceptives to a decreased microbial deconjugation resulting from antibiotic administration concurrent with contraceptive administration (Aldercreutz *et al.,* 1984; Back *et al.,* 1978; Eldere *et al.,* 1987). Therefore, the potential exists for lipophilic fat substitutes to interfere in

the enterohepatic circulation of steroid hormones if the steroids partition into the undigested fat substitute phase to a significant extent.

Results from a two-generation rat reproduction study and from an oral contraceptive study in humans provide evidence that olestra does not affect the role of the microflora in steroid hormone metabolism of the host. In the rat reproduction study (Nolen *et al.,* 1987), olestra which was fed at up to 10% of the diet for two generations had no effect on mating, conception, embryonic development, fetal or postnatal viability, or postnatal growth, suggesting that olestra did not affect microbial deconjugation and enterohepatic circulation of steroids.

If lipophilic fat substitutes interfered significantly with the microbial metabolism responsible for deconjugation, a prerequisite for steroid reabsorption, peripheral serum concentrations of the synthetic steroid hormones might be reduced compared with placebo controls. A study by Miller *et al.* (1990) showed no effects of olestra on plasma concentrations of norgesterol and ethinyl oestradiol, two lipophilic hormones contained in the oral contraceptive, Lo/Ovral-28, suggesting that this fat substitute did not affect steroid hormone deconjugation or reabsorption.

11.7.1 Microbial nitrogen metabolism

Quantitation of faecal nitrogen provides information on the status of the fermentative activity of the colonic microflora in the presence of a non-absorbable fat substitute. Stimulation of bacterial growth by increasing fermentable substrate entering the colon causes increased incorporation of nitrogen into the bacterial biomass (Wrong, 1988). This results in an increased amount of nitrogen in faeces, since 60% of faecal nitrogen is of bacterial origin (Cummings *et al.,* 1976, 1979; Stephen and Cummings, 1980). Therefore, if the fat substitute affected carbohydrate digestion and absorption or significantly affected microbial fermentation, a change in faecal nitrogen could occur.

11.7.2 Microbial metabolism of xenobiotics

Bacterial metabolism is largely degradative, hydrolytic and reductive with a potential for both metabolic activation and detoxification of xenobiotics (Ilett *et al.,* 1990). Some xenobiotics are initially absorbed and conjugated in the liver prior to their secretion into the bile. The microflora facilitate their reabsorption by deconjugating them.

A lipophilic fat substitute could interfere with xenobiotic metabolism if the compound partitioned into the undigested fat substitute phase and was thus not available to the microbial enzymes. The degree of interference would depend on the lipophilicity of the xenobiotic and the degree to which it partitions into the fat substitute.

In the case of olestra, highly lipophilic compounds, e.g. DDT, partition into olestra (Jandacek, 1982). For compounds that partition into the fat substitute, microbial metabolism would be expected to decrease in an amount proportional to the amount of fat substitute present.

Any such lipophilic xenobiotics with toxic potential would be effectively detoxified by virtue of their partitioning into the fat substitute. Therefore, the net effect of a fat substitute would be to decrease the toxic potential of highly lipophilic xenobiotics by preventing their activation, sequestering them from sites of toxic action and removing them from body pools by reduction of cycling through the enterohepatic circulation, as has been demonstrated for DDT (Jandacek, 1982).

11.8
Maintenance of colonic function

The interdependence between the mammalian host and the intestinal bacteria is based on a continual exchange of metabolic products between the intestinal epithelium and the bacteria. By-products of microbial fermentation, notably SCFA, are critical for maintaining the nutritional status of the colonic epithelial cells and the balance of water and electrolytes in the colon. The colonic epithelium, in turn, supplies the bacteria with urea, potassium, lactate and bicarbonate which are required to maintain the nutritional status, colonic pH and redox potential.

The microflora influence water and electrolyte balance in the colon because SCFA, produced by the bacteria, stimulate sodium absorption (Roediger, 1980) and therefore affect sodium concentrations in the colonic lumen. This, in turn, influences the osmotic processes that regulate the quantity of water in the colon.

Disruption of SCFA production can lead to retention of sodium in the colonic lumen, and therefore retention of water in the faecal mass (Cummings, 1984; Roediger and Moore, 1981). Therefore, faecal water content is a direct indicator of electrolyte balance in the colon and an indirect indicator of microbial function.

An estimated 70% of the energy requirements for the colonic mucosa are derived from SCFA produced solely by microbial fermentation (Roediger, 1980). Short-chain fatty acids have been shown to stimulate cell growth and cell turnover, and interruption of SCFA availability can affect the morphology of the large bowel. This is manifested as intestinal mucosal atrophy (Robeau *et al.*, 1990). Examples of this are seen in germ-free animals or in animals on a fibre-free diet.

Determining whether fat substitutes interfere with the complex interdependency of the colonic microflora and the host can come from measures of faecal water and electrolyte content and from examination of colonic epithelial morphology. Faecal water and electrolyte data can be readily acquired from human clinical studies, while an assessment of fat substitute effects on colonic morphology would need to be evaluated in chronic feeding studies conducted in animal models.

11.9
Faecal microflora composition

It is generally recognized that changes in faecal profiles of microbial species are not predictive of changes in overall microbial function, except under extremely adverse microbial conditions such as occurs with administration of antibiotics. Data on the effects of dietary changes, such as fibre or fat content of meals, are inconsistent and do not lead to any firm conclusions (Woods and Gorbach, 1993).

The interdependencies of up to 400 species of bacteria in the colon and the meaning of changes in the profiles of species with respect to changes in the diet are not understood. Determination of changes in microbial composition with the introduction of fat substitutes is likely to lead to inconclusive results. Therefore, measurement of changes in the composition of faecal bacteria is not a recommended approach to studying interactions of fat substitutes and the gut microflora.

11.10
Conclusions

Consumers are becoming more aware of the need to reduce fat intake, and the food industry continues to have an interest in developing fat substitutes. While some of these materials may not be digested and absorbed by humans, they may be susceptible to microbial transformation and represent a previously nonexistent nutrient for colonic bacteria. Hence, the lexicological as well as nutritional assessment of such materials will depend upon knowing if they are metabolized, and if so how they are metabolized and biotransformed by colonic bacteria. The research performed on olestra provides a model for how such information can be developed. Key elements of any programme would involve performing definitive laboratory experiments with sensitive and specific analyses for metabolic products and potential metabolites, and confirming these laboratory results with clinical observations. Important considerations for such studies must include an opportunity for the microflora to adapt to the compound, the ability to detect low levels of transformation, and recognition that bioavailability and nutritional conditions are important to the experimental design. Once the fate of a material in the colon is understood, only then can one begin to address other indirect and direct effects of the fat substitute and its biotransformation products on the microflora.

A thorough understanding of fat substitute effects on microflora should include experiments designed to monitor end-points of microbial metabolism, such as fermentation by-products and biotransformation products, and colonic functions supported by the presence of a viable microflora population. Although no single experiment or single end-point parameter is enough to provide a definitive answer to the effect of a fat substitute on the gut microflora community, the cumulative weight of multiple experiments and metabolic end-points can be used to assess the presence or absence of significant interactions between fat substitutes and the gut microflora.

References

Aldercreutz, H., Pulkkinen, M.O., Hamalainen, E.K. and Korpela, J.T., 1984, Studies on the role of intestinal bacteria in metabolism of synthetic and natural steroid hormones, *J. Steroid Biochem.,* **20,** 217–29.

Andrieuz, C., Gadelle, D., Leprince, C. and Sacquet, E., 1989, Effects of some poorly digestible carbohydrates on bile acid bacterial transformations in the rat, *Brit. J. Nutr.,* **62,** 103–19.

Back, D.J., Breckenridge, A.M., Challiner, M., Crawford, F.E., Orme, M.L., Rowe, P.H. and Smith, E., 1978, The effect of antibiotics on the enterohepatic circulation of ethinylestradiol and norehisterone in the rat, *J. Steroid Biochem.,* **9,** 527–31.

Bond, J.H., Engel, R.R. and Levitt, M.D., 1971, Factors influencing pulmonary methane excretion in man, *Am. J. Clin. Nutr.,* **425,** 1391–98.

Carlstedt-Duke, B., Hoverstad, T., Lingaas, E., Norin, K.E., Saxerholt, H., Steinbakk, M. and Midtvedt, T., 1986, Influence of antibiotics on intestinal mucin in healthy subjects, *Eur. J. Microbiol.,* **5,** 634–38.

Coates, M.E. and Gustafsson, B.E., 1984, The germ-free animal in biomedical research, *Laboratory Animal Handbooks* **9,** London: Laboratory Animals Ltd.

Cummings, J.H., 1984, Colonic absorption: the importance of short chain fatty acids in man, *Scand. J. Gastro.,* **19,** 89–99.

Cummings, J.H., Hill, M.J., Bone, E.S., Branch, W.J. and Jenkins, D.J.A., 1979, The effect of meat protein and dietary fiber on colonic function and metabolism, *Am. J. Clin. Nutr.,* **32,** 2094–101.

Cummings, J.H., Hill, M.J., Jenkins, D.J.A., Pearson, J.R. and Wiggins, H.S., 1976, Changes in fecal composition and colonic function due to cereal fiber, *Am. J. Clin. Nutr.,* **29,** 1468–73.

Eldere, J. van, Parmentier, G., Robben, J. and Eyssen, H., 1987, Influence of an estrone-desulfating intestinal flora on the enterohepatic circulation of estrone-sulfate in rats, *J. Steroid Biochem.*, **26**, 235–39.

Erlinger, S., 1987, Physiology of bile secretion and enterohepatic circulation, in Johnson, L.R. (Ed.) *Physiology of the Gastrointestinal Tract*, pp. 1557–80, New York: Raven Press.

Eyssen, H.J. and Parmentier, G.G., 1974, Biohydrogenation of sterols and fatty acids by the intestinal microflora, *Am. J. Clin. Nutr.*, **27**, 1329–40.

Harris, B.E., Manning, B.W., Federle, T.W. and Diasio, R.B., 1986, Conversion of 5-fluorocytosine to 5-fluorouracil by human intestinal microflora, *Antimicrob. Agents Chemother.*, **29**, 44–48.

Hofmann, A.E, 1989, The enterohepatic circulation of bile acids in health and disease, in Sleisenger, M.H. and Fordtran, J.S. (Eds) *Gastrointestinal Disease*, Philadelphia: W.B. Saunders Co.

Hoverstad, T., Carlstedt-Duke, B., Lingaas, E., Midtvedt, T., Norin, K.E., Saxerholt, H. and Steinbakk, M., 1986, Influence of ampicillin, clindamycin and metronidazole on faecal excretion of short-chain fatty acids in healthy subjects, *Scand. J. Gastroenterol.*, **21**, 621–26.

Ilett, K.F., Tee, L.B.G., Reeves, P.T. and Minchin, R.F., 1990, Metabolism of drugs and other xenobiotics in the gut lumen and wall, *Pharmac. Ther.*, **46**, 67–93.

Jandacek, R.J., 1982, The effect of nonabsorbable lipids on the intestinal absorption of lipophiles, *D. Metabolism Rev.*, **13**, 695–714.

Jandacek, R.J., Mattson, F.H., McNeely, S., Gallon, L., Yunker, R. and Bluyeck, C.J., 1980, Effect of sucrose polyester on fecal storoid excretion by 24 normal men, *Am. J. Clin. Nutr.*, **33**, 251–59.

Kay, R.M., 1981, Effect of diet on the fecal excretion and bacterial modification of acidic and neutral steroids, and implications for colon carcinogenesis, *Cancer Res.*, **41**, 3774–77.

Leijonmarck, C., Benno, P., Carlstedt-Duke, B., Monsen, U., Norin, K., Poppen, B., Saxerholt, H. and Midtvedt, T., 1990, The function of the intestinal microflora in patients with ulcerative colitis before and after colectomy, *Scand. J. Gastroenterol.*, **25**, 585–93.

Manning, B.W., Cerniglia, C.E. and Federle, T.W., 1986, Biotransformation of 1-Nitropyrene to 1-Aminopyrene by the human intestinal microflora, *J. Toxicol. Environ. Health*, **18**, 339–4B.

Manning, B.W., Federle, T.W. and Cerniglia, C.E., 1987, Use of semicontinuous culture system as a model for determining the role of human intestinal microflora in the metabolism of xenobiotics, *J. Microbiol. Methods*, **6**, 81–94.

Mattson, F.H. and Volpenhein, R.A., 1972, Hydrolysis of fully esterified alcohols containing from one to eight hydroxyl groups by the lipolytic enzymes of rat pancreatic juice, *J. Lipid Res.*, **13**, 325–28.

Midtvedt, T., 1985, The influence of antibiotics upon miroflora-associated characteristics in man and mammals, in Worstmann, B. (Ed.) Germfree Research: Microflora Control and its Application to the Biomedical Sciences, pp. 241–44, New York: Alan R.Liss, Inc.

Midtvedt, T., Johannson, G., Carlstedt-Duke, B. and Midtvedt, A.C., 1990, The effect of a shift from a mixed to a lacto-vegetarian diet on some intestinal microflora associated characteristics, *Microbial. Ecol. Health Dis.*, **3**, 33–38.

Midtvedt, T., Carlstedt-Duke, B., Hoverstad, T, Lingaas, E., Norin, E., Saxerholt, H. and Steinbakk, M., 1986, Influence of perorai antibiotics upon the biotransformation activity of the intestinal microflora in healthy subjects, *Eur. J. Clin. Invest.*, **16**, 11–17.

Miller, K.W., Williams, D.S., Carter, S.B., Jones, M.B. and Mishell, D.R., 1990, The effect of olestra on systemic levels of oral contraceptives, *Clinical Pharmacology and Therapeutics*, **48**, 34–40.

Miller, K., Lawson, K., Madison, B., Tallmadge, D., Hudson, P., Okenfuss, J., Blair, M., Thorstenson, J. and Vanderploeg, P., 1992, Absorption, distribution and elimination of olestra after oral administration in rats (abstract). Abstracts of the 31st Annual Meeting of the Society of Toxicology, *Toxicologist*, **12** (**1**).

Nolen, G.A., Wood, F.E. and Dierckmann, T.A., 1987, A two-generation reproductive and developmental toxicity study of sucrose polyester, *Fd. Chem. Toxic.*, **25**, 1–8.

Norin, K., 1985, The establishment of some microflora-associated biochemical characteristics in feces from children during the first year of Iife, *Acta Paediatr. Scand.*, **74**, 207–12.

Norin, K.E., Carlstedt-Duke, B., Hoverstad, T., Lingaas, E., Saxerholt, H., Steinbakk, M. and Midtvedt, T., 1988, Faecal tryptic activity in humans: influence of antibiotics on microbial intestinal degradation, *Microbial. Ecol Health Dis.,* **1,** 65–68.

Nuck, B.A., Schlagheck, T.G. and Federle, T.W., 1994, Inability of the human fecal microflora to metabolize the nonabsorbable fat substitute, olestra, *J. Industrial Microbiology,* **13,** 328–34.

Reddy, B.S., 1981, Diet and excretion of bile acids, *Cancer Res.,* **41,** 3766–68.

Roediger, W.E., 1980, Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man, *Gut,* **21,** 793–98.

Roediger, W.E. and Moore, A., 1981, Effect of short-chain fatty acid on sodium absorption in isolated human colon perfused through the vascular bed, *Digest. Dis. Sci.,* **26,** 100–6.

Rolfe, R.D., 1984, Interactions among microorganisms of the indigenous intestinal flora and their influence on the host, *Rev. Infect. Dis.,* **6, Supplement 1,** 573–79.

Robeau, J.L., Kripke, S.A. and Settle, R.G., 1990, Short-chain fatty acids: production, absorption, metabolism and intestinal effects, in Kritchevsky, D., Bonfield, C., and Andersen, J.W. (Eds) *Dietary Fiber: Chemistry, Physiology and Health Effects,* New York: Plenum Press.

Salvioli, G., Salati, R., Bondi, M., Fratalocchi, A., Sala, B.M. and Gibertini, A., 1982, Bile acid transformation by the intestinal flora and cholesterol saturation in bile, *Digestion,* **23,** 80–88.

Saxerholt, H., Carlstedt-Duke, B., Hoverstad, T., Lingaas, E., Norin, K.E., Steinbakk, M. and Midtvedt, T., 1986, Influence of antibiotics on the faecal excretion of bile pigments in healthy subjects, *Scand. J. Gastroenterol.,* **21,** 991–96.

Scheline, R.R., 1973, Metabolism of foreign compounds by gastrointestinal organisms, *Pharmacol. Rev.,* **25**.

Siigur, U., Tamm, A. and Tammur, R., 1990, The faecal SCFAs and lactose tolerance in lactose malabsorbers, *Eur. J. Gastroenterol. Hepatol,* **3,** 321–24.

Simon, G.L. and Gorbach, S.L., 1986, The human intestinal microflora, *Dig. Dis. Sci,* **31,** No. 9 (Sept 1986 Supplement), 1475–1625.

Spain, J.C., Pritchard, P.H. and Bourquin, A.W., 1980, Effects of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments, *Appl. Environ. Microbiol.,* **40,** 726–34.

Stephen, A.M. and Cummings, J.H., 1980, The microbial contribution to human faecal mass, *J. Med. Microbiol.,* **13,** 45–56.

Wilkins, T.D. and Hackman, A.S., 1974, Two patterns of neutral steroid conversion in the feces of normal North Americans, *Cancer Res.,* **34,** 2250–54.

Woods, M.N. and Gorbach, S.L., 1993, Influences of fiber on the ecology of the intestinal flora, in Spiller, G.A. (Ed.) *Dietary Fiber in Human Nutrition,* Boca Raton: CRC Press.

Wrong, O.M., 1988, Bacterial metabolism of protein and endogenous nitrogen compounds, in Rowland, I.R. (Ed.) *Role of the Gut Flora in Toxicity and Cancer,* New York: Academic Press.

Wrong, O.M., Edmonds, C.J. and Chadwick, V.S., 1981, *The Large Intestine: Its Role in Mammalian Nutrition and Homeostasis,* New York: John Wiley and Sons.

Section 5

Sulphur metabolism

Chapter 12
The metabolism of sulphur compounds by gut bacteria

A.G.Renwick

12.1
Introduction

Sulphur is a Group VI element which shows the expected valency of-2 in simple molecules such as H_2S, thiols (RSH) and thioethers (RSR) (where R is an alkyl group). Thiol and thioether groups may be present also in complex molecules, and as such represent potential sites for microbial metabolism. Sulphur also shows additional valencies, e.g. 4 and 6, usually by forming dative bonds with other elements especially oxygen (Figure 12.1) (Damani, 1989). The presence of oxygen on the sulphur influences its polarity and its potential for metabolism by the intestinal flora. Because of the diversity of sulphur-containing compounds, the scope of this chapter is potentially very large and therefore it will concentrate on the role of the gut bacteria in the metabolism of foreign compounds in humans.

The extent of metabolism of the sulphur moiety in a chemical by the intestinal microflora depends on two main factors:

1. the delivery of the sulphur compound to the gut bacteria,
2. the potential metabolic reactions performed by the gut bacteria.

These two areas are often inter-related, for example, polarity is crucial for delivery to the gut bacteria and when the polarity resides in the sulphur atom, then this will greatly affect the types of metabolic reaction performed.

12.2
Delivery of the sulphur compound to the intestinal bacteria

The upper part of the intestinal tract contains very few bacteria in species such as humans, which have a low gastric pH. In contrast, the lower part of the intestinal tract contains very large numbers of bacteria (about 10^{12} per gram), many of which are strict anaerobes. A mixed population of bacteria is present throughout the intestinal tract of common laboratory animals such as the rat and mouse, with predominantly aerobes in the upper intestine and anaerobes in the caecum, colon and rectum (Drasar, 1988).

Valency	-2	-1	0	+2	+4	+6
Compound	Thiol	Disulphide	Sulphenic acid	Sulphinic acid	Sulphonic acid	Sulphate ester
Structure	RSH	RS-SR	RS-OH	RS(=O)-OH	O=RS(=O)-OH	R-O-S(=O)(=O)-OH
Compound	Thioether		Sulphoxide	Sulphone		
Structure	R-S-R		R-S(=O)-R	R-S(=O)(=O)-R		

Figure 12.1 The different valency states of organo-sulphur compounds discussed in this chapter

There are two principal routes by which a compound may reach the gut flora: direct introduction into the gut lumen and secretion into the lumen (Figure 12.2).

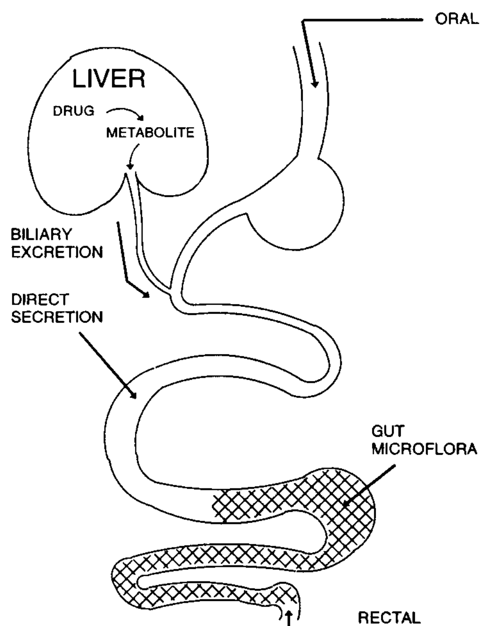


Figure 12.2 Routes of delivery of sulphur xenobiotics to the bacterial flora of the lower bowel

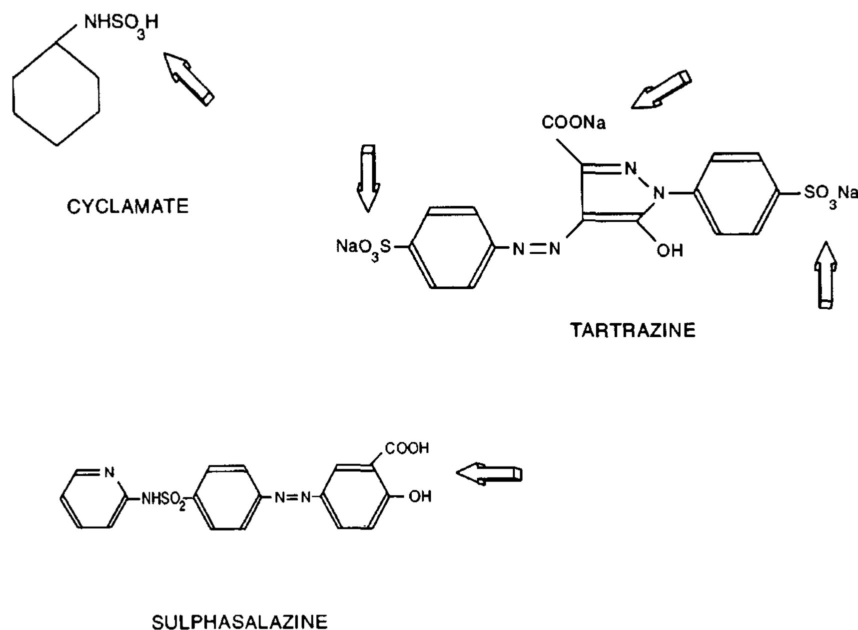


Figure 12.3 Polar sulphur compounds delivered to the gut bacteria as a result of incomplete absorption following oral administration. The polar group is indicated by an arrow

12.2.1 Oral delivery

The upper part of the intestine is designed for the digestion of food macromolecules and the absorption of nutrients such as sugars, amino acids and fats. The intestinal wall represents a permeability barrier across which low-molecular weight lipid-soluble compounds can diffuse freely. Highly water-soluble nutrients such as glucose and amino acids, including cysteine and methionine, are absorbed by active uptake processes to provide the body with its needs. Rapid absorption requires the formation of a molecular solution in the lumen of the gastrointestinal tract, and therefore a combination of water-solubility (to produce the molecular solution) plus lipid-solubility (to diffuse across cell membranes) is essential for rapid and complete absorption. The presence of a sulphur atom (such as a thiol-SH group) will provide a degree of water-solubility and may assist absorption of otherwise non-polar molecules. In contrast, when the sulphur atom is in a higher oxidation state (such as a sulphonic acid -SO_3H group) the polarity may be such as to prevent either rapid or complete absorption.

Because of the location of the gut bacteria in humans, the direct exposure of lipid-soluble compounds to microbial metabolism is usually minimal. In contrast, the gut flora may be a major site of metabolism of polar molecules which pass down the gastrointestinal tract to reach the gut bacteria. The polarity of substrates delivered to the site of microbial metabolism may be due to the sulphur atom *per se* when the chemical contains a polar sulphonic or sulphamic acid group, e.g. cyclamate in Figure 12.3. Alternatively, the polarity may reside elsewhere in the molecule and the polar group serves to deliver a non-polar sulphur centre to the intestinal bacteria. A good example of such

a compound is sulphasalazine (salicylazosulphapyridine) in which 5-amino-salicylic acid is linked to sulphapyridine via an azo bond (Figure 12.3). The salicylic acid moiety is polar and minimizes absorption, such that the majority of the oral dose passes to the gut microflora. Reduction of the azo link by the gut bacteria liberates the two active components. In this example the sulphur atom of sulphapyridine is present as a sulphonamide group (Figure 12.1) and, as such, is stable to bacterial metabolism (see later).

12.2.2 Rectal delivery

Direct delivery of a chemical of any polarity to the gut bacteria in the lower bowel is possible by rectal administration of the free compound or by the oral administration of a delayed- or controlled-release preparation. Potential advantages of rectal administration are the production of a local effect and/or the reduction in hepatic first-pass metabolism. The venous drainage from the colon and rectum is via both the hepatic portal vein (which takes the drug to the liver, which is the main site of drug metabolism) and the inferior vena cava (which delivers the drug to the general circulation without hepatic first-pass metabolism). The presence of anastomoses results in an increased absorption via the hepatic portal vein, the more proximal that the suppository is placed in the rectum.

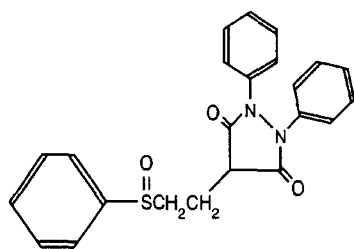
Administration via the rectal route allows lipid-soluble therapeutic drugs to be exposed to microbial metabolism and possibly novel pathways of metabolism not encountered when the drug is taken orally (Faigle, 1993). Despite examples of the increased toxicity of the microbial metabolites of a number of xenobiotics, e.g. amygdalin (Carter *et al.,* 1980), cycasin (Laqueur and Spatz, 1968) and cyclamate (Bopp *et al.,* 1986), there have been no reported cases of unique drug toxicity from the administration of suppository formulations of therapeutic drugs.

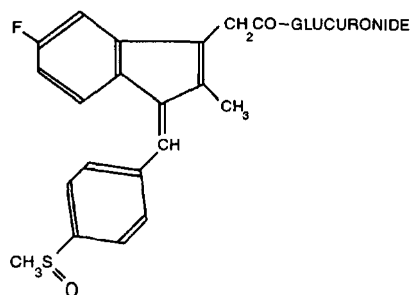
Drug delivery systems which allow the release of the chemical in the colon are usually used for a localized effect in the upper colon. Potentially successful devices include incorporating the drug in a capsule coated with a polymer containing azo cross-links which can be broken by bacterial azo-reduction, and delaying the activation of an osmotic delivery system by a slowly dissolving enteric coating (Theeuwes *et al.,* 1993). The low protease activity in the colon has stimulated research on the possible use of this site for the delivery of peptide drugs (Mackay and Tomlinson, 1993).

12.2.3 Secretion into the gut lumen

Xenobiotics can be transferred into the gut lumen and delivered to the gut flora by a variety of processes, e.g. direct secretion across the gut wall (Gardner and Renwick, 1978) or via the different secretions of the gastrointestinal tract, e.g. pancreatic fluid or bile (Smith, 1973). Such transfer results in the possibility of microbial metabolism irrespective of the route of administration of the compound, so that even intravenous doses may undergo microbial metabolism, e.g. the sulphoxide reduction of sulphinpyrazone in rats (Renwick *et al.,* 1982) and in humans (Strong *et al.,* 1984a).

The sulphur moiety may be present in the original xenobiotic excreted in bile and metabolized by the gut flora, e.g. sulphinpyrazone and sulindac (Strong *et al.,* 1985) (Figure 12.4), or it may be introduced into the xenobiotic as a result of conjugation with either sulphate or glutathione (Larsen, 1988) (Figure 12.5).

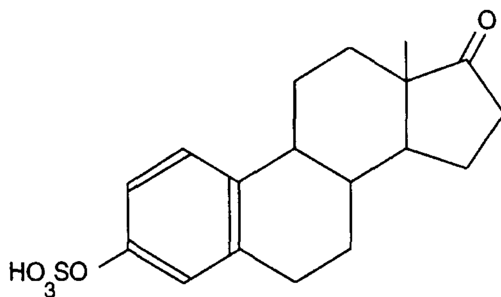




SULPHINPYRAZONE

SULINDAC GLUCURONIDE

Figure 12.4 Sulphoxides eliminated in the bile as the parent compound



OESTRONE SULPHATE

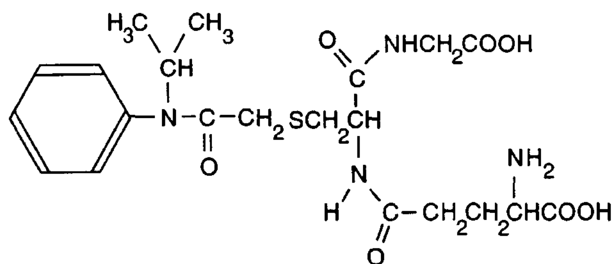


Figure 12.5 Sulphur-containing metabolites eliminated in the bile

In conclusion, these are various processes (Figure 12.2) by which sulphur atoms in a wide range of chemical configurations can be delivered to the lower bowel where they may be subjected to bacterial metabolism.

12.3
Metabolic reactions performed by the gut bacteria

In contrast to the oxidation and conjugation reactions performed by the host tissues, metabolism by the gut bacteria is largely by reduction and hydrolysis reactions. These reactions dominate the metabolism of sulphur compounds, which provide excellent examples of microbial degradation of xenobiotics and their sulphur-containing conjugates.

12.3.1
Reduction reactions

Sulphur can be found in a range of oxidation states and some sulphur-containing compounds can exist in different redox forms (Figure 12.1) which are potentially interconvertible.

Thioether, sulphoxides and sulphones

Micro-organisms are able to effect both the oxidation of thioethers and the reduction of the sulphoxide back to the thioether. The extent of the reactions depends on the organism and the environmental conditions. In addition, these oxidation/reduction reactions can be performed by tissues such as the liver and kidneys. Therefore the significance of the gut flora *in vivo* depends on the substrate specificities of the tissue and bacterial enzymes as well as the extent of delivery of the substrate to the gut bacteria.

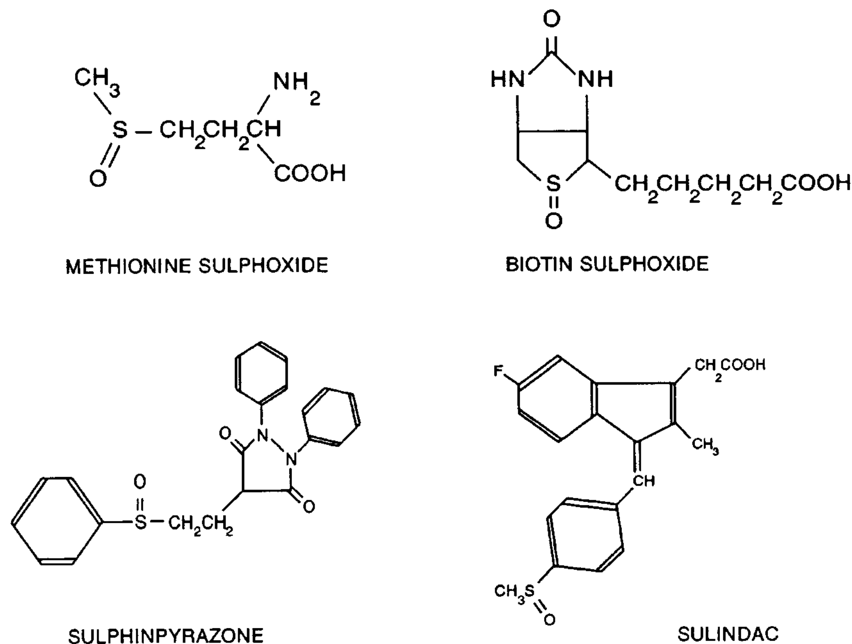


Figure 12.6 Sulphoxides studied extensively in relation to their oxidation and reduction

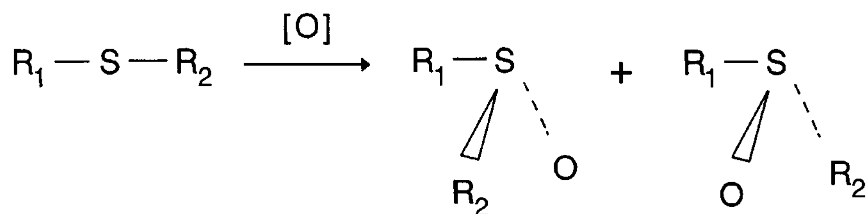


Figure 12.7 Oxidation of a thioether to a chiral sulphoxide

The different redox states are often associated with different pharmacological or toxicological properties. The sulphoxide metabolite of enoximone is probably responsible for much of the positive isotropic actions of the drug *in vivo* (Dage and Okerhjolm, 1990). The ring sulphoxide of thioridazine appears to be the most cardiotoxic metabolite compared with the sulphone and side-chain sulphoxide (Hale and Poklis, 1984; Heath *et al.,* 1985). Interestingly, the sulphoxide and sulphone metabolites of diethylthiomethylcarbamate, a metabolite of disulfiram, are potent inhibitors of both mitochondrial and cytosolic isoenzymes of aldehyde dehydrogenase (Johansson, 1992).

A number of sulphoxide and thioether substrates (Figure 12.6) have been studied extensively in relation to the enzymes involved in their oxidation and reduction.

Thioether oxidation can be performed by a wide range of animals, plants and microorganisms and the formation of a sulphoxide group is important in the production of a number of naturally-occurring compounds (Boyd *et al.,* 1989). Considerable work has been performed using moulds such as *Aspergillus* and soil organisms (Auret *et al.,* 1966, 1968a,b; Davis and Guenthner, 1985). Oxidation of a thioether bond with two different substituents produces a sulphoxide with a chiral centre (Figure 12.7). Enzyme-catalyzed oxidations frequently produce only or predominantly one of the isomeric forms (Boyd *et al.,* 1989). Although *Nocardia corallina* oxidized sulindac thioether analogue to sulindac under aerobic conditions (Davis and Guenthner, 1985), thioether oxidation was not detected with anaerobic incubations of mixed gut flora from rat caecal contents (Renwick *et al.,* 1982). Thioether oxidation *in vivo* is primarily a reaction of the tissues, catalyzed by either flavin-containing mono-oxygenases or cytochrome P450 (Souhaili *et al.,* 1987; Ziegler, 1989).

Similarly, the oxidation of sulphoxides to the corresponding sulphones has been reported for both tissues and micro-organisms. The oxidation of the sulphoxide sulindac to its sulphone is a major metabolic pathway in humans (Strong *et al.,* 1985) and in microbes such as *Nocardia* under aerobic conditions (Davis and Guenthner, 1985). The extent of microbial oxidation was dependent on the degree of aeration and this may explain the negligible oxidation of sulphoxides with incubations of mixed intestinal bacteria under anaerobic conditions (Renwick *et al.,* 1982). The enzymes involved in the oxidation of sulphoxides to sulphones have not been identified in either mammalian tissues (probably cytochrome P450) or in microbes (Davis and Guenthner, 1985).

Under the anaerobic conditions found in the lower bowel, the principal reaction on sulphoxides is reduction to the corresponding thioether. Substrates which have been studied extensively both *in vitro* and *in vivo* are sulphinpyrazone and sulindac (Figure 12.5).

Sulphinpyrazone is a uricosuric drug which is metabolized to a thioether analogue with platelet anti-aggregatory activity. Studies in experimental animals which are either germ-free or were treated with antibiotics to suppress the gut microflora indicated that the gut bacteria were the main, or

possibly the sole site of sulphoxide reduction *in vivo* (Renwick *et al.,* 1982; Strong *et al.,* 1984b). *In vitro* incubation indicated negligible reduction of sulphinpyrazone by the tissues. Studies on the metabolism of sulphinpyrazone in humans who had undergone an ileostomy (surgical removal of the colon) (Strong *et al.,* 1984a) or who had been treated with antibiotics (Strong *et al.,* 1985) indicated that the anaerobic bacteria of the lower bowel were essential for the reduction of sulphinpyrazone to its active metabolite.

Sulindac is a sulphoxide prodrug which requires reduction to the active anti-inflammatory thioether analogue. *In vitro* studies have shown that tissues such as the liver and kidneys are able to reduce sulindac to its active metabolite. The tissue reduction reactions are catalyzed by molybdenum-containing flavoenzymes, such as xanthine oxidase and aldehyde oxidase (Yoshihara and Tatsumi, 1985a,b) and by thioredoxin linked systems (Anders *et al.,* 1981). The intestinal bacteria are also able to reduce sulindac *in vitro* (Strong *et al.,* 1985) and the thioether is a major circulating metabolite *in vivo*. The contribution of the intestinal microflora to the reduction of sulindac in humans was studied using the ileostomy model applied successfully to sulphinpyrazone. The results showed two phases of sulphide formation:

(a) an initial peak (0–12 hours after the dose) corresponding to 45% of the total thioether, which was similar in normal and colectomy patients and was therefore probably due to tissue metabolism;
(b) a secondary phase (12–72 hours) corresponding to 55% of the total thioether that was present only in subjects with an intact colon (Strong *et al.,* 1985).

These data clearly indicate that the anaerobic gut flora are essential for much of the reduction of sulindac in humans.

Studies with over 200 isolated strains of human bacteria (Strong *et al.,* 1987) demonstrated significant sulphoxide reduction by many aerobic organisms, such as *Escherichia coli* under anaerobic conditions. Surprisingly, few strict anaerobes showed high reducing activity *in vitro* despite their obvious importance *in vivo,* as evidenced by the negligible reduction in patients without an intact colon, or who had been treated with metronidazole or lincomycin (Strong *et al.,* 1985,1986) which are active against anaerobes. It is possible that the strict anaerobes are important in providing the anaerobic environment in which the aerobes are able to express their sulphoxide reductase activity (Strong *et al.,* 1987).

The enzymes involved in microbial xenobiotic sulphoxide reduction have not been clearly identified. Early studies on sulphoxide reductase by *E. coli* utilized the sulphoxides of normal cell constituents such as methionine (Sourkes and Trano, 1953) and biotin (Cleary and Dykhuizen, 1974). Methionine sulphoxide and biotin sulphoxide are probably reduced by different enzyme systems since some mutants of *E. coli* are able to utilize one of the sulphoxides but not the other (Zinder and Brock, 1978). Studies with mutant strains of *E. coli* have shown a requirement for molybdenum as a cofactor for biotin sulphoxide reductase, and a thermolabile factor, possibly thioredoxin (Del Camparillo Campbell and Campbell, 1982). The enzyme or enzymes responsible for the reduction of methionine sulphoxide is not the same enzyme as that responsible for reducing this entity when it exists within peptide chains (Brot *et al.,* 1981). Recent studies using xenobiotics as substrates have indicated the presence of at least three different soluble enzymes in *E. coli* capable of reducing sulindac, one of which appeared to be a NADPH-linked thioredoxin system; only one of

the enzymes was able to reduce the more hindered sulphoxide group of sulphinpyrazone (Lee and Renwick, 1992).

Thioredoxin and its reductase, which are present in organisms such as *E. coli* (Holmgren, 1968), are involved in the interconversion of thiols and disulphides (Laurent *et al.,* 1964), and are important factors in sulphoxide reduction by renal cytosol (Anders *et al.,* 1981). However, *E. coli* thioredoxin is not structurally identical to the mammalian protein (Holmgren and Luthman, 1978) and the role of microbial thioredoxin in sulphoxide reduction remains to be defined.

Environmental micro-organisms are able to grow using thioethers as carbon sources (Kanagawa and Kelly, 1986) and as sulphur sources via their oxidation to thiols (Visscher and Taylor, 1993a,b). Dimethylsulphide can be degraded by acclimatized activated sludge (Kanagawa and Kelly, 1986). There is little evidence that intestinal micro-organisms split the carbon-sulphur bonds of xenobiotic-thioethers, except in the case of xenobiotic-cysteine conjugates (see below). The formation and urinary excretion of trace amounts of inorganic [^{35}S]sulphate in rats treated with [^{35}S] dipropylsulphide indicates some availability of thioether sulphur atoms *in vivo,* but the role of the gut flora was not defined (Nickson and Mitchell, 1994),

The interconversion of albendazole and its sulphoxide have been studied *in vitro* under anaerobic conditions with the intestinal contents of cattle and sheep (Lanusse *et al.,* 1992). With ruminal and ileal contents the oxidation of albendazole to its sulphoxide was greater in cattle than in sheep, while the opposite was found for the reduction of the sulphoxide. The sulphone was metabolically stable. The sulphoxide metabolite of cimetidine is reduced by faecal preparations *in vitro* (Mitchell *et al.,* 1982), but the significance of this reaction *in vivo* is undefined. Data on the fate of chlorpromazine N-oxide, which is excreted in rat bile as a number of metabolites including the S-oxide, indicate that the gut microflora may reduce the N-oxide group more readily than the S-oxide (Jaworski *et al.,* 1991). The inflammatory actions of SKF 105809 (6, 7-dihydro-2– (4-(methylsulfinyl) phenyl)-3–(4-pyridyl)-5(H)-pyrrolo(1, 2-a)imidazole) are probably due to its reduction to the thioether analogue, which is a potent inhibitor of 5-lipoxygenase and prostaglandin synthase activities (Hanna *et al.,* 1990). The site of reduction *in vivo* has not been defined but it is possible that both tissue and microbial enzymes could be involved.

In contrast to the extensive reduction of sulphoxides, the sulphone group is metabolically stable, both *in vitro* and *in vivo*. Mutants of *E. coli* have been isolated which express a novel sulphone-oxidizing enzyme active against heterocyclic sulphones and aliphatic sulphones (Juhl and Clark, 1990). The corresponding sulphides and thioethers are not *in vivo* metabolites of dapsone (4, 4’-diamino-diphenylsulphone) (Ellard, 1966), tolmesoxide sulphone (Greenslade *et al.,* 1981) or sulindac sulphone (Duggan *et al.,* 1978), although limited reduction of sulphinpyrazone sulphone has been reported (Renwick *et al.,* 1982). The apparent extensive formation of pentachlorothioanisole from its sulphone *in vivo* (Koss *et al.,* 1979) probably does not represent a simple sulphone reduction, but may occur via replacement of the methylsulphonyl leaving group by glutathione and the subsequent formation of a thiol, via C-S lyase, which would be methylated back to pentachlorothioanisole (Figure 12.7; see later). Sulindac sulphone is not reduced by organisms such *asAspergillus, Arthrobacter* and *Nocardia* (Davis and Guenthner, 1985) or by mixed intestinal organisms (Strong and Renwick, unpublished data), indicating that the microbial sulphoxide reductase(s) are unable to reduce sulphones. This is supported by reports that *E. coli* did not reduce dimethylsulphone (Zinder and Brock, 1978) and methionine sulphone (Ejiri *et al.,* 1979) *in vitro* and these sulphones did not inhibit the reduction of the corresponding sulphoxides to their thioethers.

Thiols and disulphides

The oxidation of thiols to disulphides can occur with simple substrates, such as glutathione, and with cysteine residues present in peptides. These interconversions are of considerable importance in the normal biochemistry of the organism since oxidation of peptide thiols to disulphides may alter the structural or catalytic characteristics of the protein. There are a series of relatively specific enzymes involved in the reduction of disulphides, e.g. glutathione reductase, and thioredoxin reductase. Thiols, which are rarely present in xenobiotics (Caldwell and Given, 1989), may dimerize to form homodimers, but are far more likely to form heterodimers with other thiols such as glutathione, cysteine and peptide thiols. For example, captopril forms mixed disulphides with cysteine and N-acetylcysteine, as well as forming an S-methyl conjugate and its sulphoxide (Migdalof *et al.,* 1984). An alternative fate for the thiol may be oxidation to the corresponding sulphinic acid, since tetrachlorophenyl sulphinic acids are metabolites of tetrachlorobenzene isomers in the squirrel monkey, and are presumably formed via cleavage of a glutathione conjugate to a thiol (Schwartz *et al.,* 1987).

Sulphenic, sulphinic and sulphonic acids and their amides

The sulphur acids and their corresponding amides represent two series of potentially interconvertible redox forms (Figure 12.1). However, there are few published data on their oxidation or, more likely, reduction by intestinal bacteria. Nucleosides of purine-6-sulphenamides, sulphinamides and sulphonamides are under development as potential anti-tumour agents (Revankar *et al.,* 1990) but there are no data on their potential for metabolic interconversion either by tissues or by the intestinal microflora. The greater potency of one such sulphonamide agent (sulfonosine) compared with its sulphenamide analogue (sulfenosine) (Finch *et al.,* 1990) suggests that there is little *in vivo* oxidation of the sulphenamide group.

Mild oxidation of thiol groups by reagents such as H_2O_2 produces a sulphenic acid group (R-SOH) which is chemically reactive and readily combines with nucleophiles, such as thiols, to produce disulphides. The sulphenic acid group of purine-6-sulphenic acid is chemically reactive and binds covalently to proteins *in vitro* (Abraham *et al.,* 1983). Additional reactions include dimerization with elimination of water to produce R-SO-S-R and oxidation to the corresponding sulphinic (RSO_2H) and sulphonic (RSO_3H) acids.

Cysteine residues in proteins may be present as cysteine disulphides or as more labile mixed disulphides, such as a cysteine-methylthiol mixed disulphide. It has been recognized recently that some enzyme cysteine residues may be oxidized to produce stable sulphenic acid groups which are implicated in the enzymic reaction (Claiborne *et al.,* 1993). Sulphenic acids may be stabilized by intramolecular hydrogen-bonding, electron-withdrawing groups and steric hindrance. It is possible that the local environment of the active site prevents reaction with other thiols, and further stabilizes the sulphenic acid. *Streptococcus faecalis,* which does not synthesize haem and lacks peroxide-metabolizing enzymes such as catalase, reduces H_2O_2 and O_2 by two flavoproteins, NADH peroxidase and NADH oxidase. In both enzymes, the non-flavin redox centre is believed to be a cysteine sulphenic acid (Ahmed and Claiborne, 1989; Poole and Claiborne, 1989), rather than a cystine disulphide, which is interconverted between sulphenic acid and thiol. The glutathione reductase and thioredoxin reductase of *E. coli* show significant sequence homology with the streptococcal NADH peroxidase, and the active cysteine of the glutathione reductase shows a

similar environment (Ross and Claiborne, 1991). Thus redox cycling between thiol and sulphenic acid appears critical to the catalytic activity of a number of bacterial redox enzymes.

In general, sulphonic acids are stable to metabolism (Williams, 1959). The sulphonic acid group is highly polar and in consequence sulphonic acids tend not to be absorbed completely from the gastrointestinal tract, and therefore may provide potential substrates for microbial metabolism. The sulphonic acid group is a common feature of many water soluble azo-dyes which are poorly absorbed and reach the gut flora; however, metabolism is at other sites such as the azo bond and not at the sulphonic acid moiety (Walker, 1970). Desulphonation was not found *in vivo* with simple alkyl sulphonates (Taylor *et al.,* 1978), with branched chain sulphonates (Michael, 1968) or with the 1- and 6-sulphonic acid derivatives of 2-naphthylamine (Batten, 1979). However, desulphonation has been found *in vitro* on incubation of alkyl sulphonates with environmental organisms such as the alga *Chlorella fusia* (Biedlingmaier and Schmidt, 1983) and *Pseudomonas* (Thysse and Wanders, 1974). The mechanism of desulphonation of n-alkane sulphonates involves an initial oxidation on carbon 1 to form a 1-hydroxy-alkane sulphonate, which is equivalent to an aldehyde bisulphite complex which can decompose to the aldehyde and bisulphite ion. The mechanisms of desulphonation of aromatic sulphonates (Ripin *et al.,* 1971) and alkyl benzene sulphonates (Willetts and Cain, 1972) are not known. Therefore, it may be possible that the gut bacterial flora could also perform desulphonation reactions; however, it is worth noting that the desulphonation reactions described above were often found under conditions in which the sulphonate was the sole source of carbon and sulphur, which are conditions of little relevance to microbial metabolism by the gut bacteria *in vivo*. Some intestinal bacteria, e.g. *E. coli, Enterobacter aerogenes* and *Serratia marcescens* are able to utilize sulphonates as sulphur sources under aerobic but not anaerobic conditions (Uria Nickelsen *et al.,* 1983). The reaction may involve the release of sulphite since studies on mutant strains have shown that sulphite reductase is essential for the utilization of alkylsulphonates.

The sulphonamide group is stable to metabolism *in vivo,* even for polar sulphonamide derivatives, such as saccharin (benzisothiazoline-1, 1-dioxide), which are poorly absorbed from the gut and reach the gut microflora in significant amounts (Renwick, 1985).

12.3.2
Hydrolysis reactions

The most important microbial lytic reactions in relation to the *in vivo* fate of foreign compounds are the hydrolysis of sulphates and sulphamates and the lysis of cysteine conjugates.

Hydrolysis of sulphate esters

Sulphate esters ($R{-}O{-}SO_3H$) are highly polar, strong organic acids. Few sulphate esters reach the gut microflora due to direct ingestion or administration of the sulphate ester *per se.* An interesting series of exceptions are the sulphate esters taken as laxatives, such as 4, 4’-dihydroxydiphenyl-(pyridyl-2)-methane disulphate (Forth *et al.,* 1972), sulisatin (Moreto *et al.,* 1977) and oxyphenisatin (Sund *et al.,* 1979). These reach the gut microflora due to a combination of poor absorption in the intestine and biliary excretion. The sulphate esters are hydrolyzed locally in the large bowel to release the active moiety.

Conjugation with sulphate is one of the most common phase II reactions, especially for substrates with alcoholic or phenolic hydroxyl groups (Figure 12.5). Sulphate conjugation occurs primarily in the gut wall and liver and the products are highly ionic, water-soluble metabolites which are frequently excreted, largely in the urine, and therefore do not pass to the gut microflora. The bile is the major route by which sulphate conjugates can pass to the gut microflora, but there is a molecular weight threshold (about 500 Daltons in humans and 325 Daltons in rats) below which there is negligible biliary excretion of anions. Sulphate conjugation increases the molecular weight of a hydroxy compound by 80 Daltons, whereas glucuronidation causes an increase of 176 Daltons.

Thus, sulphate conjugation and biliary excretion are most important in the delivery to the gut flora of larger substrates (i.e. of molecular weight 300 Daltons or more) such as triiodothyronine (Rutgers *et al.,* 1989) and steroids. Extensive hydrolysis of the sulphate conjugates of iodothyronine is catalyzed by rat caecal anaerobes such as *Lactobacillus* (De Herder *et al.,* 1985). The 3-sulphate ester, but not 7-sulphate ester groups, on sulphated bile acids are removed by anaerobic bacteria in human faeces (Pacini *et al.,* 1987) and rat microflora (Huijghebaert *et al.,* 1982). Bile acid sulphates are hydrolyzed by a strictly anaerobic strain of gut bacteria, probably a *Clostridium* (Huijghebaert *et al.,* 1982). The enzyme shows structural specificity to 5-β- and 3-α- but not 5-α-bile salt sulphates, but its activity towards xenobiotic sulphates has not been defined. Human faeces and *Bacteroides fragilis* possess mucin sulphatase which hydrolyzes the sulphate groups of sulphomucin (Tsai *et al.,* 1992).

Sulphate conjugates are major metabolites of steroids both in the general circulation and as excretory products (Briggs and Brotherton, 1970). Oestrone sulphate is hydrolyzed *in vivo* and *in vitro* by rat caecal micro-organisms (Back *et al.,* 1981); but many tissues also possess steroid-sulphatase activity which is able to release the parent steroid locally (Roy, 1970). In consequence, it is not possible to conclude that the gut microflora were responsible for the almost complete hydrolysis *in vivo* of ^{35}S-labelled oestrone sulphate to inorganic sulphate (Diczfalusy and Levitz, 1970). However, studies in experimental animals have shown that the gut microflora play an important role in the enterohepatic circulation of steroid sulphates by hydrolysis of both the sulphate moiety and also the glycosidic link of double conjugates such as oestriol-3-sulphate-16-glucuronide (Levitz and Katz, 1968).

The sulphate conjugates of a number of xenobiotics are eliminated in the bile and their subsequent hydrolysis by the gut microflora is an important part of their enterohepatic circulation, e.g. benzo[a]pyrene (Boroujerdi *et al.,* 1981), carbenoxolone (Iveson *et al.,* 1971), oxyphenisatin (Sund *et al.,* 1979) and vanillin/isovanillin (Strand and Scheline, 1975): although in many cases glucuronidation and microbial *β*-glucuronidase are also involved in the enterohepatic cycling. In most cases the hydrolysis of sulphate esters releases an active compound so that the reaction can be regarded as activation or intoxication. However, for some carcinogens such as N-hydroxyacetylaminofluorene (Grantham *et al.,* 1970; Williams *et al.,* 1970; Smith *et al.,* 1987), polycyclic aromatic hydrocarbons (Boroujerdi *et al.,* 1981; Watabe *et al.,* 1985) and the 1'-hydroxy metabolite of alkylbenzenes (Randerath *et al.,* 1984), the sulphate ester is the active carcinogenic species so that hydrolysis can be regarded as a true detoxication reaction.

Faeces may also be able to effect sulphation reactions since they contain microbial aryl sulphotransferase activity (DongHyun and Kobashi, 1986).

The sulphate released by the gut microflora sulphatase may be reduced by sulphate-reducing bacteria (Gibson *et al., 1*993a) as discussed in more detail in Chapter 13. There is wide inter-individual variability in the presence of sulphate-reducing activity in humans, which shows an

inverse relationship with methane-producing organisms (Gibson *et al.,* 1993b). A large proportion (about 25%) of humans do not show sulphate-reducing activity despite the fact that a wide range of organisms possess sulphate-reducing capacity, e.g. *Desulfovibrio* spp., *Desulfobacter* spp., *Desulfobulbus* spp., and *Desulfomonas* spp. This situation is analogous to that of cyclamate hydrolysis, and like cyclamate hydrolysis (see below), the sulphate-reducing activity is inducible by the substrate (Christl *et al.,* 1992).

Hydrolysis of sulphamates

The sulphamate group (R-NH-$SO_3^{(-)}$) is present in xenobiotics (e.g. cyclamate) and as metabolites of amino compounds (Iwasaki *et al.,* 1986; Turesky *et al.,* 1986) including the food-bone carcinogens IQ, MeIQ and MeIQX (Alexander *et al.,* 1989; Inamasu *et al.,* 1989; Turesky *et al.,* 1988, 1993). In addition, sulphamates exist endogenously as polar groups in heteropolysaccharides. The sulphamate group in amino sugars can be hydrolyzed by both mammalian enzymes (Freeman and Hopwood, 1986) and by microorganisms such as *Flavobacterium heparinum* (Bruce *et al.,* 1987).

The sulphamate group is highly polar and there is incomplete absorption of the sweetener cyclamate after oral administration, so that more than 50% of an oral dose reaches the gut microflora. Almost all information on the microbial hydrolysis of sulphamates has been developed in relation to cyclamate and its structural analogues. The hydro lysis of cyclamate to cyclohexylamine provides a number of interesting insights into the metabolism of sulphur xenobiotics by the intestinal microflora.

Cyclamate (N-cyclohexylsulphamate: Figure 12.3) has been used as an intense sweetener since the mid-1950s, frequently in combination with saccharin (Bopp *et al.,* 1986). In 1969 it was banned in a number of countries, including the UK and USA, based on the detection of bladder tumours in a study in which rats were given high dietary concentrations of the 10:1 cyclamate:saccharin mixture supplemented with cyclohexylamine in one-half of the animals (Price *et al.,* 1970). Many countries did not ban cyclamate, because of the problems of interpretation of the study, and recent studies have not confirmed the original finding (Bopp *et al.,* 1986). The metabolism of cyclamate to cyclohexylamine is central to the establishment of safe-use levels of the sweetener, since the metabolite is considerably more toxic than the parent compound (Renwick, 1990).

Early studies on the fate of cyclamate did not detect any metabolism (Taylor *et al.,* 1951, Miller *et al.,* 1966), but in 1966 it was reported that cyclohexylamine was a urinary metabolite of the sweetener in humans (Kojima and Ichibagase, 1966). This discovery was rapidly confirmed by others and resulted in the decision to add cyclohexylamine to the cocktail of cyclamate and saccharin in what was clearly expected to be a negative rat carcinogenicity bioassay (Price *et al.,* 1970).

The site of cyclamate hydrolysis *in vivo* has been shown to be the gut microflora by the negligible hydrolysis found after parenteral administration of cyclamate to rats (Senders *et al.,* 1969; Renwick and Williams, 1972; Bickel *et al.,* 1974), guinea pigs (Asahina *et al.,* 1972b) and pigs (Collings, 1971). In addition, large oral doses of antibiotics suppressed the extent of metabolism in animal species (Senders *et al.,* 1969; Asahina *et al.,* 1972b; Bickel *et al.,* 1974; Collings, 1971) and also in humans (Collings, 1971). These *in vivo* data are supported by numerous *in vitro* studies in which the conversion of cyclamate to cyclohexylamine has been demonstrated with mixed caecal or faecal organisms (Golberg *et al.,* 1969; Dalderup *et al.,* 1970; Collings, 1971; Asahina *et al.,* 1972a,b;

Drasar *et al.,* 1972; Bickel *et al.,* 1974; Tesoriero and Roxon, 1975; Renwick, 1976; Tokeida *et al.,* 1979; *Matsui et al.,* 1981;Tsuchiya, 1981; Mallet *et al.,* 1985). Isolated strains of intestinal bacteria from different mammalian hosts have been shown to possess cyclamate hydrolyzing activity, e.g. *Clostridium* (Golberg *et al.,* 1969; Drasar *et al.,* 1972; Tokieda *et al.,* 1979; Matsui *et al.,* 1981), *Pseudomonas* (Asahina *et al.,* 1972a), *Corynebacterium* (Asahina *et al.,* 1972a), *Enterobacterium* (Drasar *et al.,* 1972), *Campylobacter* (Matsui *et al.,* 1981), *E. coli* (Tokieda *et al,* 1979), *Enterococcus faecalis* (Drasar *et al.,* 1972; Tokieda *et al.,* 1979) and *Bacillus* (Tokieda *et al.,* 1979). In view of this apparent widespread occurrence of cyclamate sulphamatase activity, it is surprising that there is negligible metabolizing activity *in vivo* prior to chronic cyclamate administration and also that there is very wide inter-individual variability in both animals and humans.

Single doses of cyclamate undergo negligible hydrolysis (Renwick and Williams, 1972), but cyclohexylamine formation increases during chronic cyclamate intake to reach a relatively constant level. In humans it takes about two weeks for cyclamate metabolism to reach a plateau (Davis *et al.,* 1969; Renwick and Williams, 1972; Buss *et al,* 1992). In contrast, induction of cyclamate metabolism in rats may take considerably longer (Renwick, 1986) and some rat colonies never developed cyclamate metabolism during long-term chronic intake (Collings, 1971). The metabolism of cyclamate in humans is probably zero order, i.e. the supply of substrate is sufficient to saturate the enzyme, causing almost constant blood concentrations of cyclohexylamine over short periods, e.g. eight hours (Buss *et al.,* 1992). After the induction period, the extent of cyclamate metabolism *in vivo* shows considerable day-to-day fluctuations even during regular daily dosing in both rats and humans (Davis *et al.,* 1969; Collings, 1971; Litchfield and Swan, 1971; Renwick and Williams, 1972; Renwick, 1976; Buss *et al.,* 1992). Variations in bowel function contribute to the fluctuations in metabolism, with an increase in the extent of metabolism in an individual during periods of constipation (Davis *et al.,* 1969), probably due to the longer period of 'incubation' of substrate and enzyme(s). The fluctuations probably arise also from variations in enzyme content of the gut flora. There is little consistency in cyclamate metabolizing ability when subjects are restudied after an interval of several months (Buss *et al.,* 1992).

The cause of the intra-individual variability during chronic cyclamate intake is not known but factors known to affect cyclamate metabolism *in vitro* include the presence of a nutrient medium (Mallett *et al.,* 1985), sulphur amino acids (Tesoriero and Roxon, 1975), glucose (Dalderup *et al.,* 1970) and cyclohexylamine (Drasar *et al.,* 1972). Cyclamate metabolism *in vivo* is also affected by constipation (in humans—see above), coprophagy in animals, by neonatal exposure (Renwick, 1976) and by short-term cessation of intake (Renwick and Williams, 1972; Bickel *et al.,* 1974).

A significant aspect of cyclamate sulphamatase activity *in vivo* is the wide inter-individual variability in experimental animals (Oser *et al.,* 1968; Senders *et al.,* 1969; Collings, 1971; Renwick and Williams, 1972; Bickel *et al.,* 1974; Renwick, 1976, 1986) and also in humans (Leahy *et al.,* 1967; Davis *et al.,* 1969; Collings, 1971; Litchfield and Swan, 1971; Renwick and Williams, 1972; Wills *et al.,* 1981; Buss *et al.,* 1992). About 80% of a human population do not develop detectable metabolism (<0.2% of the daily dose) during chronic intake whereas about 20% of people metabolize some cyclamate and 3–4% can metabolize more than 20% of the daily dose. This proportion is consistent across a number of studies (Bopp *et al.,* 1986), and was also found in a recent study in almost 200 diabetic patients studied in the UK in 1989 after a period of 20 years without cyclamate in the diet (Buss *et al.,* 1992). It is likely therefore that there is some natural substrate (possibly a heteropolysaccharide) which maintains the microflora potential for cyclamate hydrolysis.

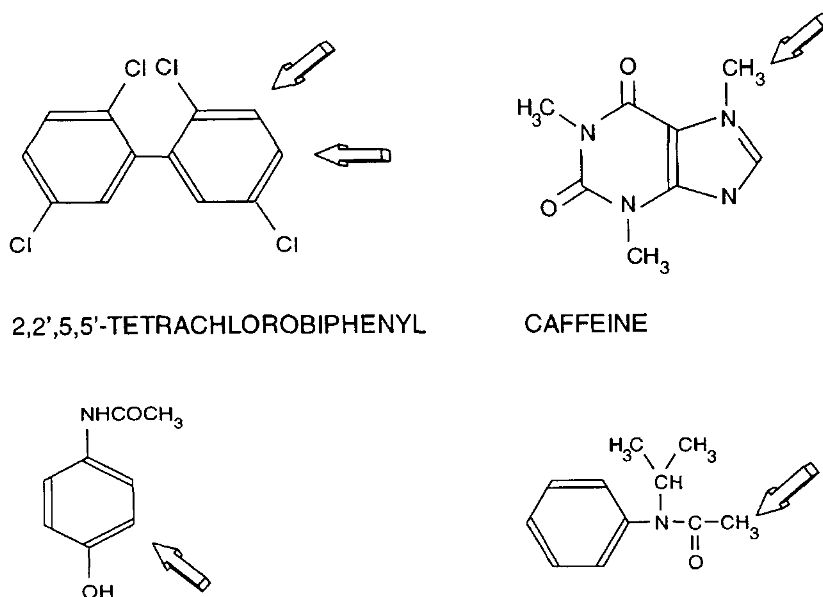


Figure 12.8 Compounds forming thiomethyl metabolites. The position of glutathione conjugation and subsequent thiomethyl substitution is indicated by an arrow

In vitro studies on the substrate specificity of the cyclamate sulphamatase in rat caecal contents (Renwick, 1976), in *Pseudomonas* (Niimura *et al.,* 1974; Tsuchiya, 1981) and in an extract of rat faeces (McGlinchey *et al.,* 1982) showed a wide substrate specificity for alkylsulphamates, and simple aryl sulphamates, but no hydrolysis of the sulphamate ion, cyclohexanol sulphate or sulphanilamide by *Pseudomonas,* or saccharin by rat caecal contents (Ball *et al.,* 1977). *In vivo* studies confirmed the wide specificity for alkylsulphamates (Renwick, 1976; McGlinchey *et al.,* 1982). It is not known if 'cyclamate sulphamatase' can hydrolyze sulphamate groups in heteropolysaccharides.

An interesting recent observation, in relation to bacterial cyclamate metabolism in humans, is the finding that the formation of cyclohexylamine *in vivo* did not cause cardiovascular effects, despite the high plasma concentrations produced in some subjects (Buss *et al.,* 1992). Similar plasma cyclohexylamine concentrations produced from a single oral dose of cyclohexylamine affected both blood pressure and heart rate (Eichelbaum *et al.,* 1974). This apparent discrepancy is explained by the indirect mechanism of action of cyclohexylamine and the slow build-up of cyclamate metabolizing ability (Buss and Renwick, 1992).

Lysis of cysteine conjugates

Recent interest in the microbial metabolism of cysteine conjugates arose from the finding of thiomethyl ethers as metabolites of compounds which do not contain a sulphur atom, such as the herbicide propachlor (Bakke *et al.,* 1980; Larsen and Bakke, 1981; Rafter *et al.,* 1983), *m*-dichlorobenzene (Kato *et al.,* 1986), caffeine (Rafter and Nilsson, 1981) and paracetamol (Gemborys

and Mudge, 1981; Warrander *et al.,* 1985; Mikov *et al.,* 1988) (Figure 12.8). These thioether metabolites were also eliminated in oxidized forms, i.e. the corresponding sulphoxides and sulphones. The excretion of the N-acetylcysteine conjugate of propachlor and its sulphoxide (Larsen and Bakke, 1981; Rafter *et al.,* 1983) pointed to cysteine as the possible source of the sulphur.

Cysteine conjugates of xenobiotics, in which the foreign compound is covalently bonded to the sulphur of cysteine, reach the intestinal bacteria in the lower bowel as a consequence of the biliary excretion of glutathione conjugates. The addition of glutathione to a foreign compound increases its molecular weight by 306 Daltons and therefore the bile is a major route for the elimination of such metabolites, although the glutathione conjugate of propachlor may also reach the gut microflora by a non-biliary route (Aschbacher and Struble, 1987). Because glutathione is a tripeptide it undergoes metabolism on passage down the gastrointestinal tract so that the product presented to the gut flora is the cysteine conjugate (Figure 12.9).

The initial loss of glutamic acid from the glutathione conjugate is catalyzed by γ-glutamyl transpeptidase and the subsequent removal of glycine is by carboxypeptidase. The enzymes are present in the liver, bile and upper intestine (Tanaka, 1974; Rosalki, 1975; Kozak and Tate, 1982). The lysis of the cysteine conjugate is catalyzed by the enzyme C-S lyase (or β-lyase). This enzyme, which purifies with the enzyme kynureninase (Stevens, 1985) but is not the same enzyme (Buckberry *et al.,* 1992a,b), is present in mammalian tissues such as the liver (Buckberry *et al.,* 1993) and kidney (Darnerud *et al.,* 1991). C-S lyase is also present in high amounts in the gut microflora. The enzyme splits the cysteine conjugate to give stochiometric amounts of the thiol, pyurate and ammonia (Figure 12.10).

The thiol metabolite may be absorbed and undergo further metabolism by S-conjugation with glucuronic acid or methylation with S-adenosyl methionine; the latter forms a methyl thioether which can be oxidized to the sulphoxide and sulphone. In the case of propachlor the thiol also binds to faecal constituents to form an unextractable residue (Rafter *et al.* 1983).

2, 2', 5, 5'-Tetrachlorobiphenyl is excreted as 3- and 4-thiomethyl metabolites and their sulphones, probably via glutathione conjugation of a 3, 4-expoxide intermediate (Preston *et al.,* 1984). Treatment of germ-free and conventional mice with 2, 4', 5-trichlorobiphenyl and ^{35}S-labelled cysteine provided direct evidence that cysteine was the source of the sulphur atom in such thiomethyl metabolites. The gut flora were shown to be the main *in vivo* site of C-S lyase activity which allowed the generation of the methyl sulphone metabolite which accumulated in the lung (Bergman *et al.,* 1982).

The importance of the C-S lyase of the bacterial flora in the formation of thiomethyl products *in vivo* has been shown by studies using germ-free animals and antibiotic treatment. In the case of propachlor, the various methyl thioether products in urine (mostly as the sulphones) represented 20–25% of the dose administered to conventional rats, with the N-acetylcysteine conjugate accounting for 21% (Rafter *et al.,* 1983). In contrast, in germ-free rats or rats given large doses of antibiotics, there were no methyl thioether products, but increased excretion of the N-acetyl cysteine conjugate and also of its sulphoxide. Similarly, in the case of caffeine, the urinary excretion of the methyl sulphoxide metabolite was at least 42-fold higher in conventional mice compared with germ-free mice (Rafter and Nilsson, 1981).

In vitro studies have demonstrated a high C-S lyase activity in caecal contents (Larsen and Bakke, 1981; Larsen *et al.,* 1983) and there is a wide distribution of the activity in different intestinal organisms (Larsen, 1985). Organisms shown to possess C-S lyase activity include *Fusobacterium* species (Larsen, 1985; Larsen *et al.,* 1983; Tomisawa *et al.,* 1984), *Bacteroides* (Larsen, 1985) and

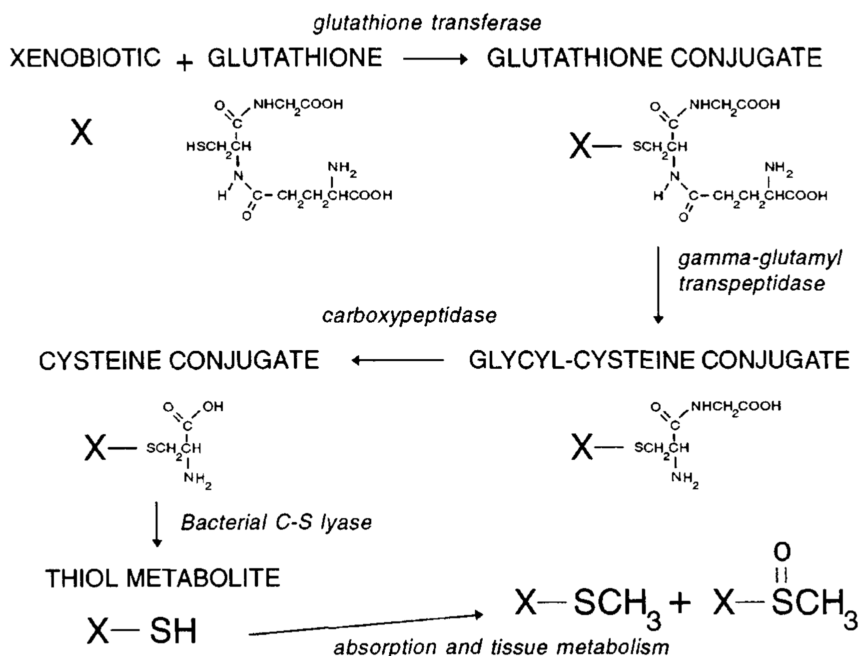


Figure 12.9 Formation of thiomethyl metabolites via conjugation with glutathione and the activity of C-S lyase

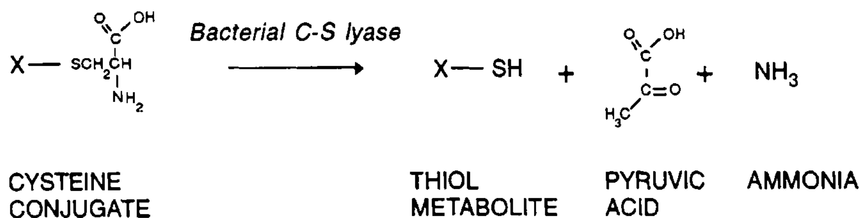


Figure 12.10 The reaction catalyzed by C-S Iyase

Eubacterium species (Larsen, 1985; Larsen and Stevens, 1985). There are at least three forms of C-S lyase showing different molecular weights and requirements for pyridoxal phosphate.

It is also possible for the sulphur atom in a xenobiotic to be replaced by sulphur from glutathione. The hepatic formation of methylsulphoxide and methylsulphone metabolites of 2-methylthiobenzothiazole produces potential leaving-groups which can be replaced by glutathione. The products of such reactions are the 2-glutathione conjugates plus sulphate and formaldehyde from the thiomethyl group (Larsen *et al.*, 1988). Further metabolism of the 2-glutathione conjugate of benzothiazole by the liver (Larsen *et al.*, 1988) and possibly by the gut flora, as described above, can give the 2-methylsulphoxide and 2-methylsulphone metabolites but with the sulphur derived from glutathione (Figure 12.9).

Meta-chlorobenzene is metabolized via glutathione conjugation and bacterial C-S lyase to 3, 5- and 2, 4-dichlorophenyl-methylsulphones (Kato *et al.*, 1986). These methylsulphone metabolites, which are inducers of drug metabolism (Kato *et al.*, 1986), are retained in the kidney bound to α-2u-

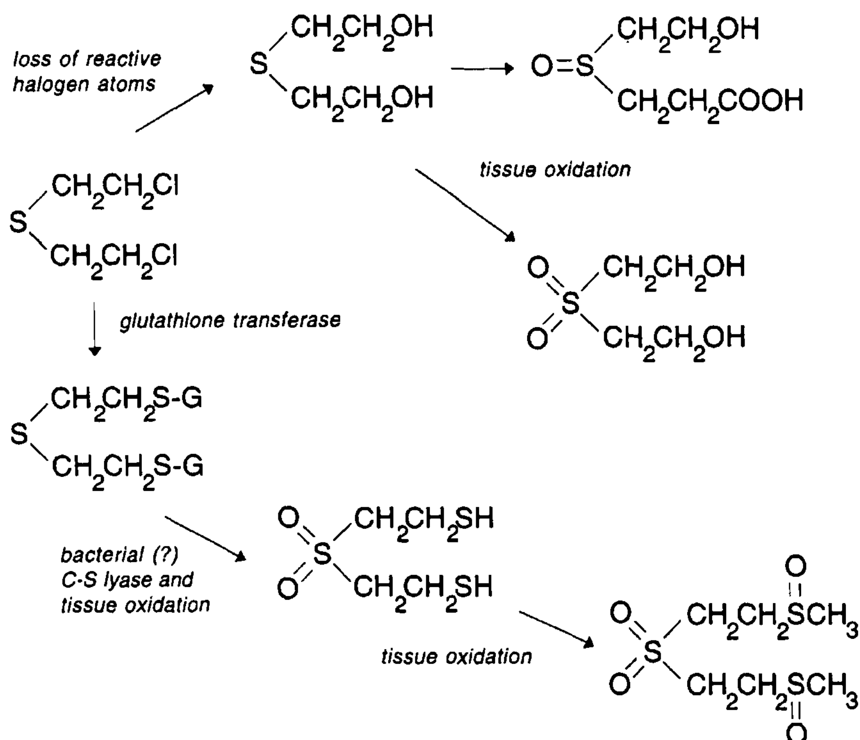


Figure 12.11 The fate of sulphur mustard

globulin (Larsen *et al.,* 1990), a process which may be linked to the renal toxicity produced by this class of compound.

An extremely interesting example of S-xenobiotic biochemistry is given by the sulphur mustard 1, 1'-thiobis(2-chloroethane) which is essentially diethylthioether with 2 and 2' chloro substitution to provide the labile/reactive alkylating centres. The urinary metabolites in the rat (Figure 12.11) show evidence of oxidation of the thioether group to sulphoxide and sulphone. There is also replacement of one or both of the chloro substituents by glutathione to produce glutathione conjugates which are converted via C-S lyase (see above) to a *bis* thiomethyl ether and the corresponding *bis* sulphoxide (Black *et al.,* 1992). The role of the gut flora C-S lyase has not been defined, but it is likely that the *bis* glutathione conjugate would be eliminated in bile. Loss of the chlorosubstituents produced (2, 2'thio-bis-ethanol) which is oxidized at the thioether, and one alcohol group to sulphoxide, sulphone and carboxylic acid respectively (Black *et al.*, 1993).

The C-S lyase pathway is of considerable interest due to the formation of a novel thiol metabolite (which may show different pharmacological or toxicological properties from the parent compound), which may introduce a sulphur site for further metabolism by conjugation and/or oxidation. Measurements of the urinary excretion of products of glutathione conjugation and C-S lyase, such as pentachlorothioanisole, may be useful measures of exposure to toxicants, such as hexachlorobenzene (ToFigueras *et al.,* 1992).

References

Abraham, R.T., Benson, L.M. and Jardine, I., 1983, Synthesis and pH-dependent stability of purine-6-sulfenic acid, a putative reactive metabolite of 6-thiopurine, *J. Med. Chem.,* **26,** 1523–26.

Ahmed, S.A. and Claiborne, A., 1989, The streptococcal NADH oxidase. I. Evidence linking NADH oxidase and NADH peroxidase cysteinyl redox centers, *J. Biol. Chem.,* **264,** 19856–63.

Alexander, J., Holme, J.A., Wallin, H. and Becker, G., 1989, Characterisation of the metabolites of food mutagens 2-amino-3-methylimidazo(4, 5-*f*)quinoline and 2-amino-3, 4– dimethylimidazo(4, 5-*f*)quinoline formed after incubation with isolated rat liver, *Chemico-Biol. Interactions,* **72,** 125–42.

Anders, M.W., Ratnayake, J.H., Hanna, P.E. and Fuchs, J.A., 1981, Thioredoxin-dependent sulfoxide reduction by rat renal cytosol, *Drug Metab. Disp.,* **9,** 307–10.

Asahina, M., Niimura, T., Yamaha, T. and Takahashi, T, 1972a, Formation of cyclohexylamine and cyclohexanone from cyclamate by microorganisms isolated from the feces of guinea pigs, *Agr. Biol. Chem.,* **36,** 711–18.

Asahina, M., Yamaha, T, Sarrazin, G. and Watanable, K., 1972b, Conversion of cyclamate to cyclohexylamine in guinea pigs, *Chem. Pharm. Bull.,* **20,** 102–8.

Aschbacher, P.W. and Struble, C.B., 1987, Evidence for involvement of non-biliary excretion into the intestine in the formation of methylsulphonyl-containing metabolites of 2-chloro-N-isopropylacetanilide (propachlor) by swine and rats, *Xenobiotica,* **17,** 1047–55.

Auret, B.J., Boyd, D.R. and Henbest, H.B., 1966, A range of stereoselectivity in the microbiological oxidation of thioethers to sulphoxides, *J. Chem. Soc. Chem. Commun.,* 66–67.

Auret, B.J., Boyd, D.R., Henbest, H.B. and Ross, S., 1968a, Stereoselectivity in the oxidation of thioethers to sulphoxides in the presence of *Aspergillus niger, J. Chem. Soc.,* **C,** 2371–74.

Auret, B.J., Boyd, D.R. and Henbest, H.B., 1968b, Stereoselectivity in the oxidation of sulphoxides to sulphones in the presence of *Aspergillus niger, J. Chem. Soc.,* **C,** 2374–76.

Back, D.H., Chapman, C.R., May, S.A. and Rowe, P.H., 1981, Absorption of oestrone sulphate from the gastrointestinal tract of the rat, *J. Steroid Biochem.,* **14,** 347–56.

Bakke, J.E., Gustafsson, J.A. and Gustafsson, B.E., 1980, Metabolism of propachlor by the germ free rat, *Science,* **210,** 433–35.

Ball, L.M., Renwick, A.G. and Williams, R.T., 1977, The fate of [^{14}C]saccharin in man, rat and rabbit and of 2-sulphamoyl[^{14}C]benzoic acid in the rat, *Xenobiotica,* **7,** 189–203.

Batten, P.I., 1979, Metabolism of 2-naphthylamine sulphonic acids, *Toxicol., Appl. Pharmacol.,* **48,** A171.

Bergman, A., Biessmann, A., Brandt, I. and Rafter, J., 1982, Metabolism of 2, 4'5-trichlorobiphenyl: role of the intestinal microflora in the formation of bronchial-seeking methyl sulphone metabolites in mice, *Chemico-Biol. Interact.,* 40, 123–31.

Bickel, M.H., Burkard, B., Meier-Strasser, E. and Van Den Broek-Boot, M., 1974, Enterobacterial formation of cyclohexylamine in rats ingesting *cyclamate, Xenobiotica,* **4,** 425–39.

Biedlingmaier, S. and Schmidt, A., 1983, Alkylsulfonic acids and some sulfur-containing detergents as sulfur sources for growth of *Chlorella fusca, Arch. Microbiol.,* **136,** 124–30.

Black, R.M., Brewster, K., Clarke, R.J., Hambrook, J.L., Harrison, J.M. and Howells, D.J., 1992, Biological fate of sulphur mustard, 1, 1'-thiobis(2-chloroethane): isolation and identification of urinary metabolites following intraperitoneal administration to rat, *Xenobiotica,* **22,** 405–18.

Black, R.M., Brewster, K., Clarke, R.J., Hambrook, J.L., Harrison, J.M. and Howells, D.J., 1993, Metabolism of thiodiglycol (2, 2'thiobis-ethanol): isolation and identification of urinary metabolites following intraperitoneal administration to rat, *Xenobiotica,* **23,** 473–81.

Bopp, B.A., Senders, R.C. and Kesterson, J.W., 1986, Toxicological aspects of cyclamate and cyclohexylamine, *CRC Crit. Rev. Toxicol,* **16,** 213–306.

Boroujerdi, M., Kung, H., Wilson, A.G.E. and Andersen, M.W., 1981, Metabolism and DNA binding of benzo (a)pyrene *in vivo* in the rat, *Cancer Res.,* **41,** 951–57.

Boyd, D.R., Walsh, C.T. and Chen, Y.C.J., 1989, S-Oxygenases, II: chirality of sulphoxidation reactions, in Damani, L.A. (Ed.) *Sulphur-Containing Drugs and Related Organic Compounds. Chemistry, Biochemistry and Toxicology,* Vol. 2, Part A, *Analytical, Biochemical and Toxicological Aspects of Sulphur Xenobiochemistry,* pp. 67–99, Chichester: Ellis Horwood Ltd.

Briggs, M.H. and Brotherton, J., 1970, *Steroid Biochemistry and Pharmacology,* London: Academic Press, pp. 80–81.

Brot, N., Weissbach, L., Worth, J. and Weissbach, H., 1981, Enzymatic reduction of protein-bound methionine sulphoxide, *Proc. Natl. Acad. Sci.,* **78,** 2155–58.

Bruce, J.S., McLean, M.W., Long, W.F. and Williamson, F.B., 1987, *Flavobacterium heparinum* sulphamidase for D-glucosamine sulphamate. Purification and characterisation, *Eur. J. Biochem.,* **165,** 633–38.

Buckberry, L.D., Blagborough, I.S. and Shaw, P.N., 1993, Cysteine conjugate toxicity in a human cell line: correlation with C-S lyase activity in human hepatic tissues, *Hum. Exp. Toxicol,* **12,** 329–35.

Buckberry, L.D., Bycroft, B.W., Shaw, P.N. and Blagborough, I.S., 1992a, Human hepatic C-S lyase: transamination reactions and significant differences between kynurenine aminotransferase and kynureninase, *Bioorgan. Med. Chem. Lett.,* **2,** 1225–30.

Buckberry, L.D., Blagborough, I.S., Bycroft, B.W. and Shaw, P.N., 1992b, Kynurenine aminotransferase activity in human liver: identity with human hepatic C-S lyase and a physiological role for this enzyme, *Toxicol. Lett.,* **60,** 241–46.

Buss, N.E. and Renwick, A.G., 1992, Blood pressure changes and sympathetic function in rats given cyclohexylamine by intravenous infusion, *Toxicol Appl Pharmacol,* **115,** 211–15.

Buss, N.E., Renwick, A.G., Donaldson, K.M. and George, C.F., 1992, The metabolism of cyclamate to cyclohexylamine and its cardiovascular consequences in human volunteers, *Toxicol Appl. Pharmacol,* **115,** 199–210.

Caldwell, J. and Given, H.M., 1989, Thioethers, thiols, dithioic acids and disulphides: Phase II reactions, in Damani, L.A. (Ed.) *Sulphur-Containing Drugs and Related Organic Compounds. Chemistry, Biochemistry and Toxicology,* Vol. 1, Part A, *Metabolism of Sulphur Functional Groups,* pp. 151–60, Chichester: Ellis Horwood Ltd.

Carter, J.H., McLafferty, M.A. and Goldman, P., 1980, Role of the gastrointestinal microflora in amygdalin (laetrile)-induced cyanide toxicity *Biochem. Pharmacol.,* **29,** 301–4.

Christl, S.U., Gibson, G.R. and Cummings, J.H., 1992, Role of dietary sulphate in the regulation of methanogenesis in the human large intestine, *Gut,* **33,** 1234–38.

Claiborne, A., Miller, H., Parsonage, D. and Ross, R.P., 1993, Protein-sulfenic acid stabilisation and function in enzyme catalysis and gene regulation, *F.A.S.E.B.,* **7,** 1483–90.

Cleary, P.P. and Dykhuizen, D., 1974, Enzymatic reduction of D-biotin-d-sulfoxide with cell-free extracts of *Escherichia coli, Biochem. Biophys. Res. Commun.,* **56,** 629–34.

Collings, A.J., 1971, The metabolism of sodium cyclamate, in Birch, G.C., Green, L.F. and Coulson, C.B. (Eds) *Sweetness and Sweeteners,* pp. 51–68, London: Applied Science.

Dage, R.C. and Okerhjolm, R.A., 1990, Pharmacology and pharmacokinetics of enoximone, *Cardiol.,* **77,** 2–13.

Dalderup, L.M., Keller, G.H.M. and Schoeten, F., 1970, Cyclamate and cyclohexylamine, *Lancet,* **i,** 845.

Damani, L.A., 1989, Aspects of sulphur chemistry, biochemistry and xenobiochemistry, in Damani, L.A. (Ed.) *Sulphur-Containing Drugs and Related Organic Compounds. Chemistry, Biochemistry and Toxicology,* Vol. 1, Part A, *Metabolism of Sulphur Functional Groups,* pp. 9–28, Chichester: Ellis Horwood Ltd.

Darnerud, P.O., Gustafson, A.-L., Törnwall, U. and Feil, V.J., 1991, Age- and sex-dependent dichlorovinyl cysteine (DCVC) accumulation and toxicity in the mouse kidney: relation to development of organic anion transport and β-lyase activity , *Pharmacol. Toxicol,* **68,** 104–9.

Davis, P.J. and Guenthner, L.E., 1985, Sulindac oxidation/reduction by microbial cultures; microbial models for mammalian metabolism, *Xenobiotica,* **15,** 845–57.

Davis, T.R.A., Adler, N. and Opsahl, J.C., 1969, Excretion of cyclohexylamine in subjects ingesting sodium cyclamate, *Toxicol. Appl. Pharmacol,* **15,** 106–16.

DeHerder, W.W., Hazenberg, M.P., Otten, M.H., Pennock-Schroder, A.M. and Visser, T.J., 1985, Hydrolysis of iodothyronine sulfates by sulfatase activity of anaerobic bacteria from the rat intestinal flora, *FEMS Microbiol. Lett.,* **27,** 79–83.

Del Camparillo Campbell, A. and Campbell, A., 1982, Molybdenum cofactor requirement for biotin sulfoxide reduction in *Escherichia coli, J. Bacterial.,* **149,** 469–78.

Diczfalusy, E. and Levitz, M., 1970, Formation, metabolism and transport of estrogen conjugates, in Bernstein, S. and Solomon, S. (Eds) *Chemical and Biological Aspects of Steroid Conjugation,* pp. 291–320, Berlin: Springer Verlag.

DongHyun, K. and Kobashi, K., 1986, The role of intestinal flora in metabolism of phenolic sulfate esters, *Biochem. Pharmacol.,* **35,** 3507–10.

Drasar, B.S., 1988, The bacterial flora of the intestine, in Rowland, I.R. (Ed.) *Role of the Gut Flora in Toxicity and Cancer,* pp. 23–38, London: Academic Press.

Drasar, B.S., Renwick, A.G. and Williams, R.T., 1972, The role of the gut flora in the metabolism of cyclamate, *Biochem. J.,* **129,** 881–90.

Duggan, D.E., Hooke, K.F., Noll, R.M., Hucker, H.B. and Van Arman, C.G., 1978, Comparative disposition of sulindac and metabolites in five species, *Biochem. Pharmacol.,* **27,** 2311–20.

Eichelbaum, M., Hengstmann, J.H., Rost, H.D., Brecht, T. and Dengler, H.J., 1974, Pharmaco-kinetics, cardiovascular and metabolic actions of cyclohexylamine in man, *Arch. Toxicol.,* **31,** 243–63.

Ejiri, S.I., Weissbach, H. and Brot, N., 1979, Reduction of methionine sulfoxide to methionine *by Escherichia coli, J. Bacterial.,* **139,** 161–64.

Ellard, G.A., 1966, Absorption, metabolism and excretion of di(p-aminophenyl)sulphone (dapsone) and di(p-aminophenyl)sulphoxide in man, *Brit. J. Pharmacol.,* **26,** 212–17.

Faigle, J.W., 1993, Drug metabolism in the colon wall and lumen, in Bieck, P.R. (Ed.) *Colonic Drug Absorption and Metabolism,* pp. 29–54, New York: Marcel Dekker Inc.

Finch, R.A., Vasquez, K.M., Hanna, N.B., Revankar, G.R., Robins, R.K. and Avery, T.L., 1990, Oxidation of 2-amino-9-beta-D-ribofuranosylpurine-6-sulfenamide to the corresponding 6-sulfonamide facilitates changes in biological characterization that include activity against thiopurine—refractory experimental leukemia, *Cancer Lett.,* **50,** 63–70.

Forth, W., Nell, G., Rummel, W. and Andres, H., 1972, The hydragogue and laxative effect of the sulfuric acid ester and the free diphenol of 4, 4'-dihydroxy-diphenyl-(pyridyl-2)-methane. *Naunyn-Schmeideberg's Arch. Pharmacol.,* **274,** 46–53.

Freeman, C. and Hopwood, J.J., 1986, Human liver sulphamate sulphohydrolase. Determinations of native protein and subunit M(r) values and influence of substrate aglycone structure on catalytic properties, *Biochem. J.,* **234,** 83–92.

Gardner, D.M. and Renwick, A.G., 1978, The reduction of nitrobenzoic acids in the rat, *Xenobiotica,* **8,** 679–90.

Gemborys, M.W. and Mudge, G.H., 1981, Formation and disposition of the minor metabolites of acetamidophen in the hamster, *Drug Metab. Disp.,* **9,** 340–51.

Gibson, G.R., Macfarlane, G.T. and Cummings, J.H., 1993a, Sulphate reducing bacteria and hydrogen metabolism in the human large intestine, *Gut,* **34,** 437–39.

Gibson, G.R., Macfarlane, S. and Macfarlane, G.T., 1993b, Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine, *FEMS Microbiol. Lett.,* **12,** 117–25.

Golberg, L., Parekh, C, Patti, A. and Soike, K., 1969, Cyclamate degradation in mammals and *in vitro, Toxicol. Appl. Pharmacol,* **14,** 654.

Grantham, P.H., Horton, R.E., Weisburger, E.K. and Weisburger, J.H., 1970, Metabolism of the carcinogen N-2-fluorenylacetamide in germ-free and conventional rats, *Biochem. Pharmacol.,* **19,** 163–71.

Greenslade, D., Havler, M.E., Humphrey, M.J., Jordan, B.L., Lewis, C.J. and Ranee, D.J., 1981, Biotransformation of tolmesoxide in animals and *man, Xenobiotica,* **11,** 89–96.

Hale, P.W. and Poklis, A., 1984, Thioridazine-5-sulphoxide cardiotoxicity in the isolated, perfused rat heart, *Toxicol. Lett.,* **21,** 1–8.

Hanna, N., Marshall, P.J., Newton, J., Schwartz, L., Kirsh, R., DiMartino, M.J., Adams, J., Bender, P. and Griswold, D.E., 1990, Pharmacological profile of SK and F 105809, a dual inhibitor of arachidonic acid metabolism, *Drugs Exp. Clin. Res.,* **16,** 137–47.

Heath, A., Svensson, C. and Martensson, E., 1985, Thioridazine toxicity—an experimental cardiovascular study of thioridazine and its major metabolites in overdose, *Vet. Human Toxicol.* **27,** 100–10.

Holmgren, A., 1968, Thioredoxin. 6. The aminoacid sequence of the protein from *Escherichia coli* B, *Eur. J. Biochem.,* **6,** 475–84.

Holmgren, A. and Luthman, M., 1978, Tissue distribution and subcellular localisation of bovine thioredoxin determined by radio immunoassay, *Biochem.,* **17,** 4071–77.

Huijghebaert, S.M., Mertens, J.A. and Eyssen, H.J., 1982, Isolation of a bile salt sulfatase-producing *Clostridium* strain from rat intestinal microflora, *Appl. Environ. Microbiol.,* **43,** 185–92.

Inamasu, T., Luki, H., Vavrek, M.T. and Weisburger, J.H., 1989, Metabolism of 2-amino-3-methylimidazo(4, 5-f)quinoline in the male rat, *Food Chem. Toxicol.,* **27,** 369–76.

Iveson, P., Lindup, W.E., Parke, D.V. and Williams, R.T., 1971, The metabolism of carbenoxolone in the rat, *Xenobiotica,* **1,** 79–95.

Iwasaki, K., Shiraga, T., Tada, K. Noda, K. and Noguchi, H., 1986, Age and sex-related changes in amine sulphoconjugations in Sprague-Dawley strain rats. Comparison with phenol and alcohol sulphoconjugations, *Xenobiotica,* **16,** 717–23.

Jaworski, T.J., Hawes, E.M., Hubbard, J.W., McKay, G. and Midha, K.K., 1991, The metabolites of chlorpromazine N-oxide in rat bile, *Xenobiotica,* **21,** 1451–59.

Johansson, B., 1992, A review of the pharmacokinetics and pharmacodynamics of disulfiram and its metabolites, *Acta. Psych. Scand.,* **86** (**Suppl.**), 15–26.

Juhl, M.J. and Clark, D.P., 1990, Thiophene-degrading *Escherichia coli* mutants possess sulphone oxidase activity and show altered resistance to sulphur-containing antibiotics, *Appl. Environ. Microbiol.,* **56,** 3179–85.

Kanagawa, T. and Kelly, D.P., 1986, Breakdown of dimethyl sulphide by mixed cultures and by *Thiobacillus thioparus, FEMS Microbiol. Lett.,* **34,** 13–19.

Kato, Y., Kogure, T., Sata, M., Murata, T. and Kimura, R., 1986, Evidence that methylsulfonyl metabolites of *m*-dichlorobenzene are causative substances of induction of hepatic microsomal drug-metabolising enzymes by the parent compound in rats, *Toxicol. Appl. Pharmacol.,* **82,** 505–11.

Kojima, S. and Ichibagase, H., 1966, Studies on synthetic sweetening agents. VIII. Cyclohexylamine, a metabolite of sodium cyclamate, *Chem. Pharm. Bull.,* **14,** 971–74.

Koss, G., Koransky, W. and Steinbach, K., 1979, Studies on the toxicology of hexachlorobenzene. IV Sulphur-containing metabolites, *Arch. Toxicol.,* **42,** 19–31.

Kozak, E.M. and Tate, S.S., 1982, Glutathione-degrading enzymes of the microvillus membranes, *J. Biol Chem.,* **257,** 6322–27.

Lanusse, C.E., Nare, B., Gascon, L.H. and Prichard, R.K., 1992, Metabolism of albendazole and albendazole sulphoxide by ruminal and intestinal fluids of sheep and cattle, *Xenobiotica,* **22,** 419–26.

Laqueur, G.L. and Spatz, M., 1968, Toxicology of cycasin, *Cancer Res.,* **28,** 2262–7.

Larsen, G.L., 1985, Distribution of cysteine conjugate β-lyase in gastrointestinal bacteria and in the environment, *Xenobiotica,* **15,** 199–209.

Larsen, G.L., 1988, Deconjugation of biliary metabolites by microflora β-glucuronidases, sulphatases and cysteine conjugate β-lyases and their subsequent enterohepatic circulation, in Rowland, I.R. (Ed.) *Role of the Gut Flora in Toxicity and Cancer,* pp. 79–107, London: Academic Press.

Larsen, G.L. and Bakke, J.E., 1981, Enterohepatic circulation in formation of propachlor (2-chloro-N-isopropylacetanilide) metabolite in the rat, *Xenobiotica,* **11,** 473–80.

Larsen, G.L. and Stevens, J.L., 1985, Cysteine conjugate β-lyase in the gastrointestinal bacterium *Eubacterium limosum, Mol. Pharmacol,* **29,** 97–103.

Larsen, G.L., Bakke, J.E. and Huwe, J.K., 1990, Methylsulphone metabolites of *m*-dichlorobenzene as ligands for $\alpha_{2\mu}$-globulin in rat kidney and urine, *Xenobiotica,* **20,** 7–17.

Larsen, G.L., Larson, J.D. and Gustafsson, J.A., 1983, Cysteine conjugate β-lyase in the gastrointestinal bacterium *Fusobacterium necrophorum, Xenobiotica,* **13,** 689–700.

Larsen, G.L., Bakke, J.E., Feil, V.J. and Huwe, J.K., 1988, *In vitro* metabolism of the methylthio group of 2-methylthiobenzothiazole by rat liver, *Xenobiotica,* **18,** 313–22.

Laurent, T.C., Moore, E.G. and Reichard, P., 1964, Enzymatic synthesis of deoxyribonucleotides. IV. Isolation and characterisation of thioredoxin, the hydrogen donor from *Escherichia coli* B, *J. Biol. Chem.,* **239,** 3436–43.

Leahy, J.S., Taylor, T. and Rudd, C.J., 1967, Cyclohcxylamine excretors among human volunteers given cyclamate, *Food Cosmet. Toxicol,* **5,** 595–96.

Lee, S.C. and Renwick, A.G., 1992, The enzymatic reduction of sulphoxide-containing drugs by *Escherichia coli, Br. J. Clin. Pharmacol.,* **33,** 212P.

Levitz, M. and Katz, J., 1968, Enterohepatic metabolism of estriol-3-sulfate-16-glucosiduronate in women, *J. Clin. Endocrinol. Metab.,* **28,** 862–68.

Litchfield, M.H. and Swan, A.A.B., 1971, Cyclohexylamine production and physiological measurements in subjects ingesting sodium cyclamate, *Toxicol. Appl. Pharmacol.,* **18,** 535–41.

McGlinchey, G., Coakley, C.B., Gestaustus-Tansey, V, Gault, J. and Spillane, W.J., 1982, *In vivo* and *in vitro* studies with sulfamate sweeteners, *J. Pharm. Sci.,* **71,** 661–65.

Mackay, M. and Tomlinson, E., 1993, Colonic delivery of therapeutic peptides and proteins, in Bieck, P.R. (Ed.) *Colonic Drug Absorption and Metabolism,* pp. 159–76, New York: Marcel Dekker Inc.

Mallett, A.K., Rowland, I.R., Bearne, C.A., Purchase, R. and Gangolli, S.D., 1985, Metabolic adaptation of rat faecal microflora to cyclamate *in vitro* , *Food Chem. Toxicol.,* **23,** 1029–34.

Matsui, M.,Tanimura, A. and Kurata, H., 1981, Identification of cyclamate-converting bacteria. (Studies on the metabolism of food additives by microorganisms inhabiting the gastrointestinal tract. VI), *J. Fd. Hyg. Soc. Jpn.,* **22,** 215–22.

Michael, W.R., 1968, Metabolism of linear alkylate sulfonate and alkyl benzene sulfonate in albino rats, *Toxicol. Appl. Pharmacol.,* **12,** 473–85.

Migdalof, B.H., Antonaccio, M.J., McKinstry, D.N., Singhvi, S.M., Lan, S.-J., Egli, P. and Kripalani, K.J., 1984, Captopril: pharmacology, metabolism and disposition, *Drug Metab. Rev.,* **15,** 841–69.

Mikov, M., Caldwell, J., Dolphin, C.T. and Smith, R.L., 1988, The role of intestinal microflora in the formation of methylthio adduct metabolites of paracetamol. Studies in neomycin-pretreated and germ-free mice, *Biochem. Pharmacol.,* **37,** 1445–49.

Miller, J.P., Crawford, L.E.M., Senders, R.C. and Cardinal, E.V., 1966, Distribution and excretion of ^{14}C-cyclamate sodium in animals, *Biochem. Biophys. Res. Comm.,* **25,** 153–57.

Mitchell, S.C., Idle, J.R. and Smith, R.L., 1982, The metabolism of (^{14}C)cimetidine in man, *Xenobiotica,* **12,** 283–92.

Moreto, M., Conalons, E., Mylonakis, N., Giraldez, A. and Torralba, A., 1977, Enterohepatic circulation of sodium sulisatin and its effects on glucose absorption in the rat, *J. Pharm. Pharmacol.,* **29,** 446–48.

Nickson, R.M. and Mitchell, S.C., 1994, The fate of dipropyl sulphide and dipropyl sulphoxide in rat, *Xenobiotica,* **24,** 157–68.

Niimura, T., Tokeida, T. and Yamaha, T., 1974, Partial purification and some properties of cyclamate sulfamatase, *J. Biochem.,* **75,** 407–17.

Oser, B.L., Carson, S., Vogin, E.E. and Senders, R.C., 1968, Conversion of cyclamate to cyclohexylamine in rats, *Nature,* **220,** 178–79.

Pacini, N., Albini, E., Ferrari, A., Zanchi, R., Marca, G. and Bandiera, T., 1987, Transformation of sulfated bile acids by human intestinal microflora, *Arzneimittel-Forschung/Drug Res.,* **37,** 983–87.

Poole, L.B. and Claiborne, A., 1989, The non-flavin redox center of the streptococcal NADH peroxidase. II. Evidence for a stabilised cysteine-sulfenic acid, *J. Biol. Chem.,* **264,** 12330–38.

Preston, B.D., Miller, J.A. and Miller, B.C., 1984, Reactions of 2, 2', 5, 5'-tetrachlorobiphenyl 3, 4-oxide with methionine, cysteine and glutathione in relation to the formation of methylthio-metabolites of 2, 2', 5, 5'-tetrachlorobiphenyl in the rat and mouse, *Chemico-Biol. Interact.,* **50,** 289–312.

Price, J.M., Biava, C.G., Oser, B.L., Vogin, E.E., Steinfeld, J. and Ley, H.L., 1970, Bladder tumors in rats fed cyclohexylamine or high doses of a mixture of cyclamate and saccharin, *Science,* **167,** 1131–32.

Rafter, J.J. and Nilsson, L., 1981, Involvement of the intestinal microflora in the formation of sulphur-containing metabolites of caffeine, *Xenobiotica,* **11,** 771–78.

Rafter, J.J., Gustafsson, J.A., Bakke, J.E., Larsen, G.E., Norin, K.E. and Gustafsson, B.E., 1983, Studies on the re-establishment of the intestinal microflora in germ-free rats with special reference to the metabolism of N-isopropyl-α-chloroacetanilide (propachlor), X*enobiotica,* **13,** 171–78.

Randerath, K., Haglund, R.E., Phillips, D.H. and Reddy, M.V., 1984, Phosphorus-32 post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes: 1. Adult female CD-1 mice, *Carcinogenesis,* **5,** 1613–22.

Renwick, A.G., 1976, Microbial metabolism of drugs, in Parke, D.V. and Smith, R.L. (Eds) *Drug Metabolism from Microbe to Man,* pp. 169–89, London: Taylor and Francis.

Renwick, A.G., 1985, The disposition of saccharin in animals and man—a review, *Food Chem. Toxicol,* **23,** 429–35.

Renwick, A.G., 1986, The metabolism of intense sweeteners, *Xenobiotica,* **16,** 1057–71.

Renwick, A.G., 1990, Acceptable daily intake and the regulation of intense sweeteners, *Food Addit. Contamin.,* **7,** 463–75.

Renwick, A.G. and Williams, R.T., 1972, The fate of cyclamate in man and other species, *Biochem. J.,* **129,** 869–79.

Renwick, A.G., Evans, S.P., Sweatman, T.W., Cumberland, J. and George, C.F., 1982, The role of the gut flora in the reduction of sulphinpyrazone in the rat, *Biochem. Pharmacol,* **31,** 2649–56.

Revankar, G.R., Hanna, N.B., Ramasamy, K., Larson, S.B., Smee, D.F., Finch, R.A., Avery, T.L. and Robins, R.K., 1990, Synthesis and *in vivo* antitumor and antiviral activities of 2'-deoxyri-bofuranosyl and arabinofuranosyl nucleosides of certain purine-6-sulfenamides, sulfinamides and sulfonamides, *J. Heterocyclic. Chem.,* **27,** 909–18.

Ripin, M.J., Noon, K.F. and Cook, T.M., 1971, Bacterial metabolism of arylsulfonates 1. Benzene sulfonate as growth substrate for *Pseudomonas testosteroni* H-8, *Appl. Microbiol.,* **21,** 495–99.

Rosalki, S.B., 1975, Gamma-glutamyl transpeptidase, *Adv. Clin. Chem.,* **17,** 53–107.

Ross, P.R. and Claiborne, A., 1991, Cloning, sequence and overexpression of NADH peroxidase from *Streptococcus faecalis* 10C1. Structural relationship with the flavoprotein disulfide reductases, *J. Mol. Biol.,* **221,** 857–71.

Roy, A.B., 1970, Enzymological aspects of steroid conjugation, in Bernstein, S. and Solomon, S. (Eds) *Chemical and Biological Aspects of Steroid Conjugation,* pp. 74–130, Berlin: Springer Verlag.

Rutgers, M., Heusdens, F.A., Bonthuis, F., De Herder, W.W., Hazenberg, M.P. and Visser, T.J., 1989, Enterohepatic circulation of triiodothyronine (T3) in rats: importance of the microflora for the liberation and reabsorption of T3 from biliary T3 conjugates, *Endocrinol.,* **125,** 2822–30.

Schwartz, H., Chu, I., Villeneuve, D.C. and Benoit, P.M., 1987, Metabolism, of 1, 2, 3, 4–, 1, 2, 3, 5– and 1, 2, 4, 5-tetrachlorobenzene in the squirrel monkey, *J. ToxicoL Environ. Health,* **22,** 341–50.

Smith, R.L., 1973, *The Excretory Function of Bile,* London: Chapman and Hall.

Smith, B.A., Springfield, J.R. and Gutmann, H.R., 1987, Solvolysis and metabolic degradation, by rat liver, of the ultimate carcinogen, N-sulfonoxy-2-acetylaminofluorene, *Mol. Pharmacol.,* **31,** 438–45.

Sonders, R.C., Netwal, J.C. and Wiegand, R.G., 1969, Site of conversion of cyclamate to cyclohexylamine, *Pharmacologist,* **11,** 241.

Souhaili, El Amri, H., Fargetton, X., Delatour, P. and Batt, A.M., 1987, Sulphoxidation of albendazole by the FAD-containing and cytochrome P-450 dependent mono-oxygenases from pig liver microsomes, *Xenobiotica,* **17,** 1159–68.

Sourkes, T.L. and Trano, Y., 1953, Reduction of methionine sulfoxides by *Escherichia coli, Arch. Biochem. Biophys.,* **42,** 321–26.

Stevens, J.L., 1985, Isolation and characterisation of a rat liver enzyme with both cysteine conjugate beta-lyase and kynureninase activity, *J. Biol Chem.,* **260,** 7945–50.

Strand, L.P. and Scheline, R.R., 1975, The metabolism of vanillin and Isovanillin in the rat, *Xenobiotica,* **5,** 49–63.

Strong, H.A., Angus, R., Gates, J., Sembi, J., Howarth, P., Renwick, A.G. and George, C.F., 1986, Effects of ischaemic heart disease, Crohn's disease and anti-microbial therapy of the pharmacokinetics of sulphinpyrazone, *Clin. Pharmacokinet.,* **11,** 402–11.

Strong, H.A., Gates, J., Sembi, J., Renwick, A.G. and George, C.F., 1984a, Role of the gut flora in the reduction of sulphinpyrazone in humans, *J. Pharmacol. Exp. Therap.* **230,** 726–32.

Strong, H.A., Renwick, A.G. and George, C.F., 1984b, The site of reduction of sulphinpyrazone in the rabbit, *Xenobiotica,* **14,** 815–26.

Strong, H.A., Renwick, A.G., George, C.F., Liu, Y.F. and Hill, M.J., 1987, The reduction of sulphinpyrazone and sulindac by intestinal bacteria, *Xenobiotica,* **17,** 685–96.

Strong, H.A., Warner, N.J., Renwick, A.G. and George, C.F., 1985, Sulindac metabolism: the importance of an intact colon, *Clin.Pharmacol. Therap.,* **38,** 387–93.

Sund, R.B., Hol, L. and Strobraten, A., 1979, Studies in the rat on the absorption, biliary excretion, laxative action and interference with intestinal transport of some oxyphenisatin derivatives, *Acta. Pharmacol. Toxicol.,* **44,** 251–59.

Tanaka, M., 1974, A histochemical study on the activity of gamma-glutamyl transpeptidase in liver disease, *Acta Path. Jap.,* **24,** 651–65.

Taylor, J.D., Richards, R.K. and Davin, J.C., 1951, Excretion and distribution of radioactive S^{35}-cyclamate sodium (sucaryl sodium) in animals, *Proc. Soc. Exp. Med.,* **78,** 530–33.

Taylor, A.J., Olavesen, A.H., Black, J.G. and Howes, D., 1978, The metabolism of the surfactants dodecylsulfonate and hexadecylsulfonate in the rat, *Toxicol. Appl. Pharmacol.,* **45,** 105–17.

Tesoriero, A.A. and Roxon, J.J., 1975, [^{35}S]Cyclamate metabolism: incorporation of ^{35}S into protein of intestinal bacteria *in vitro,* and production of volatile ^{35}S-containing compounds, *Xenobiotica,* **5,** 25–31.

Theeuwes, F., Wong, P.L., Burkoth, T.L. and Fox, D.A., 1993, Osmotic systems for colon-targeting drug delivery, in Bieck, P.R. (Ed.) *Colonic Drug Absorption and Metabolism,* pp. 137–58, New York: Marcel Dekker Inc.

Thysse, G.J.E. and Wanders, T.H., 1974, Initial steps in the degradation of n-alkane-1-sulphonates by *Pseudomonas, Antonie van Leeuwenhoek,* **40,** 25–37.

ToFigueras, J., GomezCatalan, J., Rodavnilans, M. and Corbella, J., 1992, Sulphur derivatives of hexachlorobenzene in human urine, *Hum. Exp. Toxicol.,* **11,** 271–73.

Tokeida, T., Niimura, T., Yamaha, T., Hasegawa, T. and Suzuki, T., 1979, Anaerobic deamination of cyclohexylamine by intestinal microorganisms in rabbits, *Agric. Biol. Chem.,* **43,** 25–32.

Tomisawa, H., Suziki, S., Ichihara, S., Fukazawa, H. and Tateishi, M., 1984, Purification and characterisation of C-S lyase from *Fusobacterium varium, J. Biol. Chem.,* **259,** 2588–93.

Tsai, H.H., Sunderland, D., Gibson, G.R., Hart, C.A. and Rhodes, J.M., 1992, A novel mucin sulphatase from human faeces: its identification, purification and characterisation, *Clin. Sci.,* **82,** 447–54.

Tsuchiya, T, 1981, Studies on the metabolism of sodium cyclamate by intestinal bacteria, *Memoirs Tokyo Univ. Agric.,* **23, 1**–55.

Turesky, R.J., Skipper, P.L., Tannenbaum, S.R., Coles, B. and Ketterer, B., 1986, Sulfamate formation is a major route for detoxification of 2-amino-3-methylimidazo[4, 5-f]quinoline in the rat, *Carcinogenesis,* **7,** 1483–86.

Turesky, R.J., Aeschbacher, H.U., Wurzner, H.P., Skipper, P.L. and Tannenbaum, S.R., 1988, Major routes of metabolism of the food-borne carcinogen 2-amino-3, 8-dimethylimidazo (4, 5-*f*)quinoxaline in the rat, *Carcinogenesis,* **9,** 1043–48.

Turesky, R.J., Stillwell, W.G.S., Skipper, P.L. and Tannenbaum, S.R., 1993, Metabolism of food-borne carcinogens 2-amino-3-methylimidazo(4, 5-*f*)quinoline and 2-amino-3, 8-dimethylimidazo(4, 5,-*f*) quinoxaline in the rat as a model for human biomonitoring, *Environ. Health Perspect.,* **99,** 123–28.

UriaNickelsen, M.R., Leadbetter, E.R. and Godchaux, W., 1983, Sulphonate utilisation by enteric bacteria, *J. Gen. Microbiol.,* **139,** 203–8.

Visscher, P.T. and Taylor, B.F., 1993a, A new mechanism for the aerobic catabolism of dimethyl sulfide, *Appl. Environ. Micobiol.,* **59,** 3784–89.

Visscher, P.T. and Taylor, B.F., 1993b, Aerobic and anaerobic degradation of a range of alkyl sulfides by a denitrifying marine bacterium, *Appl. Environ. Microbiol.,* **59,** 4083–89.

Walker, R., 1970, The metabolism of azo compounds: a review of the literature, *Food Cosmet. Toxicol.,* **8,** 659–76.

Watabe, T., Fujieda, T., Hiratsuka, A., Ishizuke, T, Hakamata, Y. and Ogura, K., 1985, The carcinogen, 7-hydroxymethyl-12-methylbenz[a]anthracene, is activated and covalently binds to DNA via a sulphate ester, *Biochem. Pharmacol,* **34,** 3002–5.

Warrander, A., Allen, J.M. and Andrews, R.S., 1985, Incorporation of radiolabelled amino acids into the sulphur-containing metabolites of paracetamol by the hamster, *Xenobiotica,* 15, 891–97.

Willetts, A.J. and Cain, R.B., 1972, Microbial metabolism of alkylbenzene sulphonates. Bacterial metabolism of undecylbenzene-p-sulphonate and dodecylbenzene-p-sulphonate, *Biochem. J.,* **129,** 389–402.

Williams, R.T., 1959, *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds,* pp. 497–500, London: Chapman and Hall.

Williams, J.R., Grantham, P.H., Marsh, H.H., Weisburger, J.H. and Weisburger, E.K., 1970, Participation of liver fractions and of intestinal bacteria in the metabolism of N-hydroxy-N-2-fluorenylacetamide in the rat, *Biochem. Pharmacol,* **19,** 173–88.

Wills, J.H., Serrone, D.M. and Coulston, F., 1981, A7-month study of ingestion of sodium cyclamate by human volunteers, *Regulatory Toxicol. Pharmacol.,* **1,** 163–76.

Yoshihara, S. and Tatsumi, K., 1985a, Sulfoxide reduction catalysed by guinea pig liver aldehyde oxidase in combination with one electron-reducing flavoenzyme, *J. Pharmacobio-Dyn.,* **8,** 996–1005.

Yoshihara, S. and Tatsumi, K., 1985b, Guinea pig liver aldehyde oxidase as a sulfoxide reductase: its purification and characterisation, *Archiv. Biochem. Biophys.,* **242,** 213–24.

Zinder, S.H. and Brock, T.D., 1978, Dimethylsulphoxide reduction by micro-organisms, *J. Gen. Microbiol.,* **105,** 335–42.

Ziegler, D.M., 1989, S-Oxygenases. I. Chemistry and biochemistry, in Damani, L.A. (Ed.) *Sulphur-Containing Drugs and Related Organic Compounds. Chemistry, Biochemistry and Toxicology,* Vol. 2, Part A, *Analytical, Biochemical and Toxicological Aspects of Sulphur Xenobiochemistry,* pp. 53–66, Chichester: Ellis Horwood.

Chapter 13
Sulphate-reducing bacteria

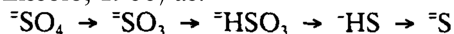
M.J.Hill, D.C.Ellwood and J.H.P.Watson

13.1
Introduction

Inorganic sulphur is ubiquitous in the environment and the element is essential for all living organisms. In consequence, in general ecology there is a sulphur cycle analogous to, and of equal importance to, the nitrogen cycle. This cycle involves both oxidation and reduction and has been reviewed by Cole and Ferguson (1988); both oxidation and reduction of sulphur compounds was described in a series of papers by Winogradsky (1887). Organisms involved in the sulphur cycle are involved in the removal of general organic detritus from the environment.

Sulphate-reduction, and sulphate-reducing bacteria (SRB) were first described in detail by Postgate (1949, 1959, 1966, 1984). The SRB form a diverse group of organisms defined solely on the basis of their ability to reduce sulphate to generate ATP and so fuel bacterial growth. Sulphate can be reduced to sulphide and then incorporated into amino acids by most bacteria in a process termed *assimilatory* sulphate reduction; this process is purely a biochemical pathway and is not energy-generating. In addition sulphate reduction can be used as an electron-acceptor and to generate energy for the oxidation of carbon substrates in general biosynthetic metabolism; this is termed *dissimilatory* metabolism and is the defining characteristic of the SRB.

Jorgensen (1977, 1982) has estimated that SRB can metabolize more than 50% of the organic detritus entering, for example, coastal marine sediments. The SRB are therefore clearly of major importance in the cleansing of water, soil and sand pollution, and in waste purification. They are also present in the gut of animals, including humans. The dissimilatory pathway is multistage and can be summarized (Peck and Lissolo, 1988) as:



$$^{=}SO_4 \rightarrow {}^{=}SO_3 \rightarrow {}^{=}HSO_3 \rightarrow {}^{-}HS \rightarrow {}^{=}S$$

The SRB act as a consortium of organisms to remove detritus from the environment; they are end-chain fermenters which use as their carbon sources the metabolic waste of other microbes, such as alcohols, short-chain fatty acids, phenols and phenolic acids produced from sources such as protein degradation, pyruvate, organic nitrogen compounds, and carbon dioxide, and which can oxidize hydrogen in the generation of energy. The human gut is a rich source of such end-chain products but there has been little work on the SRB in faeces (Postgate, 1984), most of the activity being in the fields of the microbiology of the external environment.

13.2
Isolation and identification of SRB

Working with environmental specimens, Postgate (1966) devised a series of media, particularly for the isolation of SRB able to utilize lactate as their carbon source. These were divided into spore-forming rods *(Desulfomaculum)* and non-sporing curved rods *(Desulfovibrio)*. In addition to lactate these organisms metabolize a range of fatty acids (such as pyruvate and malate) and alcohols (such as ethanol, propanol, butanol) but they do not metabolize acetate. Later Widdell (1980) described a range of other genera of SRB able to oxidize acetate and a range of higher acids. These genera include *Desulfobacter, Desulfobulbus, Desulfomonas, Desulfococcus andDesulfobacterium,* summarized in Table 13.1.

The genus *Desulphovibrio* contains thirteen species, all motile and non-sporing, two with rod-shaped and eleven with vibrio morphology, nine with a %G+C between 55 and 65%. The most usual bisulphate reductase in strains of the genus *Desulfovibrio* is desulfoviridin (present in eight of the thirteen species). *Dsv. thermophilus* is thermophilic and *Dsv. salexigens* has a requirement for salt. The genus *Desulfatomaculum* includes eight species, all motile and spore-forming; *Dsm. nigrificans* is thermophilic. Only one species of *Desulfomonas, Ds. pigra* has been described; it is a non-motile, non-sporing rod isolated from human faeces by Moore *et al.* (1976). The four species of *Desulfobacter* all have a strict requirement for salt. The two species of *Desulfobulbus* have many features in common with *Desulfovibrio* but are non-motile and do not produce desulfoviridin. Of the six species of *Desulfobacterium* two require salt and one metabolizes higher fatty acids.

The SRB are identified firstly on their ability to carry out dissimilatory reduction of sulphate, and on their Gram staining, morphology, oxidizable carbon sources, production of desulfoviridin and % G+C. More recently fluorescent antibody techniques have been applied to the SRB, as has the membrane fatty acid profile. The taxonomy of the SRB has been reviewed by Widdel (1980) and, as part of a general review, by Gibson *et al.* (1990).

13.3
Presence of SRB in human faeces

The presence of SRB in human faeces has only been studied recently and was not mentioned at all in standard early medical microbiology textbooks such as Wilson and Miles (1948). Moore *et al.* (1976) first reported the isolation of a strain of SRB that they identified as *Desulfomonas;* shortly after Beerens and Romond (1977) isolated strains of *Desulfovibrio* and *Desulfomonas*. Leclerc *et al.* (1979) studied 143 faecal samples for the presence of lactate-utilizing SRB; 83% of samples contained detectable SRB and 10% contained the organisms at levels of more than 10^9 per gram faeces.

More recently Gibson *et al.* (1988a) carried out a more detailed study of SRB in faeces from 20 British and 20 South African persons (Table 13.2). In both populations the dominant SRB were *Desulfovibrio,* but accompanied by smaller numbers of strains of other genera such as *Desulfobulbus, Desulfatomaculum, Desulfomonas, Desulfobacter* etc. The SRB were isolated from 70% of the British but only 35% of the South African persons; the organisms were able to utilize a wide range of carbon sources including the short-chain fatty acids (acetate, lactate, propionate, butyrate, succinate, pyruvate and valerate), ethanol, amino acids (serine, alanine, glutamic acid), hydrogen and carbon dioxide.

Table 13.1 The sulphate-reducing bacteria.

Organism	*Morphology*	*Spores*	*Mobility*	*Major electron donors*
Desulfovibrio	Vibrio	–	+	lactate pyruvate ethanol
Desulfatomaculum	rod	+	+	lactate pyruvate
Desulfomonas	rod	–	–	ethanol pyruvate
Desulfobulbus	ovoid	–	–	propionate lactate pyruvate
Desulfococcus	coccus	–	–	acetate lactate pyruvate
Desulfobacterium	ovoid	–	+	ethanol formate H_2
Desulfobacter	short rod	–	–	acetate

Many of these, particularly the hydrogen, are used by the methanogenic flora; it is known that the methanogens and the SRB are competitors in the environment as end-chain fermenters, and Gibson *et al.* (1988b) have evidence that the same is true in the human gut. It has been established that methane produced in the gut is in part absorbed and then excreted as breath methane, and that the latter is a reliable marker of the former (Wolin and Miller, 1983). Breath methane is detectable in significant amounts in 20–30% of British, North American and Scandinavian people and in more than 80% of black Africans (Haines *et al.*, 1977; Segal *et al.*, 1988). In their study Gibson *et al.* (1988a) only found significant numbers of SRB in those with no breath methane and vice versa.

These results have provided a considerable spur to the study of human faecal SRB, the factors controlling their presence, their function in relation to human colonic ecology and their possible role in human disease.

Gibson *et al.* (1988a) reported that, in those persons carrying SRB, the organisms tended to be present in large numbers at 10^7–10^{10} per gram in the British and at 10^9–10^{11} per gram in the South Africans. This was partly confirmed by Leclerc *et al.* (1979) who found that, in faeces from 143 European persons, 83% contained more than 10^2 and 10% more than 10^9 SRB per gram. This latter European population therefore had a smaller proportion with no SRB and a smaller proportion with high numbers of the organisms than the European population studied by Gibson and colleagues.

As in the general environment, the main factors controlling the growth of SRB in the human gut are the availability of sulphate and of oxidizable carbon sources. The gut is a rich source of short-chain fatty acids, particularly lactate, acetate, propionate and butyrate, and of hydrogen as a result of the fermentation of the large amounts of complex carbohydrate entering the colon (see Chapter 8), and so in practice the limiting factor is the amount of sulphate used as terminal electron-acceptor. However, Florin *et al.* (1991) showed that the dietary sources (mainly bread, brassica vegetables,

fermented beverages and dried fruits and vegetables) supplied up to 9 mmol/day, supplemented by sulphate from intestinal sulphomucin, small amounts of taurine from bile acid conjugates etc., and that faecal losses were negligible. This indicated that intestinal sulphate was either metabolized by SRB, or absorbed from the colon and excreted in the urine. Absorption, in their studies, reached a plateau at 5 mmol/day, leaving a similar amount for the growth of SRB when present.

Much of the dietary sulphate enters the food chain during food-processing; it is a normal additive to bread and to beers and other fermented beverages, and sulphite is used as a preservative in dried foods. Hence it is likely that the intake of sulphur in populations from the developing world will be smaller than that in western populations. Sulphate is also commonly taken as a dietary supplement in the form of iron sulphate for those who are diagnosed (or regard themselves) as iron-deficient. In particular, a high proportion of young women take iron sulphate during pregnancy and many during much of their premenopausal adult life.

The two principal terminal electron-acceptors in gut fermentation are sulphate reduction and methanogenesis, and these two routes are thought to be mutually exclusive (Gibson *et al.,* 1988a). In model studies using chemostat culture it was found that, in the presence of adequate amounts of sulphate, SRB are able to outcompete the methanogens and to displace them from faecal slurries (Gibson *et al.,* 1988b). This was confirmed *in vivo* by Christl *et al.,* (1990), who supplemented the diet of a cohort of persons with breath methane and showed that in half of the cohort previously undetectable SRB appeared in the faeces within a few days, and in those persons the breath methane level decreased. These results were interpreted as meaning that in western populations approximately 70% of persons carry only SRB, 15% carry only methanogens and the remainder carry both but have too little dietary sulphate to permit the outgrowth of the SRB in normal conditions. Christl and colleagues extended their study to include 127 persons (Christl *et al.,* 1993) of whom 43 (34%) had only methanogens, 68 (53%) had only SRB and 16 had methanogens with small numbers of SRB.

Florin (1993) has disputed that SRB have any effect on methanogenesis and his results have been supported by others (e.g. Strocchi *et al.,* 1991; Lemann *et al.,* 1990), all of whom have shown that the amount of breath methane was not decreased by dietary sulphate. Florin suggested that methanogenesis is controlled by factors other than competition for hydrogen; these include bile (Florin and Jabbar, 1992), and colonic mixing/stirring (Strocchi and Levitt, 1992).

In our own studies (described later) we have observed that all of 16 volunteers carried SRB detectable after dietary supplementation with sulphate; this contrasts with the observations of Christl *et al.* (1993) that about one-third of people carry no SRB and only methanogens. We did not measure breath methane and it is possible that, by chance, all 16 of our volunteers were non-producers of methane. However, it is difficult to see why active sulphate reductase should have any profound effect on methanogenesis or vice versa. Large amounts of hydrogen are produced in the colon and, in addition to being absorbed from the colon and excreted in breath it is also excreted from the colon in flatus. It is difficult to envisage such large amounts of hydrogen being so completely utilized by the SRB (particularly in view of the availability of large amounts of alternative oxidizable substrates) that growth of the methanogens would be restricted.

Table 13.2 Levels of SRB in faecal samples (data from Gibson *et al.,* 1988a and Leclerc *et al.,* 1979)

	Leclerc et al.	*Gibson* et al.	
	(France)	*(UK)*	*(S. Africa)*
Number of samples	143	20	20
Counts of SRB per gram			
$<10^2$	17%	30%	65%
10^2–10^5	30%	–	–
10^5–10^7	20%	5%	20%*
10^7–10^9	23%	30%	5%
10^9–10^{11}	10%	35%	10%

*Inconsistent results.

13.4
SRB and heavy metal pharmacology

It has already been noted that the growth of SRB is stimulated by dietary supplementation with sulphate. When this is in the form of ferrous sulphate the product is iron sulphide which coats the organisms, and *in vivo* this is manifest as a blackening of the stools. The black material could be removed from a faecal slurry by filtration and is clearly in particulate form; it released hydrogen sulphide on treatment with mineral acid, confirming its identity as inorganic sulphide, and was magnetic when submitted to the magnetic separation technique described by Ellwood *et al.* (1993). The material collected on the magnetic wire comprised, when studied by electron-microscopy, rod-shaped bacteria with an amorphous coating of iron sulphide.

Closer examination revealed that the amorphous coating of iron sulphide was in the form of fibrils secreted from specific points on the cell surface, and which became inter-twined to give a coating material with the appearance of spaghetti. It was extremely amorphous with a surface area of more than 150 m^2/g, which is comparable to that of activated charcoal. Further, the iron sulphide crystal lattice is very rich in defect structures and this, combined with the very high specific surface area, gives a surface which is highly absorbent for heavy metals of a size able to occupy the empty spaces in the defective lattice structure and to bind such metals with great avidity. In a model system this iron sulphide was able to decrease the mercury content of an industrial effluent by 400-fold (Ellwood *et al.,* 1993).

In the gut the normal concentration of iron sulphide associated with SRB is unlikely to be sufficient to have any effect on heavy metal pharmacology. However, in those people taking, for example, iron sulphate supplements the amounts of iron sulphide are likely to be significant and could affect the balance of the dynamic equilibrium of heavy metals across the gut wall. In our own pilot studies of two healthy volunteers fed labelled zinc, the subsequent rate of clearance of the radiolabel as measured by total-body counting was greatly increased during the period of ferrous sulphate supplementation (Bolt *et al.,* 1994).

Zinc was chosen as the transition metal that could be used safely in experiments on healthy volunteers; it is normally excreted principally through the renal route but the increased rate of clearance was presumably due to increased faecal excretion. If the technique can be optimized it could be of great clinical significance. For example, a technique to increase the rate of clearance of lead from the body could be of great value in the treatment of lead-poisoning. Similarly, methods to

clear the body load of heavy metal radionuclides from persons contaminated in nuclear accidents such as that in Chernobyl would be invaluable.

13.5
Role of SRB in disease

Although our studies have been of the possible beneficial effects of the colonic SRB, others have concentrated their attention more on the possible hazards resulting from the generation of sulphide ions in the gut. A group at the Dunn Nutrition Centre has been leading this line of research. They stress that methanogenesis and sulphate-reduction are mutually competitive, and that methanogenesis appears to be a safe route for the utilization of colonic hydrogen (Gibson *et al.,* 1988a, 1990,1993). In contrast, sulphide ions have been shown to be toxic to colonic cells in tissue culture studies (Florin *et al.,* 1991), to inhibit cytochrome oxidase activity (Smith *et al.,* 1977) and to inhibit colonocyte fat synthesis (Roediger and Nance, 1990).

On the basis of the above, Roediger and Nance (1990) have suggested that SRB are involved in the causation of ulcerative colitis. In apparent support of this view Gibson *et al.* (1993) showed higher SRB activity and higher sulphide concentrations in faeces from colitis patients than from controls. However, Florin (1993) has suggested that this latter observation may be an artefact, due to the increased bowel motility and increased bile acid loss. The data from epidemiology suggest that sulphate-rich foods such as bread and brassicas are associated with colonic health rather than disease.

In summary, although some ideas have been floated suggesting that SRB may be harmful, there is little evidence to support them. It is useful to remember that methanogenesis, currently regarded as the safe terminal electron-acceptor, was until recently regarded as being associated with colorectal carcinogenesis (Haines *et al.,* 1977).

References

Beerens, H. and Romond, C., 1977, Sulfate-reducing anaerobic bacteria in human *feces, Am. J. Clin.Nutr.,* **30,** 1770–76.

Bolt, L., Ellwood, D.C., Hill, M.J., Wootton, S. and Watson, J., 1994, The role of colonic sulphate-reducing bacteria in the pharmacology of heavy metals, *Eur. J. Cancer Prev.,* **3,** 357–60.

Christl, S.U., Gibson, G.R., Florin, T.H.J. and Cummings, J.H., 1990, Role of dietary sulfate in the regulation of methanogenesis in the human large intestine, *Gastroenterology,* **98,** A164.

Christl, S.U., Gibson, G.R. and Cummings, J.H., 1993, Methanogenesis in the human large intestine, *Gut,* **34,** 573.

Cole, J.A. and Ferguson, J., 1988, *The Nitrogen and Sulphur Cycles,* Cambridge: Cambridge University Press.

Ellwood, D.C., Hill, M.J. and Watson, J.H.P., 1993, Pollution control using microorganisms and magnetic separation, in Fry, J.C., Gadd, G.M., Herbert, R.A. *et al.* (Eds) *Microbial Control of Pollution,* pp. 89–112, Cambridge: Cambridge University Press.

Florin, T., 1993, Methanogenesis in the human large intestine, *Gut,* **34,** 573.

Florin, T., Neale, G., Gibson, G.R., Christl, S.U. and Cummings, J.H., 1991, Metabolism of dietary sulphate: absorption and excretion in humans, *Gut,* **32,** 766–73.

Florin, T. and Jabbar, I., 1992, Factors controlling human bowel metabolism: the effect of bile on methanogenesis, *J. Gastro. Hepatol.,* **7,** A21.

Gibson, G.R., Macfarlane, G.T. and Cummings, J.H., 1988a, Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut, *J. Appl. Bact.,* **65,** 103–11.

Gibson, G.R., Cummings, J.H. and Macfarlane, G.T., 1988b, Competition for hydrogen between sulphate-reducing bacteria and methanogenic bacteria from the human large intestine, *J. Appl. Bact.,* **65,** 241–47.

Gibson, G.R., Cummings, J.H., Macfarlane, G.T, Allison, C., Segal, I., Vorster, H.H., Walker, A.R.R., 1990, Alternative pathways for hydrogen disposal during fermentation in the human colon, *Gut,* **31,** 679–83.

Gibson, G.R., Macfarlane, G.T. and Cummings, J.H., 1993, Sulphate-reducing bacteria and hydrogen metabolism in the human large intestine, *Gut,* **34,** 437–39.

Haines, A., Metz, G., Diliwari, J., Blendis, L. and Wiggins, H., 1977, Breath methane in patients with cancer of the large bowel, *Lancet,* **ii,** 481–83.

Jorgensen B.B., 1977, The sulfur cycle of a coastal marine sediment, *Limnology and Oceanography,* **22,** 814–32.

Jorgensen B.B., 1982, Mineralization of organic matter in the sea bed—the role of sulphate reduction, *Nature,* **296,** 643–45.

Kieth, S.M., Herbert, R.A. and Harfoot, C.G., 1982, Isolation of new types of sulphate-reducing bacteria from estuarine and marine sediments using chemostat enrichments, *J. Appl. Bact.,* **53,** 29–33.

Leclerc, H., Oger, C., Beerens, H. and Mossel, D.A., 1979, Occurrence of sulphate-reducing bacteria in the human intestinal flora and in the aquatic environment, *Water Res.,* **14,** 253–56.

Lemann, F., Pochart, P., Pelier, P., Fiourie, B. and Ramband, J.C., 1990, Does sulfate availability in human colonic contents control methanogenesis in man? *Gastroenterology,* **98,** A251.

Moore, W.E.C., Johnson, J.L. and Holdeman, L.V., 1976, Emendation of *Bacteroidaceae* and *Butyrivibrio* and descriptions of *Desulfomonas* gen. nov. and ten new species of the genera *Desulfomonas, Butyrivibrio, Eubacterium, Clostridium* and *Ruminicoccus, Int. J. Syst. Bact.,* **26,** 238–52.

Peck, H.D. and Lissolo, T., 1988, Assimilatory and dissimilatory sulphate reduction, in Cole, J.A. and Ferguson, J. (Eds) *The Nitrogen and Sulphur Cycles,* Cambridge: Cambridge University Press.

Postgate, J., 1949, Competitive inhibition of sulphate reduction by selenate, *Nature,* **164,** 670–72.

Postgate, J., 1959, A diagnostic reaction of *Desulphovibrio desulphuricans, Nature,* **183,** 481–82.

Postgate, J., 1966, Media for sulphur bacteria, *Lab. Practice,* **15,** 1239–44.

Postgate, J., 1984, *The Sulphate-Reducing Bacteria,* Cambridge: Cambridge University Press.

Roediger, W.E.W. and Nance, S., 1990, Selective reduction of fatty acid oxidation in colonocytes: correlation with ulcerative colitis, *Lipids,* **25,** 646–52.

Segal, I., Walker, A.R.P., Lord, S. and Cummings, J.H., 1988, Breath methane and large bowel cancer risk in contrasting African populations, *Gut,* **29,** 608–13.

Smith, L., Kruszyna, H. and Smith, R.P., 1977, The effect of methaemoglobin on the inhibition of cytochrome c oxidise by cyanide, sulfide or azide, *Biochem. Pharmacol,* **22,** 47–50.

Strocchi, A., Furne, J., Ellis, J. and Levitt, M.D., 1991, Competition for hydrogen by faecal bacteria: evidence for the predominance of methane-producing bacteria, *Gut,* **32,** 1498–501.

Strocchi, A. and Levitt, M.D., 1992, Factors affecting hydrogen production and consumption by human fecal flora, *J. Clin. Invest.,* **89,** 1304–11.

Widell, F. 1980, Anaeroberabbau von Fettsauren und Benzoesaure durch neuisolierte Arten sulfat reduzierender Bacterien, Doctorial Dissertation, Gottingen University.

Wilson, G.S. and Miles, A.A., 1948, *Principles of Bacteriology and Immunity,* 4th Edition, London: Edward Arnold.

Winogradsky, S., 1887, Uber Schwefelbakterien, *Bot. Zeitung.,* **45,** 489–507, and subsequent papers in the same volume.

Wolin, M.J. and Miller, T.L., 1983, Carbohydrate fermentation, in Hentges, D. (Ed.) *Human Intestinal Microflora in Health and Disease,* pp. 147–65, London: Academic Press.

Section 6

Metabolism of miscellaneous compounds

Chapter 14
The interaction of the gut flora with metal compounds

I.R.Rowland

14.1
Introduction

In this review two aspects of the interaction of metals with intestinal bacteria will be considered: the influence of essential metals on the gut microflora and the metabolism of toxic metals by the microflora. In the case of essential metals the major consideration is the differential requirements of intestinal micro-organisms for metals. Dietary supplementation with certain metals could in theory lead to alterations in the microflora, perturbing the balance of the flora and potentially leading to a change in colonization resistance. This process can be exacerbated if a potential enteric pathogen is capable of binding the essential metal, thus stimulating its growth.

The metal which has been the subject of most intense study in this respect is iron.

14.2
Effect of iron on the intestinal microflora

Bacterial species can have very different requirements for iron. *Lactobacilli* and *Bifidobacteria* are among the few organisms that can survive in the absence of iron (Archibald, 1983). In contrast, bacteroides and potential pathogens such as *Escherichia coli* require iron for their multiplication, and some strains produce iron-chelating agents to facilitate their growth when free ionic iron concentration is low (Marcelis, 1980; Neilands, 1974; Weinberg, 1978; Rogers, 1973). Conversely, the ready availability of iron can enhance the growth and virulence of several microbial species (Bullen *et al.,* 1978; Griffiths, 1987).

The possibility exists therefore that high levels of dietary iron may stimulate growth of potential pathogens in the gut; this is of greatest concern in neonates and infants receiving cow's milk formulas, particularly since enteritis caused by *E. coli* is more frequent in infants fed cow's milk than in those consuming breast milk. Despite the low concentration of iron (0.7–1.5 mg/l) in human breast milk, it is present in a highly bioavailable form bound to lactoferrin. It has been suggested that the low ionic iron concentration in breast milk is one of the factors contributing to the low enterobacteria concentration in breast-fed infants (Bullen *et al., 1978*). The iron in cow's milk infant formulas is poorly available and so iron is added to provide levels of 5–7 mg/l (Balmer and Wharton, 1991). The possibility exists, therefore, that the addition of iron to these preparations may influence

colonization of the neonatal alimentary tract. The effect of supplemental iron on the development of the infant faecal microflora has been investigated with somewhat inconsistent results.

Mevissen-Verhage and colleagues in The Netherlands studied the colonization, over the first three months of life, of the gut of neonates fed cow's milk preparations with and without added iron. During the first week of life, neonates fed the iron-fortified milk (iron concentration 5 mg/l) exhibited more rapid colonization of the gut with *E. coli* than infants fed the milk preparation without added iron, in which the concentration was less than 0.5 mg/l (Mevissen-Verhage *et al.,* 1985a), although it was also reported that the isolation frequency of bifidobacteria was lower in the latter group. However, in a subsequent study of infant gut flora over the first three months of life, no significant differences in counts of *E. coli* between iron-supplemented and non-supplemented infants were seen, although the count in breast-fed infants was lower (Mevissen-Verhage *et al.,* 1985b). The most frequently isolated anaerobe group was bifidobacteria in the infants fed breast milk or non-supplemented cow's milk, whereas clostridia were the most frequently isolated anaerobes in the neonates fed iron-supplemented milk. The mean count of bacteroides was similar in all three dietary groups, but the isolation frequency was highest (77%) in the iron-supplemented group by comparison with the infants fed milk without iron (53%) or breast milk (38%).

Balmer and Wharton (1991) reported a study of infants up to 15 weeks old given breast milk or whey and casein-based formulas with (6.7 mg/l) or without (0.6 mg/l) iron. In contrast to the results of Mevissen-Verhage's group, the colonization frequency and mean count of bacteroides were lower in infants given iron-supplemented formulas. Staphylococci counts and colonization frequency were also lower in babies on the iron-supplemented milks. However, the addition of iron was associated with both higher mean counts and increased isolation frequency of enterococci and clostridia. For example, of the babies fed casein-based formula, 88% of those given iron were colonized with clostridia, in contrast to 45% of those fed the non-supplemented diet.

Overall, these studies suggest that not supplementing formula milks with iron gives a faecal flora closer to that of babies fed breast milk, although there are still major differences between the floras of infants on these two diets. It seems that the evidence, that consumption of iron-supplemented formula milks results in increased colonization of the infant gut with enterobacteria, is inconsistent, although several studies indicate that clostridial numbers are increased by dietary iron supplements. A recent study in rats fed an iron supplementation product, iron albumin succinylate, at levels up to 1500 mg/kg/day in the diet, reported no significant effects on faecal microflora (Rowland *et al.*, 1993).

In an attempt to assess the clinical significance of iron supplementation, Figueroa *et al.* (1990) performed a prospective study of Chilean infants (3–12 months old) living in poor sanitary conditions. The infants were fed either an iron-fortified milk formula (12 mg $FeSO_4$/l) or a non-supplemented formula. No diet-related differences in incidence of diarrhoea or in carriage of enteric pathogens were seen, suggesting that increased provision of iron confers no ecological advantages for pathogens in the gut.

14.3
Role of the gut flora in the metabolism of toxic metals

The 'heavy' metals, particularly mercury, lead, cadmium and the metalloid arsenic, exert potent toxic effects in many living organisms, including man, and their levels in food are regularly monitored. It is important to consider not only the total amount of the metal ingested but also its

chemical form, since the latter can have a profound influence on the rate of metal absorption, excretion and tissue distribution. This in turn can lead to differences in metal concentration at target sites and hence toxic effects.

In view of the importance of chemical form to the disposition and toxicity of metals, knowledge of the interconversion of the different forms of a metal in the body is clearly crucial for understanding the mechanisms of action of a metal and for evaluating its potential health effects. Several toxic metals undergo biotransformation in mammals including man. The mammalian transformation reactions relating to these toxic metals and metalloids (mercury, arsenic, selenium, lead and tin) have been reviewed recently by Mushak (1983). Of these metals, the role of the intestinal microflora in the biotransformation processes has been studied in detail only in the cases of mercury and arsenic compounds.

14.4
Arsenic

14.4.1
Chemical forms, exposure and toxic effects

Arsenic occurs in the environment and in food in various chemical forms, notably inorganic salts (arsenate and arsenite) and organo-arsenicals such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). The organic forms appear to predominate in fish and shellfish (Penrose, 1974) whereas in drinking water and in wine arsenic is present mainly as inorganic salts, the exact ratio of arsenate to arsenite being affected by the prevailing redox potential of the solution (Clement and Faust, 1973; Crecelius, 1977).

As with many metals, the toxicity of arsenic is dependent on its chemical form. A number of studies in laboratory animals, fish and *in vitro* cell systems have shown that arsenite (AsIII) is more toxic than arsenate (AsV) (Byron *et al.,* 1967; Nakamura and Sayato, 1981). The organoarsenicals MMA and DMA are in turn less toxic than both the inorganic salts.

14.4.2
In vitro metabolism of arsenicals by the gut flora

In incubations with suspensions of rat caecal contents under anaerobic conditions, sodium arsenate is rapidly reduced to arsenite and subsequently methylated first to MMA then to DMA (Rowland and Davies, 1981). The role of bacteria in these metabolic changes was demonstrated by showing that reduction and methylation were absent or markedly decreased in the presence of sterilized caecal contents or gut contents from germ-free rats. The rate of arsenate metabolism by gut contents was modified by a number of factors. For example, the addition of cholic, taurocholic, or deoxycholic acid increased the rate of reduction to arsenite by caecal contents without affecting its methylation. Arsenate was almost completely reduced to arsenite in the presence of the reducing agent hydrogen sulphide and furthermore the rate of arsenic methylation by caecal contents was also increased (Rowland and Davies, 1981). Other reducing agents, cysteine and thioglycollate, also reduced arsenate. It seems possible therefore that the gut flora generates reducing conditions in the intestinal tract which facilitate chemical reduction of arsenate. Methylation of arsenic by bacteria

from the environment is mediated by S-adenosyl methionine (McBride *et al.,* 1978) and involves reduction of arsenate to arsenite before methylation takes place. The pattern of the metabolism of arsenate by the gut microflora described above suggests that a similar pathway of reduction and sequential methylation operates in these organisms.

14.4.3 Reduction and methylation of arsenic *in vivo*

Arsenate is readily reduced to arsenite in biological systems, and methylation of both trivalent and pentavalent forms of inorganic arsenic occurs in humans and experimental animals (Vahter, 1983; Mushak, 1983). In humans not exposed to high levels of arsenic, the metabolites in urine comprise about 60% DMA, 20% MMA and 20% inorganic arsenic (Crecelius, 1977; Smith *et al.,* 1977; Tam *et al.,* 1979; Buchet *et al.,* 1981). In other animal species, however, DMA is the major metabolite found in urine and most tissues (Lakso and Peoples, 1975; Vahter, 1983; Odanaka *et al.,* 1980; Rowland and Davies, 1982). Since the organoarsenicals are much less toxic than arsenite, biomethylation can be considered a detoxification reaction.

Despite the ability of gut bacteria to reduce and methylate sodium arsenate *in vitro,* the prevailing evidence from studies in laboratory animals and man suggests that the gut microflora does not play a major role in arsenic metabolism in the intact animal.

Both arsenate and arsenite are rapidly and virtually completely absorbed from the mammalian gut, thus limiting the opportunity for interaction between the large bowel microflora and ingested inorganic arsenicals (see review by Vahter, 1983).

Studies *in vivo* have shown that reduction and methylation of inorganic arsenic in the rat is extremely rapid (Rowland and Davies, 1982) and it seems unlikely that the rates of methylation measured in gut bacteria *in vitro* could account for such a rapid appearance of MMA and DMA in tissues *in vivo*. Furthermore, the rate of arsenate metabolism after intravenous injection was almost identical to that after administration of the arsenical directly into the intestine, indicating that reduction and methylation of inorganic arsenic *in vivo* was independent of the gut microflora (Rowland and Davies, 1982). Confirmatory evidence for the relative unimportance of the gut flora in arsenic metabolism in animals comes from a study in germ-free and conventional mice (Vahter and Gustafsson, 1980) in which orally administered arsenate was methylated to the same extent in both groups.

The most likely site of methylation of inorganic arsenic in mammals appears to be the liver, since Shirachi *et al.* (1981) have reported the conversion of arsenate to DMA by rat liver preparations *in vitro,* while Lerman and Clarkson (1983) have demonstrated methylation of arsenite by isolated rat hepatocytes.

14.4.4 Microbial metabolism of other arsenicals

A number of organic arsenicals based on arsanilic acid have been used as growth-promoting agents and for disease control in farm animals. When incubated anaerobically with hen faeces, 4-nitrophenylarsonic acid was converted to arsanilic acid (the nitroreduction product), 4-nitrophenylarsenoxide and 4-aminophenylarsenoxide (Moody and Williams, 1964). It was suggested that the growth-promoting effects of these organoarsenicals is due to their reduction in the gut to

arsenoxide derivatives which are more toxic to parasites. It should be noted however that *in vivo* metabolic studies have shown that, in hens, only the nitroreduction products of nitrophenylarsonic acid can be detected in faeces, and arsanilic acid itself appears metabolically stable (Moody and Williams, 1964; Overby and Fredrickson, 1963).

14.5
Mercury

14.5.1
Chemical forms, exposure and toxic effects

Mercury exists in a variety of chemical forms: the elemental (metallic) state, as inorganic salts in the monovalent (mercurous) and divalent (mercuric) forms, and as organic compounds, most importantly short-chain alkyl-, alkoxyalkyl- and phenyl-mercury compounds. The gut microflora does not appear to be involved in metabolism of elemental mercury, which if ingested, is eliminated rapidly in the faeces. Alkoxy- and phenyl-mercury compounds have been used as antifungal dressings for seeds. Although they are known to be unstable in the body, decomposing to mercuric mercury, the role of the gut flora in this reaction has not been elucidated. There is, however, evidence for gut bacterial metabolism of mercuric salts and methylmercury compounds.

Mercuric salts, which are toxicologically more important than mercurous compounds, are widely distributed in the environment and enter the body via food and beverages. The daily intake by this route is, however, low, being less than 10 μg/day (Berlin, 1979). Mercuric salts are poorly absorbed from the gut and their distribution is confined mainly to the kidneys, which are the main site of toxic action on chronic exposure.

The high toxicity of methylmercury (MeHg) is due to a combination of its efficient absorption from the gut (over 90% of an oral dose is absorbed), long retention-time in the body and its ability to penetrate the blood-brain barrier and accumulate in the lipidrich neuropile of the central nervous system where it exerts its major toxic effects (Berlin, 1979). The main route of exposure of the general population to MeHg is via food since it is accumulated in food chains, particularly those in aquatic environments, leading to high levels in predatory fish such as tuna. Moderate consumption of relatively uncontaminated fish is estimated to result in a daily intake of 1–20 μg/day (Berlin, 1979). In populations whose diets consist mainly of fish, intakes of MeHg can reach 300 μg per day and may attain toxic levels in communities which consume large quantities of fish caught in badly polluted areas (Berlin, 1979). The disastrous outbreaks of mercury poisoning in the populations of Minimata and Niigata in Japan in the 1950s and 1960s (reviewed by Takeuchi, 1972) resulted from this route of high-level exposure. Other notable cases of organomercurial poisoning occurred when people consumed grain intended for sowing and treated with MeHg as an antifungal agent.

14.5.2
Metabolism of mercury compounds by gut bacteria

Methylation of mercuric chloride—in vitro *studies*

Evidence that inorganic mercury salts could undergo bacterially catalyzed methylation was found for anaerobic sediments of lakes and rivers (Jenson and Jernelov, 1969; Matsumura *et al.,* 1972). Such biological methylation was thought to be responsible, at least in part, for the high levels of MeHg in the mercury contaminated waters of Minimata Bay which led to the outbreak of mercury poisoning in that area (see above).

The ability of bacteria in anaerobic sediments to catalyze methylation suggests that a similar reaction might occur in the anaerobic milieu of the mammalian intestine and indeed this has been found to be the case. Incubation of suspensions of human faeces or rat caecal contents with mercuric chloride under anaerobic conditions results in the production of small quantities (5–15 ng/g faeces or gut contents) of MeHg (Edwards and McBride, 1975; Rowland *et al.,* 1977a). MeHg formation was virtually abolished by sterilization of the gut contents (Rowland *et al.,* 1977a) indicating that bacteria participated in the reaction. Microbial involvement was further confirmed by studies of MeHg synthesis by pure cultures of intestinal bacteria and yeast derived from rats and humans (Rowland *et al.,* 1975; Rowland *et al.,* 1977a). The highest levels of methylating activity were seen among streptococci, *E. coli,* yeasts and staphylococci. Only a small proportion of the obligate anaerobes and lactobacilli formed MeHg.

The methylation reaction is thought to require the presence of a carbanion, i.e. CH] (Bertilsson and Neujahr, 1971; Ridley *et al.,* 1977). Methylvitamin B_{12} (methylcobalamine), which is synthesized by several bacterial species, is the only biological methylating agent known to have the capacity to transfer carbanions and does so *in vitro* to mercury, lead, tin, platinum, palladium and gold (Bertilsson and Neujahr, 1971; Wood, 1974; Agnes *et al.,* 1971). However, methylation of inorganic mercury also occurs in organisms which are unable to synthesize methylcobalamine (Vonk and Sijpesteijn, 1973) and so other mechanisms for methylation must operate in these cells. Lander (1971) has suggested that methylation of mercury by bacteria derived from sediments may involve one or more steps in the pathway of methionine biosynthesis, such that the methyl group is transferred to a mercuric ion complexed to homocysteine.

Mercuric mercury methylation in vivo

The bacterial methylation of mercuric mercury converts the metal from a form poorly absorbed from the gut to one which is virtually completely absorbed and so, potentially, should increase the uptake of mercury from an oral dose of mercuric mercury. Evidence that this occurs, however, is very limited and has been obtained only from experiments conducted in animals with abnormal gastro-intestinal conditions. Abdulla *et al.* (1973) measured the mercury content (total and methylmercury) of various tissues after giving 1 mg mercuric chloride per day, by gastric intubation for three weeks, to rats with jejunal blind loops and to non-surgically-treated control animals. Retroperistaltic intestinal blind loops are known to harbour a more abundant microflora than the normal intestine. In the rats with blind loops, total mercury and MeHg content of all tissues taken, including the brain, were much higher than in control animals. The rats with blind loops also developed symptoms of neurological damage. It seems unlikely, however, that these were induced by MeHg, since brain

levels of mercury reported in this study (0.17 μg Hg/g) were considerably below those needed to be achieved (5–10 μg Hg/g) before overt neurotoxicity becomes apparent (Syverson, 1982). Conditions which permit a microbial population to develop in the upper gastrointestinal tract are not uncommon in man, e.g. achlorhydria, partial gastrectomy, Crohn's disease (Drasar and Hill, 1974), and whilst these may increase the potential for bacterial methylation, the amounts of mercuric mercury ingested are normally too small for the reaction to be of any toxicological consequence.

Methylmercury metabolism in vitro

In the studies on methylation of mercuric salts by gut bacteria *in vitro* described above (Edwards and McBride, 1975; Rowland *et al.,* 1977a) MeHg formation reached a peak and then declined if incubation was continued, suggesting that the organomercurial was further metabolized. This was confirmed by incubating ^{203}Hg-labelled methylmercuric chloride with gut contents from the rat or mouse, or with suspensions of faeces from humans, which resulted in extensive metabolism of the organomercurial over a period of 12 days (Rowland *et al.,* 1978; 1983).

A range of metabolites appears to be produced under these conditions depending on the source of the gut contents. When ^{203}Hg-labelled MeHg is incubated with small intestinal or caecal contents from Wistar rats, 50–70% of the radioactivity is lost from the incubation mixture, indicating that volatile metabolites are produced (Rowland *et al.,* 1978). Differences in the rates of volatilization of MeHg labelled with ^{203}Hg and ^{14}C by rat caecal contents suggested that the carbon-mercury bond was cleaved and that at least one of the volatile products was elemental mercury (Rowland *et al.,* 1978). Although mercury-resistant enteric bacteria can effect such a reaction (see below), it is unlikely that these organisms are responsible for MeHg volatilization in gut contents, since the time-course of the reaction (12 days, Rowland *et al.,* 1978) is much slower than that for volatilization by mercury-resistant bacteria (Schottel *et al.,* 1974; Summers and Silver, 1972). Incubation of MeHg with pure cultures of organisms isolated from the rat or human gut revealed that demethylating activity was present in 50% of the lactobacilli and obligate anaerobes tested (Rowland *et al.,* 1978).

In addition to the formation of metallic mercury, there is also evidence that MeHg reacts with hydrogen sulphide, produced by intestinal bacteria, leading to volatilization by the formation of bis-methylmercuric sulphide $(CH_3Hg)_2S$, which decomposes to mercuric sulphide and dimethylmercury (Rowland *et al.,* 1977b; 1978; Craig and Bartlett, 1978).

Volatilization of MeHg to metallic Hg also occurs in the presence of gut contents from mice and faecal suspensions from humans. However, in contrast to incubations of MeHg with suspensions of rat gut contents in which no mercuric mercury was detected (Rowland *et al.,* 1978), both human faeces and mouse caecal suspensions produce substantial amounts of the mercuric ion from MeHgCl (Rowland *et al.,* 1983). Furthermore, with caecal contents from mice, the rate of demethylation of methylmercury glutathione (the main form of the organomercurial in bile) is similar to that of methylmercuric chloride (Rowland *et al.,* 1983).

The conversion of MeHg to mercuric mercury by the human and mouse gut floras alters with age. Caecal contents from adult (3-month-old) mice demethylated MeHg rapidly, but in 10-day-old animals the reaction was very slow. The rate of demethylation by the caecal microflora of 20-day-old mice was similar to that in adult animals (Rowland *et al.,* 1983; Table 14.1) indicating that the underlying metabolic change occurred in mice during weaning (15–18 days).

If mice are not weaned on to a solid diet, but instead are maintained on milk, there is little if any change in the rate of MeHg demethylation, which remains at the slow rate characteristic of the

Table 14.1 Effect of age and diet on MeHg demethylation by mouse caecal and human faecal suspensions

Species	*Age*	*Diet*	*% MeHg demethylated*
Mouse	10 days	Milk	94
	20 days	Stock	50
	3 months	Stock	46
Man	2 days	Milk	97
	4.5 months	Milk	90
	10 months	Milk	88
	8 months	Solid mixed diet	29
	4.5 years	Solid mixed diet	18

The faecal suspensions were incubated with MeHg for 24 hours at 37°C, and % MeHg remaining determined by benzene extraction. (After Rowland *et al.,* 1983.)

unweaned mouse (Rowland *et al.,* 1983). These metabolic changes during the weaning period in mice coincide with a major alteration in the bacterial composition of the gut microflora with lactobacilli decreasing and obligate anaerobes such as bacteroides increasing (Schaedler, 1973).

It was shown that corresponding developmental changes in demthylating capacity occur in the human gut microflora, since weaned and unweaned children of similar ages exhibit markedly different faecal demethylation capacities (Table 14.1).

Methylmercury metabolism in vivo

The rapid and virtually complete absorption of MeHg from the gut makes it unusual among toxic metal compounds (Miettinen, 1973; Walsh, 1982). The cumulative body-burden of mercury after methylmercury exposure is determined not only by the quantity taken in, but also, critically, by its rate of elimination. The main route of excretion is via the faeces in man and laboratory animals (Clarkson, 1979). In rats and mice given MeHg, the majority (50–90%) of the mercury in faeces is in the mercuric form (Norseth and Clarkson, 1970; Norseth, 1971a; Landry *et al.,* 1979; Clarkson, 1979; Rowland *et al.,* 1980; 1983). Therefore demethylation of methylmercury would appear to be a rate-determining step in the excretion of the organomercurial from the body. In mice given a single oral dose of MeHg, about 10% of the body-burden of mercury at six days was present in the mercuric form (Rowland *et al.,* 1984) although the proportion was not identical in all tissues. In brain and blood, about 5% of the mercury content was in the mercuric form whereas in liver and kidney the mercuric mercury content was much higher (21–24%). These results largely confirm those of an earlier study (Norseth, 1971a). In rats, the distribution of inorganic mercury after MeHg exposure appears to be similar to that seen in mice (Norseth and Clarkson, 1970; Magos and Butler, 1976; Hargreaves *et al.,* 1985). *In vitro* experiments have identified a number of potential sites for MeHg demethylation (Lefevre and Daniel, 1973; Ishihara and Suzuki, 1976). However, comparisons between germ-free and conventional flora animals (described below) indicate a major role for the gut microflora in the demethylation of methylmercury *in vivo,* a suggestion first made by Norseth and Clarkson (1971a).

MeHg gains access to the gut flora via its secretion in bile in adult rats; a small proportion (up to 3% in two hours) of an absorbed dose of MeHg is excreted in the bile. The majority (60–80%) of

this mercury in bile is found as a methylmercury-glutathione complex while only 4–5% of mercury in bile is inorganic (Norseth and Clarkson, 1971; Ohsawa and Magos, 1974; Norseth, 1973; Refsvik and Norseth, 1975; Klaasen, 1976). The methylmercury-glutathione complex and its derivatives formed by the action of pancreatic enzymes on the conjugate (Hirata and Takahashi, 1981) are largely reabsorbed, leading to an enterohepatic circulation of mercury (Norseth, 1973). Conversion of MeHg in bile to the poorly-absorbed, mercuric form would interrupt this enterohepatic circulation, resulting in increased mercury excretion in faeces. The high proportion of mercuric mercury in faeces of animals given MeHg and the low proportion of the inorganic form in bile lend support to the theory that demethylation occurs in the gut. However, direct evidence for the hypothesis comes from studies in germ-free rodents and animals treated with antibiotics to suppress their gut bacteria. Four such studies which reported mercury excretion data showed that the suppression or absence of the gut microflora was associated with decreased excretion of total mercury in faeces by comparison with control animals with an intact microflora (Nakamura *et al.,* 1977; Rowland *et al.,* 1980, 1984; Seko *et al.,* 1981). Furthermore, the amount of mercuric mercury in faeces or colon contents was markedly lower in the germ-free or antibiotic-treated animals. One study (Norseth, 1971b) was at variance with the rest in finding no differences in mercury level in blood or in mercuric mercury in faeces between germ-free and conventional rats, but this may be a consequence of administering MeHg by the subcutaneous rather than the oral route. The balance study of Rowland *et al.* (1984) demonstrated that the decreased faecal excretion of mercury in mice treated with antibiotics was reflected in significantly higher body-burdens of mercury. The half-time of mercury elimination was increased from 10 days in the conventional flora mice to more than 100 days in the antibiotic-treated animals. Furthermore, in the animals given antibiotics, the proportion of the total mercury body-burden present as mercuric mercury was smaller than in the mice with intact gut microfloras. In general, suppression of the gut microflora in mice results in higher total mercury levels in most tissues (Table 14.2), and also higher proportions of MeHg in those tissues. It is the mercury concentration in the central nervous system which is of particular importance since this is the target site for toxicity of MeHg. The concentration of mercury in the brain after MeHg exposure was found to be 25–45% greater in germfree or antibiotic-treated animals than in controls (Table 14.2). The consequences of this altered level of Hg in the central nervous system were elucidated by Rowland *et al.* (1980), who investigated the effect of eliminating the microflora on the neurotoxicity of MeHg. MeHg-induced behavioural signs of neurotoxicity and the severity of the histopathological lesions in the cerebellum were much greater in antibiotic-treated rats than in their conventional flora counterparts.

It should be noted that in animals lacking a gut flora, mercuric mercury is still found in faeces and in tissues, although the proportion of the total mercury in the inorganic form is usually lower. This implies that there are other (non-bacterial) sites of demethylation in the body. However, the observation that antibiotic treatment of mice leads to almost complete retention of a dose of MeHg (half-time of mercury elimination greater than 100 days; Rowland *et al.,* 1984) would suggest that non-bacterial demethylation does not play a significant role in determining the body-burden of mercury after MeHg-exposure.

Table 14.2 Effect of germ-free status or antibiotic treatment on mercury concentration in tissues of rodents given MeHgCI

	Hg concentration in tissue (AB or GF as % of CV)				
Species	*Blood*	*Brain*	*Liver*	*Kidney*	*Reference*
Rat	135	127	95	142	Rowland *et al.*, 1980
Mouse	ND	145	160	186	Nakamura *et al.*, 1977
Mouse	210	125	165	102	Rowland *et al.*, 1984

CV Conventional flora
GF Germ-free
AB Antibiotic treated
ND Not determined

14.5.3
Diet-gut microflora interactions in methylmercury metabolism

Landry *et al.* (1979) reported differences in MeHg retention and tissue concentration of mercury in mice fed different diets. In comparison to a stock laboratory chow diet, a liquid synthetic diet decreased mercury retention after an oral dose of MeHg and a milk diet appeared to increase it. Rowland *et al.* (1984) extended this work and provided evidence that these diet-related differences in mercury excretion are a consequence of diet-induced changes in demethylation of MeHg by the intestinal microflora. It was found that six days after mice were given a single oral dose of MeHg, the percentage of the total mercury body-burden present in the mercuric form was highest (35.5%) in the animals fed the synthetic diet (which exhibited the highest rate of mercury excretion). Mercuric mercury made up 10.4% of the mercury body-burden in mice fed the stock diet and only 6.6% in the milk-fed animals, which had the lowest rate of mercury excretion. Treatment of the mice with antibiotics decreased both the rate of mercury excretion and the proportion of mercuric mercury found, and at the same time abolished the dietary differences in mercury excretion, demonstrating that the diets per se were not directly affecting MeHg metabolism.

The feeding of dietary fibre has also been demonstrated to influence body-burden of mercury after MeHg exposure of mice (Rowland *et al.*, 1986). Wheat bran (15% or 30% in the diet) increased mercury excretion and decreased total mercury concentration in the brain and blood. The proportion of mercury present in the inorganic form in the tissues was higher in the bran-fed animals than in those fed the fibre-free diet (Table 14.3). The differences in proportions of mercuric mercury in tissues of the control and bran-fed mice strongly suggests that bran exerts its effects on mercury retention via modification of the metabolic activity of the gut microflora, rather than simply by binding MeHg or reducing transit time.

14.5.4
Implications of bacterial demethylation of MeHg

Dramatic differences have been found in the rate of excretion of MeHg between neonatal and weaned mice. Suckling mice absorb and retain the majority of an oral dose of MeHg (half-time of mercury elimination, T½, greater than 100 days (Doherty and Gates, 1973) whereas older mice excrete the mercurial much more rapidly (T½ 6–10 days)). This developmental change in rate of

Table 14.3 Effect of dietary wheat bran on mercury concentration in tissues of mice given MeHgCl

Tissue	*μg mercury/g tissue (% mercuric)*		
	Control (fibre-free)	*15% bran*	*30% bran*
Blood	9.2 (2.1)	8.4 (2.5)	7.3** (2.2)
Liver	14.0 (5.8)	13.5 (8.3***)	12.6 (9.2***)
Kidney	77.1 (9.7)	86.2 (11.0*)	82.4 (11.4*)
Brain	4.9 (3.8)	4.4 (4.1)	3.8*** (4.6**)
Colon (wall & contents)	5.4 (25.60)	5.2 (49.8***)	4.9 (53.6***)

Mice were given a single oral dose of Me^{203} HgCl. Total Hg content of tissues was determined by gamma-counting, and proportion of mercuric mercury assessed by benzene extraction. Values shown are means of at least 9 mice with values for percentage mercuric mercury (means of 4 mice) shown in parentheses.
Significant differences from controls shown by asterisks- *$P<0.05$; **$P<0.01$; ***$P<0.001$. After Rowland *et al.* (1986).

excretion occurs at 16–18 days after birth, coinciding with the time of weaning to a pelleted rodent diet, and has been linked to a number of possible mechanisms (reviewed by Rowland *et al.,* 1983). The most likely of these are a change in the rate of biliary secretion of MeHg into the gut at weaning (Ballatori and Clarkson, 1982) and an increase in the demethylation activity of the gut microflora. The latter has been demonstrated *in vitro* (see above) and *in vivo* where it has been shown that the mercuric mercury excretion is much greater in 20-day-old mice than in 4- or 10-day-old animals (Rowland *et al.,* 1983). Since faecal suspensions from unweaned human babies also exhibit little ability to demethylate MeHg (Table 14.1), it seems likely that the latter would also absorb and retain more of an oral dose of MeHg than adults. Thus the lack of bacterial demethylating activity in the gut may make babies and unweaned infants more susceptible to MeHg neurotoxicity than adults.

It is also tempting to speculate that the wide range of mercury elimination rates seen in humans (Shahristani and Shibab, 1974) may be related to the variations in the composition of the gut flora between individuals. In addition, if the major differences in gut flora that have been reported in populations in different parts of the world (Drasar and Hill, 1974) are reflected in their MeHg demethylation rates, it is conceivable that there are inter-regional as well as inter-individual differences in susceptibility to MeHg poisoning.

Mercury metabolism by mercury-resistant enteric bacteria

Resistance to the toxic effects of inorganic and organic mercury compounds has been found in a wide range of enteric organisms including *Escherichia, Proteus, Klebsiella, Staphylococcus* and *Pseudomonas* (Schottel *et al.,* 1974). Mercury-resistant (Hg^{r}) strains of bacteria are often resistant to other heavy metals, e.g. arsenic, lead and cadmium, and also to antibiotics. In both cases the resistance is determined by plasmids (Novick and Roth, 1968; Summers and Silver, 1972).

All Hg^{r} plasmids confer resistance to mercuric mercury, but plasmid-mediated resistance to other mercury compounds, namely phenylmercuric acetate (PMA), p-dihydroxymercuribenzoate, MeHg and ethylmercury also occurs (Robinson and Tuovinen, 1984). Resistance to alkylmercurials, however, has not been reported in enterobacteria or staphylococci.

The mechanism of resistance to the mercury compounds appears to be via their biotransformation to elemental mercury which, being volatile, is lost from solution. The kinetics and enzymology of these reactions have been reviewed in detail by Robinson and Tuovinen (1984) and so will not be discussed in the present paper. However, it is relevant to consider the possible biochemical and lexicological consequences of the presence of Hg^r bacteria in the gut microflora.

The increased frequency of occurrence of Hg^r bacteria in clinical specimens appears to coincide with the increased frequency of isolation of antibiotic-resistant organisms. It seems possible therefore that exposure of individuals to antibiotics may select for Hg^r bacteria as well as those resistant to antibiotics. However, the converse, namely that exposure to mercury compounds selects for Hg^r and antibiotic-resistant bacteria, does not seem to occur. A study in Iraq (Groves *et al.*, 1975) revealed a similar incidence of Hg^r *staphylococci* in people whether or not they were exposed to methylmercury-coated grain. Oral administration of mercuric chloride (2 mg/day) to rats did not increase the numbers or proportion of Hg^r *E. coli* in faeces (Rowland and Davies, unpublished observations, 1981). When rats were given, by gavage, large numbers of an *E. coli* strain carrying a plasmid (pUB932), carrying resistance to Hg and tetracycline, the numbers of Hg^r *E. coli* recovered in faeces increased during the dosing period, but declined rapidly when dosing stopped. Oral administration of 2 mg mercuric chloride per day did not prevent the decline, although when the animals were given tetracycline in drinking water, Hg^r *E. coli* persisted in the faeces (Davies *et al.*, 1982). It would appear therefore that exposure of animals to mercuric salts does not increase the carriage of Hg^r plasmids by members of the host microbial flora. There is also evidence that the presence of a large population of Hg^r bacteria in the gut does not increase the volatilization of mercuric mercury by the flora. Incubation of $^{203}HgCl_2$ with faecal suspensions from rats dosed orally with Hg^r *E. coli* (at levels which increased the population of Hg^r *E. coli* in faeces) resulted in low rates of volatilization of ^{203}Hg, which were similar to those seen in the presence of faeces from untreated rats (Rowland and Davies, unpublished observation, 1981). Furthermore, in the presence of a suspension of rat faeces, the ability of a pure culture of an Hg^1 *E. coli* to volatilize Hg from solution was inhibited (Rowland and Davies, unpublished observation, 1981). It seems likely that faecal components bind mercuric mercury, making it unavailable for metabolism by Hg^r bacteria.

14.6
Conclusions

In vitro studies have shown that the intestinal microflora can biotransform a number of metal compounds leading, in some cases, to compounds exhibiting greater toxicity (e.g. methylation of mercuric salts) and in others to less toxic derivatives (e.g. demethylation of MeHg, or methylation of inorganic arsenic). In the case of MeHg demethylation there is evidence that the microbial metabolism is of considerable lexicological importance to the host animal.

Other metals, including selenium and lead, have inorganic and organic salts with different toxicity and are subject to biotransformation in mammals (see review by Mushak, 1983) and furthermore, are known to undergo microbial metabolism in the environment (Wood *et al.*, 1978). The possibility of intestinal microbial biotransformation of metals in addition to mercury and arsenic should perhaps be investigated.

References

Abdulla, M., Arniesjo, B. and Ihse, I., 1973, Methylation of inorganic mercury in experimental jejunal blind-loop, *Scand. J. Gastroent.,* **8,** 565–67.

Agnes, G., Bendle, S., Hill, H.A.O., Williams, F.R. and Williams, R.J.P., 1971, Methylation by methyl vitamin B12, *Chemical Communications,* **15,** 850–51.

Archibald, F.S., 1983, *Lactobacillus plantorum,* an organism not requiring iron, *FEMS Microbiol. Lett.,* **19,** 29–32.

Ballatori, N. and Clarkson, T.W., 1982, Developmental changes in the biliary excretion of methylmercury and glutathione, *Science,* **261,** 61–63.

Balmer, S.E. and Wharton, B.A., 1991, Diet and faecal flora in the newborn: *iron,Arch. Dis. Child.,* **66,** 1390–94.

Berlin, M., 1979, Mercury, in Friburg, L., Nordbery, G.F and Vouk, V.B. (Eds) *Handbook on Toxicology of Metals,* pp. 503–30, Amsterdam: Elsevier.

Bertilsson, L. and Neujahr, H.J., 1971, Methylation of mercury compounds by methylcobalamin, *Biochemistry,* **10,** 2805–88.

Buchet, J.P., Lauwerys, R. and Roels, H., 1981, Comparison of the urinary excretion of arsenic metabolites after a single oral dose of sodium arsenite, monomethyl-arsonate, or dimethylarsenite in man, *Int. Arch. Occup. Environ. Health,* **48,** 71–79.

Bullen, J.J., Rogers, H.J. and Griffiths, E., 1978, Role of iron in bacterial infection, *Curr. Topics Microbiol. Immunol.,* **80,** 1–35.

Byron, W.R., Bierbower, G.W., Brouwer, J.B. and Hanson, W.H., 1967, Pathologic changes in rats and dogs from two-year feeding sodium arsenite and sodium arsenate, *Toxicol. Appl. Pharmacol.,* **10,** 132–47.

Clarkson, T.W., 1979, Effect—general principles underlying the toxic action of metals, in Friburg, L. (Ed.) *Handbook on the Toxicology of Metals,* pp. 99–117, Amsterdam: Elsevier.

Clement, W.H. and Faust, S.D., 1973, A new convenient method for determining arsenic (+3) in natural waters, *Environ. Lett.,* **5,** 155.

Craig, P.J. and Bartlett, P.D., 1978, The role of hydrogen sulphide in environmental transport of mercury, *Nature,* **275,** 635–37.

Crecelius, E.A., 1977, Arsenite and arsenate levels in wine, *Bull. Environ. Contam. Toxicol,* **18,** 227–30.

Davies, M.J., Coutts, T.M. and Rowland, I.R., 1982, Survival in the gut of bacteria bearing plasmids coding for mercury and tetracycline resistance, *Eur. J. Chemother. Antibiotics,* **2,** 144–47.

Doherty, R.A. and Gates, A.H., 1973, Epidemic methylmercury poisoning: application of a mouse model, *Pediat. Res.,* **7,** 319.

Drasar, D.S. and Hill, M.J., 1974, *Human Intestinal Flora,* London: Academic Press.

Edwards, T. and McBride, G.C., 1975, Biosynthesis and degradation of methylmercury in human faeces, *Nature,* **253,** 462–64.

Figueroa, G., Troncoso, M., Galeno, H. and Faundez, G., 1990, Oral iron supplementation and diarrhoeal disease in infants: a prospective bacteriological study, *Microb. Ecol. Health Dis.,* **3,** 139–43.

Griffiths, E., 1987, The iron-binding proteins and host defence, in Bullen, J.J. and Griffiths, E. (Eds) *Iron and Infection,* pp. 171–99, New York: Wiley-Interscience Publication.

Groves, P.J., Short, H., Thewaini, A.J. and Young, F.E., 1975, Epidemiology of antibiotic and heavy metal-resistance in bacteria: resistance patterns in *staphylococci* isolated from populations in Iraq exposed and not exposed to heavy metals or antibiotics, *Antimicrob. Agents Chemother.,* **7,** 622–28.

Hargreaves, R.J., Foster, J.R., Pelling, D.P., Moorhouse, S.R., Gangolli, S.D. and Rowland, I.R., 1985, Changes in the distribution of histochemically-localized mercury in the CNS and in tissue levels of organic and inorganic mercury during the development of intoxication in methylmercury treated rats, *Neuropathol. Appl. Neurobiol.,* **11,** 383–401.

Hirata, E. and Takahaski, H., 1981, Degradation of methylmercury glutathione by the pancreatic enzymes in bile, *Toxicol Appl. Pharmacol.,* **58,** 483–91.

Ishihara, N. and Suzuki, T., 1976, Biotransformation of methylmercury *in vitro, Tohoku. J. Exp. Med.,* **120,** 361–63.

Jensen, S. and Jernelov, A., 1969, Biological methylation of mercury in aquatic organisms, *Nature,* **223,** 753–54.

Klaasen, C.D., 1976, Biliary excretion of metals, *Drug Metab. Rev.,* **5,** 165–96.

Lakso, J.V. and Peoples, S.A., 1975, Methylation of inorganic arsenic by mammals, *Agric. Food Chem.,* **23,** 674–76.

Lender, L., 1971, Biochemical model for the biological methylation of mercury suggested from methylation studies *in vivo* with *Neurospora crassa, Nature,* **230,** 452–54.

Landry, T.D., Doherty, R.A. and Gates, A.H., 1979, Effects of three diets on mercury excretion after methylmercury administration, *Bull. Environ. Contam. Toxicol.,* **22,** 151–58.

Lefevre, P.A. and Daniel, J.W., 1973, Some properties of the organomercury-degrading system in mammalian liver, *FEBS Lett.,* **35,** 121–23.

Lerman, S. and Clarkson, T.W., 1983, The metabolism of arsenite and arsenate by the rat, *Fund. Appl Toxicol.,* **3,** 309–14.

McBride, B.C., Merilees, H., Cullen, W.R. and Pickett, W., 1978, in Brinkman F.E. and Ballama, J.M. (Eds) *Organometals and Organometalloids: Occurrence and Fate in the Environment,* pp. 94–115, Washington DC: American Chemical Soc.

Magos, L. and Butler, W.H., 1976, The kinetics of methylmercury administered repeatedly to rats, *Arch. Toxicol.,* **35,** 25–39.

Marcelis, J.H., 1980, Interactions between bacteria and iron-binding proteins, *Vet. Res. Commun.,* **4,** 151–64.

Matsumura, F., Gotoh, Y. and Boush, G.M., 1972, Factors influencing translocation and transformation of mercury in river sediment, *Bull. Environ. Contam. Toxicol.,* **8,** 267–72.

Mevissen-Verhage, E.A.E., Marcelis, J.H., Harmsen, Van Amerongen, W.C.M., de Vos, N.M., Berkel, J. and Vehoef, J., 1985a, Effect of iron on development of the neonatal gut flora during the first week of life, *Eur. J. Clin. Microbiol.,* **4,** 14–18.

Mevissen-Verhage, E.A.E., Marcelis, J.H., Harmsen-Van Amerongen, W.C.M., de Vos, N.M. and Verhoef, J., 1985b, Effect of iron on neonatal gut flora during the first three months of life, *Eur. J. Clin. Microbiol.,* **4,** 273–78.

Miettinen, J.K., 1973, Absorption and elimination of dietary mercury (Hg2+) and methylation in man, in Miller, N.W. and Clarkson, T.W. (Eds) *Mercury, Mercurials and Mercaptans,* pp. 233–43, Springfield, Illinois: Charles C.Thomas.

Moody, J.P. and Williams, R.T., 1964, The fate of 4-nitrophenylarsonic acid in hens, *Toxicol. Appl. Pharmacol.,* **2,** 692–706.

Mushak, P., 1983, Mammalian biotransformation processes involving various toxic metalloids and metals, in Brown, S.S. and Savory, J. (Eds) *Chemical Toxicology and Clinical Chemistry of Metals,* pp. 227–45, London: Academic Press.

Nakamura, K. and Sayato, Y, 1981, Comparative studies of chromosomal aberration induced by trivalent and pentavalent arsenic, *Mutation Res.,* **88,** 73–80.

Nakamura, I., Hosokawa, K., Tamura, H. and Miura, T., 1977, Reduced mercury excretion with feces in germ-free mice after oral administration of methylmercury chloride, *Bull. Environ. Contam. Toxicol.,* **17,** 528–33.

Neilands, J.B., 1974, Iron and its role in microbial physiology, in Neilands, J.B. (Ed.) *Microbial Iron Metabolism,* pp. 3–34, New York: Academic Press.

Norseth, T., 1971a, Biotransformation of methyl mercuric salts in the mouse: studies by specific determination of organic mercury, *Acta Pharmacol. Toxicol.,* **29,** 375–84.

Norseth, T., 1971b, Biotransformation of methyl mercuric salts in germ-free rats, *Acta Pharmacol. Toxicol.,* **30,** 172–76.

Norseth, T., 1973, Biliary excretion and intestinal reabsorption of mercury in the rat after injection of methylmercuric chloride, *Acta Pharmacol. Toxicol.,* **33,** 280–88.

Norseth, T. and Clarkson, T.W., 1970, Studies on the biotransformation of ^{203}Hg-labelled methylmercury chloride in rats, *Arch. Environ. Health,* **21,** 717–27.

Norseth, T. and Clarkson, T.W., 1971, Intestinal transport of ^{203}Hg-labelled methylmercuric chloride. Role of biotransformation in rats, *Arch. Environ. Health,* **22,** 568–72.

Novick, R.P. and Roth, C, 1968, Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus, J. Bacterial.,* **95,** 1335–42.

Odanaka, Y, Matano, O. and Goto, S., 1980, Biomethylation of inorganic arsenic by the rat and some laboratory animals, *Bull. Environ. Contam. Toxicol.,* **24,** 452–59.

Ohsawa, M. and Magos, L., 1974, The chemical form of methylmercury complex in the bile of the rat, *Biochem. Pharmacol.,* **23,** 1903–5.

Overby, L.R. and Fredrickson, R.L., 1963, Metabolic stability of radioactive arsanilic acid in chickens, *Agric. Fd. Chem.,* **11,** 378–81.

Penrose, W.R., 1974, Arsenic in the marine and aquatic environments: analysis, occurrence and significance, *CRC Crit. Rev. Environ. Control,* **4,** 465–82.

Refsvik, T. and Norseth, T., 1975, Methylmercuric compounds in the *bile, Acta Pharmacol. Toxicol.,* **36,** 67–68.

Ridley, W.P., Dizikes, L.J. and Wood, J.M., 1977, Biomethylation of toxic elements in the environment, *Science,* **197,** 329–32.

Robinson, J.B. and Tuovinen, O.H., 1984, Mechanisms of microbial resistance and detoxification of mercury acid organomercury compounds: physiological, biochemistry and genetic analysis, *Microbiol. Rev.,* **48,** 95–124.

Rogers, H.J., 1973, Iron-binding catechols and virulence in *Escherichia coli, Infection Immunity,* **7,** 443–56.

Rowland, I.R. and Davies, M.J., 1981, *In vivo* metabolism of inorganic arsenic by the gastrointestinal microflora of the rat, *J. Appl. Toxicol.,* **1,** 278–83.

Rowland, I.R. and Davies, M.J., 1982, Reduction and methylation of sodium arsenate in the rat, *J. Appl. Toxicol.,* **2,** 294–99.

Rowland, I.R., Davies, M.J. and Evans, J.G., 1980, Tissue content of mercury in rats given methylmercuric chloride orally: influence of intestinal flora, *Arch. Environ. Health,* **35,** 155–60.

Rowland, I., Davies, M. and Grasso, P., 1977a, Biosynthesis of methylmercury compounds by the intestinal flora of the *rat, Arch. Environ. Health,* **32,** 24–28.

Rowland, I.R., Davies, M.J. and Grasso, P., 1977b, Volatilisation of methylmercuric chloride by hydrogen sulphide, *Nature,* **265,** 718–19.

Rowland, I.R., Davies, M.J. and Grasso, P., 1978, Metabolism of methylmercuric chloride by the gastrointestinal flora of the rat, *Xenobiotica,* **8,** 37–43.

Rowland, I.R., Grasso, P. and Davies, M.J., 1975, The methylation of mercuric chloride by human intestinal bacteria, *Experientia,* **31,** 1064.

Rowland, I., Li, M. and Forster, R., 1993, Effect of iron albumin succinylate on rat faecal microflora, *Microbial. Ecol.,Hlth Dis.,* **6,** 129–31.

Rowland, I.R., Mallett, A.K., Flynn, J. and Hargreaves, R.J., 1986, The effect of various dietary fibres on tissue concentration and chemical form of mercury after methylmercury exposure in mice, *Arch. Toxicol.,* **59,** 94–98.

Rowland, I.R., Robinson, R.D. and Doherty, R.A., 1984, Effects of diet on mercury metabolism and excretion in mice given methylmercury: role of gut *flora, Arch. Environ. Health,* 39, 401–8.

Rowland, I.R., Robinson, R.D., Doherty, R.A. and Landry, T.D., 1983, Are developmental changes in methylmercury metabolism and excretion mediated by the intestinal microflora? in Clarkson, T.W.,

Nordberg, G.F. and Sager, P.R. (Eds) *Reproductive and Developmental Toxicity of Metals,* pp. 745–58, New York: Plenum Press.

Schaedler, R.W., 1973, The relationship between the host and its intestinal microflora, *Proc. Nutr. Soc.,* **32,** 41–47.

Schottel, J., Mandal, A., Clark, D., Silver, S. and Hedges, R.W., 1974, Volatilisation of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria , *Nature,* **251,** 335–37.

Seko, Y., Miura, T., Takashi, M. and Koyama, T., 1981, Methylmercury decomposition in mice treated with antibiotics, *Acta Pharmacol. Toxicol.,* **49,** 259–65.

Shahristani, H. and Shihbab, K.M., 1974, Variation of biological half-life of methylmercury in *man, Arch. Environ. Health,* **28,** 324–44.

Shirachi, D.Y., Lakso, J.V. and Rose, L.J., 1981, Methylation of sodium arsenate by the rat liver *in vitro, Proc. West Pharmacol Soc.,* **24,** 159–60.

Smith, T.J., Crecelius, E.A. and Reading, J.C., 1977, Airborne arsenic exposure and excretion of methylated arsenic compounds, *Environ. Health Perspect.,* **19,** 89–93.

Summers, A.O. and Silver, S., 1972, Mercury resistance in a plasmid-bearing strain of *Escherichia coli, J. Bacterial.,* **112,** 1228–36.

Syverson, T.L.M., 1982, Effects of methyl mercury on rat brain protein synthesis, *Ph.D. Thesis University ofTrondheim* , NTH-Trykk Trondheim.

Takeuchi, T., 1972, Biological reactions and pathological changes in human beings and animals caused by organic mercury contamination, in Hartin, R. and Dinman, B.D. (Eds) *Environmental Mercury Contamination,* Ann Arbor: Ann Arbor Science Publishers.

Tam, G.K.H., Charbonneau, S.M., Bryce, F., Promroy, C. and Sardi, E., 1979, Metabolism of inorganic arsenic (74As) in human following oral ingestion, *Toxicol Appl. Pharmacol.,* **50,** 319–22.

Vahter, M., 1983, Metabolism of inorganic arsenic in relation to chemical form and animal species, *Ph.D. Thesis,* Karolinska Institute, Stockholm.

Vahter, M. and Gustafsson, B., 1980, Biotransformation of inorganic arsenic in germ-free and conventional mice, in Anke, M., Schneider, H.-J. and Bruckner, C. (Eds) *Proceedings of the Third Symposium on Trace Elements, Arsenic,* pp. 123–29, Abteilung Wissenschaftliche Publikationen der Friedrich-Schiller Universität, Jena.

Vonk, J.W. and Sijpesteijn, A.K., 1973, Studies on the methylation of mercuric chloride by pure cultures of bacteria and fungi, *Antonie van Leeuwenhoek,* **39,** 505–13.

Walsh, C.T., 1982, The influence of age on the gastrointestinal absorption of mercuric chloride and methylmercury chloride in the rat,*Environ. Res.,* **27,** 412–20.

Weinberg, E.D., 1978, Iron and infection, *Microbiol. Rev.,* **42,** 46–66.

Wood, J.M., 1974, Biological cycles for toxic elements in the environment, *Science,* **183,** 1049–52.

Wood, J.M., Cheh, A., Dizikes, L.J., Ridley, W.P., Rakow, S. and Lakowicz, J.R., 1978, Mechanisms for the biomethylation of metals and metalloids, *Fed. Proc.,* **37,** 16–21.

Chapter 15
Vitamin K and selected water-soluble vitamins: roles of the gut bacteria in nutrition and pharmacology

J.C.Mathers

15.1
Introduction

In the summer of 1925, a group of Danish scientists made an accidental and puzzling observation (Fridericia *et al.,* 1927). They were studying B vitamin deficiency induced by feeding a diet devoid of vitamin B (Table 15.1) to weanling rats. Normally rats fed this diet stopped growing after 1–3 weeks, developed weakness of the hind legs and died within four to six weeks. Two of the rats in the study behaved in the expected way but a third which had developed hind-leg weakness in week three 'suddenly started growing at a normal rate…whilst its faeces became white and bulky'. Fridericia *et al.* (1927) named the phenomenon 'refection' to mean 'restoring change'. Adding some fresh white faeces from refected rats to the diet of deficient animals promoted recovery from deficiency and allowed normal growth. The white colour of the faeces of refected rats was due to its unusually high starch content and the Danish group speculated on the possibility that the animals had become infected with a transmissible virus of refection, but no such organism was isolated. Alternatively, defective starch digestion may have resulted from 'bacterial processes' in the gut. Whatever the cause, Fridericia *et al.* (1927) concluded that in refected animals vitamin B was produced in the gut by bacteria or other microorganisms in amounts adequate for the tissue needs of the rats.

The Danish observations were rapidly confirmed by Roscoe (1927) from Dame Harriet Chick's group at the Lister Institute. In the introduction to her paper, Roscoe (1927) wrote that, having heard that Professor Fridericia had made a communication on the subject to the Physiological Congress in Stockholm the previous August, 'the writer's observations were sent to him and publication withheld until he should publish his results in full'. I wonder if such honourable behaviour would occur today? Within the year, Kon and Watchorn (1927) reported that the type of starch in the diet was critical in determining the appearance of refection. Rats fed readily-digested rice starch rarely refected but those given uncooked potato starch or arrowroot (both relatively resistant to pancreatic α-amylase) spontaneously recovered from vitamin B deficiency.

This was not the first indication that gut bacteria may play a role in vitamin supply but it was the first to provide good systematic evidence of the phenomenon. These early reports provided reason to believe that in certain circumstances the gut bacteria could make good any dietary deficiencies of water soluble vitamins but that their capacity to do so was dependent upon macro-constituents of the diet, particularly the amount and type of carbohydrate.

Table 15.1 Composition of diet 'devoid of vitamin B' used by Fridericia *et al.* (1927)

Component	*g/100 g*
Caseinogen	20
Rice starch	57
Butter fat	15
Agar	3
Salt Mixture	5

Intestinal synthesis is not the only way in which bacteria interact with vitamins. In addition, bacteria may:

(a) provide, directly or indirectly, the vitamins in foods;
(b) cause destruction or reduce availability of vitamins within the intestine;
(c) influence the rate and/or site of absorption of vitamins from the gut.

This chapter will comment on all of these phenomena in relation to selected vitamins from both the water- and fat-soluble groups, with an emphasis on the relevance to human nutrition and health. Interactions with commonly prescribed drugs will also be considered.

Vitamins are a structurally and metabolically heterogeneous group of organic substances required in small (milligram or less) amounts in the diet of man and other animals. The conventional separation into water- and fat-soluble groups is no longer particularly helpful in understanding the functions of these substances and the definition as a dietary essential is true only if the target animal is specified. Most species synthesize adequate amounts of ascorbic acid in their tissues (higher primates including man are among a small group of exceptions) and ruminant animals are independent of a dietary supply of the water-soluble vitamins provided they have a normal functioning rumen. Vitamin D is not a nutrient in normal circumstances, being produced in the skin by the photochemical action of ultraviolet sunlight on the precursor 7-dehydrocholesterol (Frazer, 1992), and niacin can be synthesized in the tissues if tryptophan supplies are adequate.

15.2
Vitamin K

My interest in vitamin K started nearly ten years ago with some quite unexpected observations which were both puzzling and distressing at the time. A Nigerian student, Joseph Agbo, and I were carrying out a nutritional experiment on gastrointestinal responses to increased non-starch polysaccharide (NSP) intake in growing rats given foods widely eaten by humans in W. Africa. The basal diet was based on white rice (which is very low in NSP) to which we added black-eyed beans (*Vigna unguiculata:* a rich source of NSP). Within 23 days of starting on the test diets all five animals on the basal diet had died or were humanely killed because they had haemorrhaged. We collected blood from the fifth animal before death and discovered that it had a prothrombin (Factor II) clotting time in excess of 60 seconds (the normal value is about 15 seconds). None of the animals given the beans-containing diets became unwell (Mathers *et al.,* 1990).

Compounds with vitamin K activity have a 2-methyl-1, 4-naphthoquinone ring and two vitamers occur naturally. Phylloquinone (K_1) from higher plants, especially green leafy vegetables, has a 3-phytyl side chain, whilst the bacterial isomers (menaquinones, MK) have a rather variable 3-poly-isoprenyl side-chain with up to 15 isoprenyl units (usually 6–10, MK6-MK10) and are known as vitamin K_2. Menadione (K_3) is a synthetic vitamin lacking a side-chain on position 3 (Bender, 1992).

Vitamin K is required as the coenzyme for γ-glutamyl carboxylase which post-translationally modifies specific precursor proteins to yield their functional forms. For normal haemostasis, at least ten glutamic acid residues in four vitamin K-dependent proteins (Factors II, VII, IX and X) are converted into calcium-binding γ-carboxyglutamic acid (Gla) residues in the liver. The chelated calcium is then able to form an ion bridge between the active clotting factors and phospholipids in the cell membranes of platelets and endothelial cells (Conly and Stein, 1992). Vitamin K deficiency results in prolonged prothrombin clotting time and eventually in haemorrhage. Gla-containing proteins are also found in many tissues, including bone matrix (osteocalcin), the inter-membrane space of mitochondria, the kidney cortex, hydroxyapatite and calcium oxalate-containing urinary stones, atherosclerotic plaque and various pathological conditions which involve mineralization of soft tissue (Bender, 1992).

15.2.1 Gut bacteria as a source of vitamin K

Bacterial respiratory quinones include the menaquinones and the ubiquinones (coenzyme Q) of which only the menaquinones have vitamin K activity. There is a considerable literature on these isoprenoid quinones, largely because of their value in bacterial classification and identification (Jeffries *et al.*, 1967; Collins and Jones, 1981; Collins *et al.*, 1985; Collins, 1985). Some species of enteric bacteria contain menaquinones (Collins and Jones, 1981; Ramotar *et al.*, 1984) (Table 15.2) with characteristic patterns of isoprenalogues and the concentration of MK varies between species. Growth of bacteria under anaerobic conditions appears to increase MK concentrations by a factor of two to ten and yields of 0.05–6.6 µmol menaquinone/g bacterial dry weight have been reported (Conly and Stein, 1992). If human daily stool output is equivalent to approximately 30 g dry weight of which half is bacterial cells (Stephen and Cummings, 1980), then the daily menaquinone production by the colonic bacteria could be 1–100 µmoles depending upon the species of bacteria present. Actual faecal outputs appear to be only 17–21 *µg* (0.04–0.05 µmol) menaquinones/day (Conly and Stein, 1992). For comparison, the adult human requirement for vitamin K (phylloquinone) is approximately 0.1 *µmol/day*. Following early studies which indicated that several animal species were resistant to dietary vitamin K deficiency, it has frequently been assumed that half or more of human vitamin K needs can be provided by gut bacteria (Passmore and Eastwood, 1986). Compared with the other fat-soluble vitamins, body stores of phylloquinone are low, yet up to four weeks' dietary deficiency of vitamin K (O'Reilly, 1971) or total starvation (Frick *et al.*, 1967) in healthy human adults does not result in symptoms of deficiency. The detection in liver from man (Duello and Matchiner, 1972; Usui *et al.*, 1990) and other animals (Kindberg *et al.*, 1987; Mathers *et al.*, 1990; Shearer *et al.*, 1991; Will and Suttie, 1992) of a range of menaquinones indicates that these substances have been absorbed but leaves open several questions. Are the menaquinones derived from the diet or from the gut bacteria? If they are derived from gut bacteria, from where in the intestine are they absorbed? As electron transporters, bacterial menaquinones are

Table 15.2 Menaquinones (MK) produced by the main species of the enteric flora*

	MK					
Species	*Major components*			*Minor components*		
Bacteroides fragilis	MK-11	MK-10†	MK-12	MK-9	MK-8	MK-7
Bacteroides vulgatus	MK-11	MK-10†	MK-12	MK-9	MK-8	MK-7
Bacteroides distasonis	MK-11	MK-10				
Bacteroides ovatus	MK-11	MK-10				
Bacteroides thetaiotamicron	MK-11	MK-10				
Fusobacterium sp.	‡			‡		
Veillonella sp.	MK-7		MK-6			
Eubacterium sp.	‡			‡		
Eubacterium lentum	MK-6	MMK-6	DMMK-6			
Clostridium sp. Lec+ve	‡			‡		
Clostridium sp. Lec-ve	‡			‡		
Lactobacillus sp.	‡			‡		
Enterobacteria sp.	Q8	MK-8	DMK-8			
Enterococcus sp.	DMK-9		DMK-8		DMK-7	DMK-6

Q-n, ubiquinone; MK-n, menaquinone; DMK-n,demethylmenaquinone; MMK-n, methylmenaquinone; DMMK-n, dimethylmenaquinone, where *n* indicates the number of isoprene units in the side-chain; Lee+ve, lecithinase positive. Lee-ve, lecithinase-negative.

*Data from Collins & Jones (1981), Ramotar *et al.* (1984), Collins *et al.* (1985) and Fernandez & Collings (1987)

†Present in comparable amounts.

‡Lacks MK.

§Except *Eubacterium lentum*

presumed to be tightly bound to the cytoplasmic membrane, so how could they become available for absorption in a free form in the absence of coprophagy?

15.2.2
Vitamin K absorption

In a systematic series of studies published in the 1970s, Hollander and colleagues (Table 15.3) used the everted gut sac technique to investigate the absorption of phylloquinone, menaquinones and menadione by rats. Phylloquinone appeared to be absorbed by an energy-mediated saturable transport system which was more active in the duodenum than in the ileum (Hollander, 1973). Bile acids and the short-chain fatty acid butyrate stimulated phylloquinone absorption whilst polyunsaturated fatty acids seemed to be inhibitory (Hollander and Rim, 1978). A passive noncarrier-mediated diffusion process was responsible for MK-9 absorption from both small and large bowels (Hollander and Rim, 1976; Hollander *et al.*, 1976) with the proximal small bowel showing consistently higher rates than the ileum. Butyric acid stimulated absorption and neither phylloquinone nor menadione competed with MK-9 for absorption. In contrast Ishihashi *et al.* (1992) infused radio-labelled menaquinones into isolated loops of rat intestine *in vivo* and reported that when MK-9 was administered into the colon, almost all was recovered from the loop and there was no transfer of MK-9 from the colon into lymph or blood for up to six hours after dosing. MK-4 was absorbed from both the jejunum and colon and Ishihashi *et al.* (1992) suggested that absorption rates of menaquinones decrease strongly as the number of isoprenoid units in the side-chain increase. Conly and Stein (1992) have attempted to resolve this apparent conflict in results by pointing out

Table 15.3 Summary of selected studies of vitamin K absorption in rats

Vitamer	*Site studied*	*Absorption*	*Absorption mechanism*	*Reference*
1. *Phylloquinone*				
	Duodenum	Yes	energy-mediated saturable transport	Hollander (1973)
	Ileum	Yes	energy-independent transport	Hollander (1973)
2. *Menaquinones*				
MK-9	Duodenum	Yes	Passive diffusion	Hollander + Rim (1976)
MK-9	Ileum	Yes	Passive diffusion	Hollander + Rim (1976)
MK-9	Colon	Yes	Passive diffusion	Hollander + Rim (1976)
MK-4	Jejunum	Yes	n.d.	Ishihashi *et al.*, 1992
MK-4	Colon	Yes	n.d.	Ishihashi *et al.*, 1992
MK-9	Colon	No	n.d.	Ishihashi *et al.*, 1992
3. *Menadione*				
	Duodenum	Yes	Passive diffusion	Hollander + Truscott (1974a)
	Ileum	Yes	Passive diffusion	Hollander + Truscott (1974a)
	Colon	Yes	Passive diffusion	Hollander + Truscott (1974b)

n.d., not determined

the facilitatory role of the bile salts in menaquinone absorption and that their absence in the study of Ishihashi *et al.* (1992) may explain the lack of menaquinone absorption. An alternative approach to this problem was employed by Kindberg *et al.* (1987) who inoculated different groups of germ-free rats with groups of organisms which were known to produce menaquinones (*Bacteroides vulgatus* and *Escherichia coli*) or did not produce menaquinones (*Bifidobacterium longum* and *Clostridium ramosum*). Menaquinones were not detected in faeces or livers from animals given the latter two inoculants, but rats colonized with *Bacteroides vulgatus* had high concentrations of MK-10 and significant amounts of MK-9 and MK-11 in faeces, whilst the E. *coli*-inoculated rats had MK-8 and MK-7 as major faecal menaquinones. Liver concentrations of menaquinones mirrored faecal concentrations and were higher in animals housed in wire-bottomed cages than in those in cages designed to prevent coprophagy. Orally administered, bacterially synthesized menaquinones are absorbed and are bioactive in man (Conly and Stein, 1993). The synthetic analogue menadione is absorbed by passive diffusion throughout the gut (Table 15.3) and transported mainly via the portal vein to the liver where it is alkylated to the active MK-4 (Bender, 1992).

In colonic bacteria, menaquinones are present intracellularly, tightly bound to the cytoplasmic membrane as an integral component of the respiratory system. For absorption to take place it would be necessary for the menaquinones to become available in a 'free' form perhaps through the natural process of cell senescence, death and bacterial lysis (Conly and Stein, 1992) or following lysis by bacteriophage. Recent studies reviewed by Conly and Stein (1992) have demonstrated that menaquinones are released into the external medium from menaquinone-containing bacteria placed within a dialysis bag. If this release occurred *in vivo* it could provide an explanation for the apparent absorption of colonic-derived menaquinones in rats in which coprophagy was prevented (Kindberg *et al.*, 1987) and in man (in the absence of coprophagy). Alternatively, since menaquinones have been detected in human ileal contents (Conly and Stein, 1992; although their

source was not established) it may be that absorption occurs in the distal small intestine where pancreatic enzymes are still active and lipids including bile acids and menaquinones are known to be absorbed. Retrograde movement of digesta from the caecum could provide a source of bacterial menaquinones as could bacteria colonizing the mucosa or lumen of the ileum.

15.2.3 Human infants

Whilst adult humans appear to be very resistant to the development of a primary deficiency of vitamin K (Suttie, 1985), haemorrhagic disease of neonates (HDN) is occasionally reported and seems to be more common in breast-fed than bottle-fed infants (Motohara *et al.,* 1984; von Kries *et al.,* 1988). Vitamin K prophylaxis at birth is strongly protective. The development of improved high-performance liquid chromatographic methods with electrochemical detection has permitted the assay of phylloquinone and menaquinones in physiological fluids and tissue samples. In Japanese mother: infant pairs, Hiraike *et al.* (1988) found very low concentration of phylloquinone in umbilical cord plasma compared with matched maternal plasma (0.11 and 1.54 ng/ml respectively). Greer *et al.* (1988) reported similar concentrations of phylloquinone in maternal serum but higher concentrations in cord blood in mother: infant pairs at Madison General Hospital, USA. Menaquinones with four, six and seven isoprene units in the side-chain were found in maternal plasma (with mean concentrations of 0.05, 0.22 and 1.08 ng/ml for MK-4, MK-6 and MK-7 respectively) but only MK-4 (0.04 ng/ml) was found in umbilical cord plasma. Hiraike *et al.* (1988) suggested that the very low concentrations of vitamin K in the foetal circulation may be one of the reasons why bleeding occurs frequently in neonates. Intravenous injection of phylloquinone shortly before delivery can increase cord plasma vitamin K_1 (Shearer *et al.,* 1982) but a more practical means of supplementation would be via the diet. Fermented soybeans, which are a traditional Japanese food, contain high levels of MK-7 and supplementation of two pregnant women with 30 g fermented soybeans on alternate days during the last month of pregnancy resulted in a significant increase in both maternal plasma and placental MK-7, compared with a control group of women, but no significant change in umbilical cord plasma vitamin K was detected (Hiraike *et al.,* 1988). If dietary menaquinones provided by fermented soybeans or similar products improve the vitamin K status of the mother then these authors suggest that this may increase the vitamin K in breast milk and so benefit the neonate.

Human colostrum and mature breast milk contain similar concentrations of phylloquinone (Canfield *et al.,* 1991) but, being fat-soluble, this vitamin is in higher concentration in hind- than in fore-milk (von Kries *et al.,* 1985) and has been localized in the lipid core of the milk fat globule (Canfield *et al.,* 1991). The reasons why bottle-fed infants are less susceptible to HDN than are breast-fed infants are not fully established but differences in phylloquinone content may be crucial. Most infant formulae are based upon cows' milk, which has a much higher concentration of phylloquinone than does breast milk, and some infant formulae are supplemented with vitamin K_1 (Shearer *et al.,* 1980). In addition, the bacteria which colonize the large bowel of the two groups of infants also differ, with the flora of breast-fed babies characterized by organisms devoid of menaquinones (bifidobacteria and lactobacilli) whilst formula-fed babies develop a more adult type of flora with significant *Bacteroides* populations (Bullen *et al.,* 1977) which are good sources of menaquinones (Table 15.2). In summary, the human infant is born with low body-stores of vitamin

K because of limited transport across the placenta and, if breast-fed, receives only a modest intake of the vitamin in milk and little (or none) from its gut flora.

Neonatal haemorrhage, particularly after the first week of extra-uterine life, appears particularly high in Japan with a reported incidence of one in 4,500 babies compared with one per 50,000 to 100,000 in Germany (von Kries *et al.,* 1988). Given the important role of white rice in the traditional Japanese diet and the observation that white rice-based diets have been used to establish vitamin K deficiency in human subjects (Udall, 1965; O'Reilly, 1971), chicks (Almquist, 1936) and rats (Mathers *et al.,* 1990) it is tempting to speculate that this may contribute to a lower vitamin K status among Japanese women.

15.2.4 Effects of drugs and vitamin K nutrition

The classical studies of Black *et al.* (1942) and Kornberg *et al.* (1944) in rats, and of Frick *et al.* (1967) and O'Reilly (1971) in man, demonstrated convincingly that antibiotics against the intestinal flora can precipitate vitamin K deficiency if dietary intakes of the vitamin are low. Indeed, 'unexpected' bleeding has been a clinical feature of patients given enteral or parenteral broad-spectrum antibiotics particularly if food intake is poor (Pineo *et al.,* 1973; Colvin and Lloyd, 1977; Hooper *et al.,* 1980; Krasinski *et al.,* 1985). The mechanism(s) by which specific antibiotics interfere with the gut synthesis or tissue utilization of vitamin K are not fully understood. Clearly, oral antibiotics reaching the colon may be active against menaquinone-synthesizing bacteria and may reduce MK production, as has been reported for short-chain fatty acids, the major end-products of anaerobic catabolism of carbohydrates (Hoverstad *et al.,* 1986). With intravenously administered antibiotics, it is possible that the drug itself or a metabolite may be excreted in bile and reach the colon (Bender, 1992). Green leafy vegetables and liver are the richest vitamin K sources among commonly-eaten foods (Conly and Stein, 1992) and when food intake is reduced because of illness, these foods may be eaten less frequently. Low food intake may also exacerbate vitamin K deficiency by reducing the amount of substrate (undigested food fragments) reaching the large bowel, which is necessary for the growth of the menaquinone-producing bacteria (Mathers *et al.,* 1990).

Warfarin and other 4-hydroxy-coumarin drugs are well-recognized vitamin K antagonists which modulate the hepatic biosynthesis of prothrombin (Factor II) and other vitamin K-dependent factors. The primary action of warfarin and related substances seems to be to inhibit vitamin K-2, 3-epoxide reductase, preventing the recycling of the epoxide to vitamin K and thus reducing the effective concentration of the vitamin (Olson, 1984). High doses of salicylate can also induce hypoprothrombinaemia in humans, an effect which can be counteracted by high doses of vitamin K.

Broad-spectrum antibiotics containing an N-methylthiotetrazole (NMTT) side-chain decrease concentrations of vitamin K-dependent plasma clotting factors (Hooper *et al.,* 1980; Weitekamp and Aber, 1983) and it has been suggested that this is due to a direct effect of NMTT on the vitamin K-dependent post-translational carboxylation of these clotting factors, rather than to a suppression of menaquinone production by the colonic microflora. This hypothesis has been tested in both mice (Shirakawa *et al.,* 1990) and humans (Allison *et al.,* 1987). Young healthy male volunteers consumed a vitamin K-free diet for two weeks, and for the final ten days received daily therapeutic doses of antibiotics containing or not containing NMTT. Serum phylloquinone concentrations declined to 50% of initial values within three days of starting the vitamin K-free diet, reflecting the low body-stores and rapid turnover of the plasma pool, but there was no impairment of normal

vitamin K function. There was no evidence in this study that the NMTT-containing antibiotics had more of an effect on vitamin K-dependent processes than did other broad-spectrum antibiotics (Allison *et al.,* 1987). Whilst these authors demonstrated the expected fall in faecal phylloquinone in the volunteers consuming the vitamin K-free diet, they did not measure faecal menaquinones and so were unable to comment on the potential effects of the antibiotics on menaquinone production.

To determine whether gut bacteria play a role in NMTT-containing antibiotic-induced vitamin K deficiency, Shirakawa *et al.* (1990) administered sodium latamoxef (LMOX) intraperitoneally to both conventional and germ-free mice for at least eight days, whilst the mice consumed a vitamin K-deficient diet (K-def) or one supplemented with MK-4. Germ-free animals fed K-def showed the most severe symptoms of deficiency and LMOX administration caused rapid mortality. With conventional animals longer-term (15 or 30 days) administration of LMOX increased plasma prothrombin clotting times when the K-def was fed but had no apparent effect in animals fed the MK-4 supplemented diet (Shirakawa *et al.,* 1990). The authors concluded that vitamin K deficiency is amplified by LMOX administration even in the absence of gut bacteria and so re-opens the possibility of a tissue-level effect of antibiotics with an NMTT side- chain.

15.2.5
Pharmacological uses of vitamin K

The only accepted pharmacological uses for vitamin K are the treatment of vitamin K deficiency, prophylaxis in new-born infants (to prevent HDN) and as an antidote to overcome anticoagulant toxicity. Since vitamin K-dependent proteins (osteocalcin and bone matrix γ-carboxylglutamate protein) are essential for the formation of normal bone structure, vitamin K supplements may be of benefit in the healing of bone fractures or in retarding osteoporosis—patients with osteoporosis may have low blood concentrations of vitamin K (Bender, 1992). In high doses menadione is cytotoxic and has been used in cancer chemotherapy (Bender, 1992). There have been suggestions that disturbed vitamin K metabolism may contribute to malignancy in general (Egilsson, 1977) and that menaquinones (from a *Bacteroides-dominated* gut flora) could participate in colorectal carcinogenesis by participating with bile acids and iron (II) in oxygen radical generation, leading to an overwhelming of stem-cell antioxidant defence mechanisms and hence hydroxyl radical-mediated DNA damage (Blakeborough *et al.,* 1989).

15.3
Biotin

Biotin is a bicyclic compound with fused ureido (imidazolidone) and thiophene rings and an aliphatic carboxylate side-chain. It is widely distributed in foods where it is probably mainly in the form of biocytin (N-biotinyl-ε-lysine) in which the ε-amino group of a lysine residue is covalently bound to the carboxyl group of the biotin side-chain (Bender, 1992). There are four biotin-dependent carboxylases in mammals and birds: the cytoplasmic acetyl CoA carboxylase catalyzes the first and rate-limiting step in fatty acid synthesis; methylcrotonyl CoA carboxylase participates in the catabolism of leucine, propionyl CoA carboxylase catalyzes the carboxylation of propionyl CoA to methylmalonyl CoA and pyruvate to oxaloacetate. The latter three enzymes are all located in the mitochondria. In this way biotin is vital in lipogenesis, gluconeogenesis and in the catabolism of certain amino acids (Bender, 1992). Primary deficiency of biotin in humans is extremely rare but

has occurred in people consuming large amounts of raw egg white and in patients with major intestinal resection who are receiving total parenteral nutrition (Velázquez *et al.,* 1990). The glycoprotein avidin in egg white binds biotin with great affinity and the resulting complex is not digested by small bowel enzymes (Bender, 1992). Deficiency syndromes in man include scaly erythematous dermatitis, alopecia, glossitis, ataxia, irritability and depression. Feeding a biotin-deficient diet containing 245 g spray-dried egg white/kg diet (to provide avidin and prevent biotin absorption) to mice during pregnancy provoked gross congenital malformations including micrognathia, cleft palate and micromelia. Quantitative human requirements for biotin have not been established with any certainty because of the difficulty in determining the contribution of biotin synthesis by the gut bacteria to tissue needs. For those on total parenteral nutrition, where the contribution from intestinal bacterial synthesis is assumed to be negligible, 60 μg biotin/day is recommended (Bender, 1992).

15.3.1 Gut bacteria and biotin supply

Many micro-organisms including yeasts, lower fungi, e.g. *Aspergillus* and *Penicillium* and bacteria, are capable of synthesizing biotin. In some cases, biotin is synthesized under aerobic but not anaerobic conditions (Ohsugi *et al.,* 1990) and some micro-organisms are net consumers of this vitamin (Sugita *et al.,* 1992). However, the observation that faecal output of biotin is independent of dietary intake and can be several times greater than its intake in both man (Bender, 1992) and experimental animals (Kopinski *et al.,* 1989a) is strong evidence for net synthesis within the gut. A detailed study of biotin flows through the gut of young pigs demonstrated that dietary biotin was absorbed in the upper small intestine with biotin concentrations reaching a minimum in the second quarter of the small bowel. Thereafter biotin concentrations and flow rates increased particularly in the caecum and colon (Kopinski *et al.,* 1989b,c). While there is now no doubt that the gut flora are potentially an important source of biotin, and that biotin can be absorbed from the large bowel (Bowman and Rosenberg, 1987; Kopinski *et al.,* 1989c; Barth *et al.,* 1986), quantifying uptake from the distal intestine has been more difficult.

In vitro several intestinal bacteria release biotin into the surrounding medium (Ohsugi *et al.,* 1990). Fractionation of biotin in caecal digesta from chickens showed that 40% was ‘free’ in the supernatant (after centrifugation at 3,500 g for 20 minutes) and of this about half was not bound to protein (Bryden, 1989). Although the rate of absorption of biotin from isolated loops of rat intestine was lower for caecum and colon than for the jejunum (Bowman *et al.,* 1986; Bowman and Rosenberg, 1987), given the relatively high concentrations of total biotin in large bowel contents (Bryden, 1989; Kopinski *et al.,* 1989b) there would seem to be the potential for a significant contribution from this source to tissue needs. Mosethin *et al.* (1990) infused starch into the caecum of pigs (to stimulate bacterial fermentation) and observed a net increase in faecal biotin output. However, there was no significant change in plasma biotin concentration or urinary biotin output and Mosethin *et al.* (1990) concluded that microbial-derived biotin has a low efficiency of absorption from the pig large bowel. Feeding lactulose (a non-absorbable disaccharide which flows to the large intestine and is readily fermented by the colonic flora) also significantly increased faecal biotin output, but again had no significant effect on urinary biotin excretion (Scholtissek *et al.,* 1990). In the same study, intracaecal infusion of avidin almost doubled faecal biotin excretion and

urinary biotin output was decreased by 21%. Scholtissek *et at.* (1990) suggested that only 2–17% of the pig's tissue requirements for biotin could be supplied by the gut flora.

15.3.2
Drugs and biotin metabolism

Long-term anticonvulsant drug therapy has been associated with impaired biotin status (Krause *et al.,* 1985) and two anticonvulsant drugs, carbamazepine and primidone, have been shown to inhibit competitively the energy-dependent, carrier-mediated transport of biotin by the human jejunal brush-border membrane (Said *et al.,* 1989). Transport by the enterocyte basolateral membrane appeared unaffected. Of more general interest is the finding that chronic ethanol ingestion inhibits intestinal biotin transport by the carrier-mediated process (Said *et al.,* 1990). This may be a contributor to the reduced plasma biotin concentrations reported in alcoholics, but poor dietary intake and altered gut flora may also be important (Persson, 1991). Absorption of pharmacological doses of biotin did not seem to be affected by ethanol (Said *et al.,* 1990). High doses of biotin have a hypoglycaemic effect in insulin-dependent diabetics and in a mouse model of non-insulin-dependent diabetes mellitus, pharmacological doses of biotin improved several aspects of blood glucose control (Bender, 1992). Koutsikos *et al.* (1990) have suggested that regular biotin administration in pharmacological amounts may help in the management of diabetic peripheral neuropathy.

Oral antibiotics which are active against the gut flora might be expected to reduce large bowel biotin synthesis and indeed there are reports of lower faecal biotin excretion after such treatment (Oppel, 1942; Grundy *et al.,* 1947). However, this is not a universal finding and Scholtissek *et al.* (1990) observed no effect of oral dosing with Veomycin sulphate and Bacitracin on faecal biotin output in minipigs. It is probable that the divergence in observations relates to differences in the balance between biotin-producing and biotin-consuming bacteria.

15.4
Vitamin B_{12}

Vitamin B_{12} is a generic descriptor for the cobalamins which have biological activity as a vitamin. These large organic molecules have a central cobalt atom within a corrin ring and are the only established form in which cobalt is essential for animal metabolism. Vitamin B_{12} is synthesized only by bacteria. Several vitamers are known as well as non-cobalamin corrinoids, which are not vitamins for man but have activity in microbiological assays and so can cause overestimation of B_{12} content of foods. There are three vitamin B_{12}-dependent enzymes in mammals: methionine synthetase, methylmalonyl CoA mutase and leucine aminomutase (Bender, 1992). Despite being found only in animal foods and some bacteria, dietary deficiency of vitamin B_{12} is rare and occurs only in strict vegetarians (vegans). Pernicious anaemia is mainly caused by failure of gastric secretion of intrinsic factor (IF), a glycoprotein (M_r 44,000) produced in the parietal cells and required for B_{12} absorption.

15.4.1 Gut bacteria and vitamin B_{12} absorption

Prior to absorption from the distal ileum, vitamin B_{12} undergoes a series of associations with proteins and is rarely found as the free cobalamin. This may be a means of preventing its uptake by intestinal bacteria. Protein-bound B_{12} in foods is released by acid pepsin hydrolysis in the stomach and binds to cobalophilin (previously known as R-protein) from saliva. In the duodenum, pancreatic proteases hydrolyze cobalophilin, releasing B_{12}which then binds to IF and in this form is carried in digesta to the distal ileum. Absorption from this site requires B_{12} to be bound to IF but it is not known whether the IF-vitamin B_{12} complex crosses the apical membrane intact (Bender, 1992).

The normal process of ageing in a healthy human has no demonstrable effects on vitamin B_{12} absorption (McEvoy *et al.,* 1982) but atrophic gastritis associated with bacterial overgrowth could prevent B_{12} release from food proteins or impair binding to IF. In elderly subjects (>60 years of age), those with atrophic gastritis absorbed significantly less protein-bound vitamin B_{12} than did controls. Treatment with tetracycline for five days normalized B_{12} absorption (Suter *et al.,* 1991). These authors found that bacteria derived from intestinal intubations of atrophic gastritis patients avidly bound free (crystalline) B_{12}, effectively making the vitamin unavailable for absorption, whereas there was no bacterial binding to protein-bound B_{12}. Small bowel bacteria might interfere with B_{12} absorption in other ways, e.g. by producing analogues of B_{12} without vitamin activity but which are recognized by receptors on the ileal mucosa and compete with the IF-B_{12} complex for absorption.

It may take 20–30 years for B_{12} deficiency to appear in strict vegetarians (vegans) with no apparent dietary source of the vitamin. The relatively large hepatic store of vitamin B_{12} and efficient conservation as a result of entero-hepatic circulation may explain this prolonged protection, but there have been suggestions that significant amounts of B_{12} could be provided by the gut flora. Certainly B_{12} analogues equivalent to about 5 μg vitamin B_{12}/day and capable of curing B_{12} deficiency are found in faeces, but it is not known if any B_{12} can be absorbed from the large bowel (Herbert, 1988). Passive diffusion is a possibility. Bender (1992) concluded that bacterial contamination of soil, water and foods with B_{12}-producing organisms could provide small, but adequate, amounts of the vitamin.

15.5 Folic acid

Folic acid is a conjugated pterin in which the pteridine ring is linked to p-aminobenzoyl-poly-γ-glutamate and which is required for transfer of one-carbon fragments in several biosynthetic and catabolic reactions (Bender, 1992). Several vitamers have been characterized. Free folate is released from folate conjugates within the intestinal lumen by the action of pteroyl-polyglutamate hydrolase (a pancreatic enzyme) and is readily absorbed from the upper small intestine by a carrier-mediated mechanism. There is a significant enterohepatic circulation of folate equivalent to about one-third of daily intake, but reabsorption is very efficient, faecal output is small and probably mainly a result of large bowel bacterial synthesis (Bender, 1992). Recent studies have demonstrated that bacterially derived [^{3}H]-folate administered into the caecum of rats was absorbed and incorporated into liver- and kidney-specific folate polyglutamates (Rong *et al.,* 1991). The quantitative significance of large bowel-derived folate in supplying tissue needs is unknown, but the observation that individuals with small bowel bacterial overgrowth have significantly higher serum folate concentrations than age-

matched controls (Russell *et al.,* 1986a,b) prompts the question of the role of small bowel organisms in folate supply in humans in developing countries, where bacterial colonization of the small intestine is much more common.

15.5.1
Iatrongenic folate deficiency

Folate antimetabolites have been developed for clinical use in cancer therapy (e.g. methotrexate) and as antibacterial (trimethoprim) and antimalarial (pyrimethamine) agents. Prolonged use of the latter two drugs may induce folate deficiency (Bender, 1992). As with biotin, some anti-epileptic drugs, e.g. diphenylhydantoin (phenytoin) can cause folate deficiency. Possible mechanisms include reduced absorption, increased tissue catabolism and increased excretion (Bender, 1992).

15.6
Riboflavin

The metabolically-active coenzymes riboflavin 5'-phosphate and flavin adenine dinucleotide act as redox cofactors in energy-yielding metabolism. For a substance with such a central importance and for which tissue stores are modest, it is surprising that, although widespread, dietary deficiency is apparently never fatal (Bender, 1992). As with the other B vitamins, riboflavin is synthesized by the intestinal flora—so to what extent are we protected from dietary deficiency by our symbiotic bacteria?

15.6.1
Gut bacteria and riboflavin supply

The early studies of Fridericia *et al.* (1927) had established that deliberate induction of vitamin B deficiency could be thwarted if the animals' diet contained raw potato starch and the phenomenon of refection occurred. Further studies by Mannering *et al.* (1944) and Czaczkes and Guggenheim (1946) in rats and by Gershoff *et al.* (1959) in cats confirmed that diets rich in carbohydrate, particularly if that carbohydrate was poorly digested in the small intestine, exerted a sparing effect on oral riboflavin requirements, probably because of intestinal synthesis of the vitamin. More recently, Prentice and Bates (1980) reported refection in rats fed on a sucrose-based, riboflavin-deficient diet. Fitting the rat with tail-cups to inhibit coprophagy prevented refection so it is probable that the riboflavin-deficient animal without tail-cups had 'learned' to consume their faeces and so augment their deficient diet. Prentice and Bates' (1980) success in inducing deficiency when coprophagy was prevented argues against the importance of intestinal bacterial supply as a significant source of riboflavin in man, but it would be interesting to explore riboflavin supply in situations where small bowel bacterial overgrowth is common, e.g. in developing countries or in the elderly. A comparison of biochemical responses to oral riboflavin supplementation in adolescent and elderly Gambian subjects has been published (Bates *et al.,* 1989) but riboflavin intake from food was not measured or controlled and no information was reported on whether any subjects had bacterial contamination of the small bowel.

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References

Allison, P.M., Mummah-Schendel, L.L., Kindberg, C.G., Harms, C.S., Bang, N.U. and Suttie, J.W., 1987, Effects of a vitamin K-deficient diet and antibiotics in normal human volunteers, *J. Lab. Clin. Med.,* **110,** 180–88.

Almquist, H.J., 1936, Factors influencing the incidence of dietary hemorrhagic disease in chicks, *J.Nutr.,* **12,** 329–35.

Barth, C.A., Frigg, M. and Hagemeister, H., 1986, Biotin absorption from the hindgut of the pig, *J. Animal Physiol., Animal Nutr.,* **55,** 128–34.

Bates, C.J., Powers, H.J., Downes, R., Brubacher, D., Sutcliffe, V. and Thurnhill, A, 1989, Riboflavin status of adolescent *vs* elderly Gambian subjects before and during supplementation, *Am. J. Clin. Nutr.,* **50,** 825–29.

Bender, D.A., 1992, *Nutritional Biochemistry of the Vitamins,* Cambridge: Cambridge University Press.

Black, S., Overman, R.S., Elvehjem, C.A. and Link, K.P., 1942, The effect of sulphaguanidine on rat growth and plasma prothrombin, *J. Biol. Chem.,* **145,** 137–43.

Blakeborough, M.H., Owen, R.W. and Bilton, T.F., 1989, Free radical generating mechanisms in the colon: their role in the induction and promotion of colorectal cancer, *Free Rad. Res. Commun.,* **6,** 359–67.

Bowman, B.B. and Rosenberg, I.H., 1987, Biotin absorption by distal rat intestine, *J. Nutr.,* 117, 2121–26.

Bowman, B.B., Selhub, J. and Rosenberg, I.H., 1986, Intestinal absorption of biotin in the rat, *J.Nutr.,* **116,** 1266–71.

Bryden, W.L., 1989, Intestinal distribution and absorption of biotin in the chicken, *Brit. J. Nutr.,* **62,** 389–98.

Bullen, C.L., Tearle, P.V. and Stewart, M.G., 1977, The effect of ‘humanised’ milks and supplemented breast feeding on the faecal flora of infants, *J. Med. Microbiol.,* **10,** 403–13.

Canfield, L.M., Hopkinson, J.M., Lima, A.F., Silva, B. and Garza, C., 1991, Vitamin K in colostrum and mature human milk over the lactation period—across-sectional study, Am. *J. Clin. Nutr.,* **53,** 730–35.

Collins, M.D., 1985, Isoprenoid quinone analyses in bacterial classification and identification, in Goodfellow, M. and Minnikin, D.E. (Eds) *Chemical Methods in Bacterial Systematics,* pp. 267–87 London: Academic Press.

Collins, M.D., Fernandez, F. and Howarth, O.W., 1985, Isolation and characterization of a novel vitamin-K from *Eubacterium lentum, Biochim. Biophys. Res Commun.,* **133,** 322–28.

Collins, M.D. and Jones, D., 1981, Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications, *Microbiol. Rev.,* **45,** 316–54.

Colvin, B.T. and Lloyd, M.J., 1977, Severe coagulation defect due to a dietary deficiency of vitamin K, *J. Clin. Pathol.,* **30,** 1147–48.

Conly, J.M. and Stein, K., 1992, The production of menaquinones (vitamin K.) by intestinal bacteria and their role in maintaining coagulation homeostasis, *Prog. Food Nutr. Sci.,* **16,** 307–43.

Conly, J.M. and Stein, K.E., 1993, The absorption and bioactivity of bacterially-synthesized menaquinones, *Clin. Invest. Med.,* **16,** 45–57.

Czaczkes, J.W. and Guggenheim, K., 1946, The influence of diet on the riboflavin metabolism of the rat, *J. Biol. Chem.,* **162,** 267–74.

Duello, T.J. and Matchiner, J.T., 1972, Characterization of vitamin K from human liver, *J. Nutr.,* **102,** 331–36.

Egilsson, V., 1977, Cancer and vitamin *K, Lancet,* **i,** 254–55.

Fernandez, F. and Collins, M.D., 1987, Vitamin K composition of anaerobic gut bacteria, *FEMS Microbiol. Lett.,* **41,** 175–80.

Frazer, D.R., 1992, Fat soluble vitamins, in Widdowson, E.M. and Mathers, J.C. (Eds) *The Contribution of Nutrition to Human and Animal Health,* pp. 184–90, Cambridge: Cambridge University Press.

Frick, P.G., Riedler, G. and Brögli, H., 1967, Dose-response and minimal daily requirement for vitamin K in man, *J. Appl. Physiol.,* **23,** 387–89.

Fridericia, L.S., Freudenthal, P., Gudjonnsson, S., Johansen, G. and Schoubye, N., 1927, Refection, a transmissible change in the intestinal content, enabling rats to grow and thrive without vitamin B in the food, *J. Hygiene,* **27,** 70–102.

Gershoff, S.N., Andrus, S.B. and Hegsted, D.M., 1959, The effect of carbohydrate and fat content of the diet upon the riboflavin requirement of the cat, *J. Nutr.,* **68,** 75–88.

Greer, F.R., Mummah-Schendel, L.L., Marshall, S. and Suttie, J.W, 1988, Vitamin K, (phylloquinone) and vitamin K, (menaquinone) status in newborns during the first week of life, *Pediatrics,* **81,** 137–40.

Grundy, W.E., Freed, M., Johnson, H.C., Henderson, C.R., Berryman, G.H. and Friedmann, T.E., 1947, The effect of phthalysulfathiazole (sulfathalidine) on the excretion of B-vitamins by normal adults, *Arch. Biochem. Biophys.,* **15,** 187–94.

Herbert, V., 1988, Vitamin-B_{12}: plant sources, requirements, and assay, *Am. J. Clin. Nutr.,* **48,** 852–58.

Hiraike, H., Kimura, M. and Itokawa, Y, 1988, Distribution of K vitamins (phylloquinone and menaquinones) in human placenta and maternal and umbilical cord plasma, *Am. J. Obstet. Gynecol.,* **158,** 564–69.

Hollander, D., 1973, Vitamin K_1 absorption by everted intestinal sacs of the *rat, Am. J. Physiol.,* **225,** 360–64.

Hollander, D., Muralidhara, K.S. and Rim, E., 1976, Colonic absorption of bacterially synthesized vitamin K_2 in the *rat, Am. J. Physiol.,* **230,** 251–55.

Hollander, D. and Rim, E., 1976, Vitamin K_2 absorption by rat everted small intestinal sacs, *Am. J. Physiol.,* **231,** 415–19.

Hollander, D. and Rim, E., 1978, Effect of luminal constituents on vitamin K_1 absorption into thoracic duct lymph, *Am. J. Physiol.,* **234,** E54–9.

Hollander, D. and Truscott, T.C., 1974a, Mechanism and site of vitamin K-3 small intestinal transport, *Am. J. Physiol.,* **226,** 1516–22.

Hollander, D. and Truscott, T.C., 1974b, Colonic absorption of vitamin K-3, *J. Lab. Clin. Med.,* **83,** 648–56.

Hooper, C.A., Haney, B.B. and Stone, H.H., 1980, Gastrointestinal bleeding due to vitamin K deficiency in patients on parenteral cefamandole, *Lancet,* **i,** 39–40.

Hoverstad, T., Carlstedt-Duke, B., Lingaas, E., Norin, E., Saxerholt, H., Steinbakk, M. and Midtvedt, T., 1986, Influence of oral intake of seven different antibiotics on faecal short-chain fatty acid excretion in healthy subjects, *Scand. J. Gastroenterol.,* **21,** 997–1003.

Ishihashi, T., Takagishi, Y., Uchida, K. and Yamada, H., 1992, Colonic absorption of menaquinone–4 and menaquinone-9 in rats, *J. Nutr.,* **112,** 506–12.

Jeffries, L., Cawthorne, M.A., Harris, M., Diplock, A.T., Green, J. and Price, S.A., 1967, Distribution of menaquinones in aerobic *Micrococcaceae, Nature,* **215,** 257–59.

Kindberg, C., Suttie, J.W., Uchida, K., Hirauchi, K. and Nakao, H., 1987, Menaquinone production and utilization in germ-free rats after inoculation with specific organisms, *J. Nutr.,* **117,** 1032–35.

Kon, S.K. and Watchorn, E., 1927, Relation between the nature of the carbohydrate in the diet and refection rats, *J. Hygiene,* **27,** 321–27.

Kopinski, J.S., Leibholz, J., Bryden, W.L. and Fogarty, A.C., 1989a, Biotin studies in pigs 1. Biotin deficiency in the young pig, *Brit. J. Nutr.,* **62,** 751–59.

Kopinski, J.S., Leibholz, J. and Bryden, W.L., 1989b, Biotin studies in pigs 3. Biotin absorption and synthesis, *Brit. J. Nutr.,* **62,** 767–72.

Kopinski, J.S., Leibholz, J. and Love, R.J., 1989c, Biotin studies in pigs 5. The post-ileal absorption of biotin, *Brit. J. Nutr.,* **62,** 781–89.

Kornberg, A., Daft, F.S. and Sebrell, W.H., 1944, Mechanism of production of vitamin K deficiency in rats by sulfanomides, *J. Biol. Chem.,* **155,** 193–200.

Koutsikos, D., Agroyannis B. and Tzanatos-Exarchou, H., 1990, Biotin for diabetic peripheral neuropathy, *Biomed. Pharmacotherapy,* **44,** 511–14.

Krasinski, S.D., Russell, R.M., Furie, B.C., Kruger, S.F, Jacques, P.F and Furie, B., 1985, The prevalence of vitamin K deficiency in chronic gastrointestinal disorders, *Am. J. Clin. Nutr.,* **41,** 639–43.

Krause, K.H., Berlit, P., Bonjour, J.P., Berlit, P. and Kochen, W., 1985, Biotin status of epileptics, *Ann. N.Y. Acad. Sci.,* **447,** 297–313.

von Kries, R., Göbel, U., Shearer, M.J. and McCarthy, P.T., 1985, Vitamin K deficiency in breastfed infants, *J. Pediat.,* **107,** 650–51.

von Kries, R., Shearer, M.J. and Göbel, U., 1988, Vitamin K in *infancy, Eur. J. Pediat.,* **147,** 106–12.

Mannering, G.J., Orsini, D. and Elvehjem, C.A., 1944, Effect of the composition of the diet on the riboflavin requirement of the rat, *J. Nutr.,* **28,** 141–56.

Mathers, J.C., Fernandez, F., Hill, M.J., McCarthy, P.T., Shearer, M.J. and Oxley, A., 1990, Dietary modification of potential vitamin K supply from enteric bacterial menaquinones in rats, *Brit. J. Nutr.,* **63,** 639–52.

McEvoy, A.W., Fenwick, J.D., Boddy, K. and James, O.F.W., 1982, Vitamin B_{12} absorption from the gut does not decline with age in normal elderly humans, *Age Aging,* **11,** 180–83.

Mosethin, R., Sauer, W.C., Völker, L. and Frigg, M., 1990, Synthesis and absorption of biotin in the large intestine *of pigs, Livestock Prod. Sci.,* **55,** 95–103.

Motohara, K., Matsukura, M., Matsuda, I., Iribe, K., Ikeda, T., Kondo, Y., Yonekubo, A., Yamamoto, Y. and Tsuchiya, F, 1984, Severe vitamin K deficiency in breast-fed infants, *J. Pediat.,* **105,** 943–45.

Ohsugi, M., Imanishi, Y., Teraoka, T., Nishimura, K. and Makao, S., 1990, Biosynthesis of biotinvitamers by family Enterobacteriaceae, *J. Nutr. Sci. Vitaminol.,* **36,** 447–56.

Olson, R.E., 1984, The function and metabolism of vitamin K, *Ann. Rev. Nutr.,* **4,** 281–337.

Oppel, T.W., 1942, Studies of biotin metabolism in man, *Am. J. Med. Sci.,* **204,** 856–75.

O'Reilly, R.A., 1971, Vitamin K hereditary resistance to oral anticoagulent drugs, *Am. J. Physiol.,* **221,** 1327–30.

Passmore, P. and Eastwood, M.A., 1986, *Davidson and Passmore Human Nutrition and Dietetics,* 8th Edn, pp. 140–42, Edinburgh: Churchill Livingstone.

Persson, J., 1991, Alcohol and the small intestine, *Scand. J. Gastroenterol.,* **26,** 3–15.

Pineo, G.F., Gallus, A.S. and Hirst, J., 1973, Unexpected vitamin K deficiency in hospitalized patients, *Can. Med. Assoc. J.,* **109,** 880–83.

Prentice, A.M. and Bates, C.J., 1980, Refection in rats fed on a sucrose-based, riboflavin-deficient diet, *Brit. J. Nutr.,* **43,** 171–77.

Ramotar, K., Conly, J.M., Chubb, H. and Louie, T.J., 1984, Production of menaquinones by intestinal anaerobes, *J. Infect. Dis.,* **150,** 213–18.

Rong, N., Selhub, J., Goldin, B.R. and Rosenberg, I.H., 1991, Bacterially-synthesised folate in rat large intestine is incorporated into host tissue folyl polyglutamates, *J. Nutr.,* **121,** 1955–59.

Roscoe, M.H., 1927, Spontaneous cures in rats reared upon a diet devoid of vitamin B and antineuritic vitamin, *J. Hygiene,* **27,** 103–7.

Russell, R.M., Krasinski, S.D., Samloff, I.M., Jacob, R.A., Hartz, S.C. and Brovender, S.R., 1986a, Folic acid malabsorption in atrophic gastritis, *Gastroenterology,* **91,** 1476–82.

Russell, R.M., Krasinski, S.D., Samloff, I.M., Jacob, R.A., Dallal, C.E., McCandy R.B. and Hartz, S.C., 1986b, Fundic atrophic gastritis in an elderly population, *J. Am. Geriatric Soc.,* **34,** 800–6.

Said, H.M., Redha, R. and Nylander, W, 1989, Biotin transport in the human intestine: inhibition by anticonvulsant drugs, *Am. J. Clin. Nutr.,* **49,** 27–31.

Said, H.M., Sharifan, A., Bagherzadeh, A. and Mock, D., 1990, Chronic ethanol feeding and acute ethanol exposure *in vitro:* effect on intestinal transport of biotin, *Am. J. Clin. Nutr.,* **52,** 1083–86.

Scholtissek, J., Barth, C.A., Hagemeister, H. and Frigg, M., 1990, Biotin supply by large bowel bacteria in minipigs: evidence from intracaecal avidin, *Brit. J. Nutr.,* **64,** 715–20.

Shearer, M.J., Kazim, N. and Mathers, J.C., 1991, Measurement and significance of hepatic menaquinones in rats fed on low-fibre diets, *Proc. Nutr. Soc.,* **50,** 71A.

Shearer, M.J., Allan, V, Haroon, Y. and Barkhan, P., 1980, Nutritional aspects of vitamin K in the human, in Suttie, J.W. (Ed.) *Vitamin K Metabolism and Vitamin K-Dependent Proteins,* pp. 317–32, Baltimore: University Park Press.

Shearer, M.J., Barkhan, P., Rahim, S. and Stimmler, L., 1982, Plasma vitamin K^1 in mothers and their newborn babies, *Lancet,* ii, 460–63.

Shirakawa, H., Komai, M. and Kimura, S., 1990, Antibiotic-induced vitamin K deficiency and the role of the presence of intestinal flora, *Int. J. Vitaminol. Nutr. Res.,* **60,** 245–51.

Stephen, A.M. and Cummings, J.H., 1980, The microbial contribution to human faecal mass, *J. Med. Microbiol.,* **13,** 45–56.

Sugita, H., Takahashi, J. and Deguchi, Y, 1992, Production and consumption of biotin by the intestinal microflora of cultured freshwater fishes, *Biosci. Biotechnol. Biochem.,***56,** 1678–79.

Suter, P.M., Golner, B.B., Goldin, B.R., Morrow, F.D. and Russell, R.M., 1991, Reversal of protein-bound vitamin B_{12} malabsorption with antibiotics in atrophic gastritis, *Gastroenterology,* **101,** 1039–45.

Suttie, J.W., 1985, Vitamin K, in Diplock, A.T. (Ed.) *The Fat-Soluble Vitamins,* pp. 225–311, London: William Heinemann.

Thijssen, H.H.W and Drittij-Reijnders, M.J., 1994, Vitamin K distribution in rat tissues: dietary phylloquinone is a source of tissue menaquinone-4, *Brit. J. Nutr.,* **72,** 415–25.

Udall, J.A., 1965, Human sources and absorption of vitamin K in relation to anticoagulation stability, *J. Am. Med. Assoc.,* **194,** 107–9.

Usui, Y, Tanimura, H., Nishimura, N., Kobayashi, N., Okanoue, T and Ozawa, K., 1990, Vitamin K concentrations in the plasma and liver of surgical patients, *Am. J. Clin. Nutr.,* **51,** 846–52.

Velázquez, A., Zamundio, S., Báez, A., Murguía-Corral, R., Rangel-Peniche, B. and Carrasco, A., 1990, Indicators of biotin status: a study of patients on prolonged total parenteral nutrition, *Eur. J. Clin. Nutr.,* **44,** 11–16.

Weitekamp, M.R. and Aber, R.C., 1983, Prolonged bleeding times and bleeding diathesis associated with moxalactam administration, *J. Am. Med. Assoc.,* **249,** 69–71.

Will, B.H. and Suttie, J.W., 1992, Comparative metabolism of phylloquinone and menaquinone-9 in rat liver, *J. Nutr.,* **122,** 953–58.

Section 7

Biliary excretion and enterohepatic circulation

Chapter 16
Factors governing biliary excretion

R.Coleman and J.K.Chipman

16.1
General aspects of bile formation and enterohepatic cycling

Bile is formed in the liver and delivered to the intestines via the duodenum. It is a complex fluid and its composition varies with the nutritional state and xenobiotic challenge of the individual. In addition to its secretory role of delivering bile acids and phospholipids for the facilitation of lipid digestion and absorption, bile serves the excretory role of delivering waste products to the intestines for elimination from the body. Biliary excretion plays a major role in the elimination of many compounds, both xenobiotics and endobiotics, and the principles balancing biliary excretion against other excretory routes are now becoming better understood.

The haemocirculatory position of the liver in the body is of great importance in its role as an organ of xenobiotic elimination. In many mammalian species the hepatic portal vein delivers approximately 75% of the blood supply and thus the liver is the first port of call for nutrients and xenobiotics leaving the intestines. This gating function, supported by a battery of powerful biliary-directed transport systems, means that extraction from hepatic afferent blood may be extensive. In some cases much of the absorbed compound may be removed on first pass through the liver, thereby avoiding exposure of the remainder of the body to high systemic blood concentrations.

Subsequent to their uptake from the blood many biliary-directed compounds undergo hepatic metabolism, e.g. hydroxylation, conjugation etc., which serve to make the molecules more polar, water-soluble and of higher molecular weight. In this form they are secreted into the bile, and the absence of specific intestinal transport systems militates against passive permeation from the gut lumen, and favours elimination from the body in the faeces.

In some cases, however, the gut flora makes modifications to the molecules in the intestinal lumen; these modifications may involve deconjugation, further metabolism, reconversion to the parent compound, and so on. The resulting molecules, often being more hydrophobic, may then be subject to a greater degree of absorption by passive permeation and pass back to the liver in the portal blood, i.e. an enterohepatic circulation (this is discussed in detail in Chapter 17).

For recent reviews on general aspects of bile formation and enterohepatic cycling see Klaasen and Watkins, 1984; Gregus and Klaasen, 1986; Coleman, 1987; Meijer, 1987; Dobrinska, 1989; Nathanson and Boyer, 1991 and Burwen *et al.,* 1992.

16.2
Functional organization of the hepatobiliary system

Hepatic portal and arterial blood flow together in the hepatic sinusoids and then exit the liver via the hepatic vein. The sinusoids are lined with cells that do not quite form a continuous sheet; fenestrations are present of a size which allow plasma, but not blood cells, into the Space of Disse to bathe the hepatocytes below. Hepatocytes, the parenchymal cells of the liver, are arranged in plates between the sinusoids.

Those hepatocytes first exposed to the afferent blood supply are said to be in Zone 1 and are termed periportal cells; they are exposed to the highest levels of oxygen (from hepatic artery and hepatic portal vein) and of nutrient and xenobiotics taken up from the intestines (hepatic portal vein). Periportal cells are smaller than other hepatocytes and have well-developed mitochondria; they possess higher levels of oxidative metabolism and gluconeogenesis than cells at the other end (Zone 3) of the sinusoid. The cells in Zone 3 are often termed centrilobular, pericentral, or perivenous cells and are larger, have a better developed endoplasmic reticulum and are more active in glycolysis, biotransformation of xenobiotics and in ammonia detoxification. Cells between the two extremes, i.e. Zone 2 cells, have less well defined differences (Klaasen and Watkins, 1984; Burwen *et al.*, 1992).

The hepatocyte plasma membrane facing the Space of Disse is at the sinusoidal pole of the cell and it is here that most of the molecules destined for bile are removed from the plasma. At the biliary pole of the hepatocyte the plasma membranes of at least two adjacent cells are 'zipped' together at the junctional complex to enclose an extracellular secretory lumen, the bile canaliculus. This leads as an anastomosing channel between successive hepatocytes (in the direction perivenous to periportal), until hepatocytes give way to ductular cells and eventually the ductular lumen opens into the common bile duct and subsequently the intestine.

The bile canalicular membrane is seen under higher magnification to be a series of microvilli; these are more pronounced in periportal than in perivenous areas. Microvilli appear to be capable of movement and this may contribute to the flow of bile down the biliary tree. Movement is probably controlled by the molecules of the pericanalicular cytoplasm, since depolymerization or disruption of microfilaments causes cessation of contractions, dilation of the bile canaliculi and reduction in bile flow. The role for other cytoskeletal components, e.g. microtubules, in the processes of bile formation is more controversial (Coleman, 1987; Nathanson and Boyer, 1991; Crawford and Gollan, 1991; Burwen *et al.*, 1992; Arias *et al.*, 1993).

16.3
Composition of bile

From the anatomy of its formation it is clear that the composition of bile may be continuously modified as it passes along the biliary tree, from the canaliculi of pericentral hepatocytes onwards to the intestines. Canalicular bile is difficult to obtain; much of our knowledge of composition is based upon ductular (hepatic) or gall bladder biles. Hepatic biles from several species contain of the order of 3% solids; storage of bile in the gall bladder results in removal of water, inorganic salts etc. and the total solids content may rise to 10–15%. Since such storage times and mixing in the gall bladder are variable, compositions are best discussed in relation to hepatic bile (Klaasen and Watkins, 1984; Coleman, 1987).

Bile is an iso-osmotic extracellular fluid, its ionic composition basically reflecting that of the fluid bathing the cells of the biliary tree, i.e. blood plasma. Like plasma its predominant cation is Na^+ and its principal inorganic anions are Cl^- and HCO^-_3. Here the resemblance to plasma ends as, whilst plasma is protein-rich, bile contains much smaller amounts of protein. Some of these proteins are derived from plasma (albumin, IgA), others originate more specifically from liver cells, e.g. 5'-nucleotidase and secretory component. Superimposed upon the basic composition are the molecules added to bile by the activities of the cells of the biliary tree (Coleman, 1987).

Bile acids are present up to about 35 mM concentration in hepatic bile and there are a wide variety of other organic anions, e.g. endobiotics such as bilirubin glucuronides, steroid sulphates, and the conjugates of drugs, other xenobiotics, and their metabolites. The precise balance and concentrations of all of these will depend upon the metabolic state, and dietary or xenobiotic history of the individual. Bile also contains organic cations and neutral molecules and significant amounts of lipids, e.g. phospholipids and cholesterol (Klaasen and Watkins, 1984, Coleman, 1987; Burwen *et al.,* 1992).

Where material exists in bile below its plasma concentration, biliary excretion is usually only a minor route of elimination from the body, and probably represents either limited passage across tight junctions sealing the canaliculus or other low-specificity mechanisms such as pinocytosis and subsequent transcytosis. Where the material is concentrated in bile relative to plasma this demonstrates a more important excretory route employing the presence of specific concentrative mechanisms in hepatobiliary cells, and also that there is little leakage of the material out from the biliary tree across the tight junctions (Coleman, 1987; Dobrinska, 1989).

16.4
Generation of bile flow

Biliary volume is generated by the secretion of osmotically-active solutes into the bile canaliculus leading to an inward passive movement of water and inorganic ions, mainly across the tight junctions. Fluid-phase endocytosis may also contribute a small amount of biliary volume but hydrostatic filtration, though important in kidney function, appears to have no role in bile formation (Klaasen and Watkins, 1984; Burwen *et al.,* 1992; Boyer *et al.,* 1992).

Bile acids are important generators of biliary volume in all species and changes in rates of bile acid secretion are paralleled by changes in biliary flow. Retention of the negatively charged bile acids within the biliary tree by the negatively charged tight junctions is thus very important. The amount of water (apparent choleretic activity) generated by different bile acid species ranges from 6 to 30 µl/µmole, and is largely determined by the micelle-forming ability (non-micellar more choleretic) and hydrophilicity, e.g. deoxycholate is less choleretic than cholate (Coleman, 1987; Hofmann, 1989; Burwen *et al.,* 1992).

Reduction in bile acid secretion brings about reduction in bile flow. In some cases however, significant bile flow remains even at low rates of bile acid secretion (e.g. in perfused livers) or after extrapolation of bile acid secretion rates to zero. This bile acid independent flow (BAIF) contributes a different proportion in various species; in the rat it makes up about 50% of the total flow whereas in man the proportion is appreciably smaller. The driving force for this flow is likely to be the secretion of osmotically-active organic ions other than bile acids, and which are also retained by the tight junctions. Such can be seen by presenting certain drugs to bile acid-free perfused livers, resulting in a choleresis, and in observations that certain drugs, e.g. phenobarbitone, will increase

bile flow without altering bile acid output. BAIF will therefore rise and fall according to the xenobiotic load on the liver (Klaasen and Watkins, 1984; Hofmann, 1989; Boyer *et al.,* 1992).

In rats, glutathione and its conjugates appear to be responsible for much of the normal BAIF, and agents which modulate glutathione secretion affect bile flow in a parallel fashion. The apparent choleretic activity of glutathione is higher than that of bile acids. Extrapolation of the glutathione output to zero does not abolish all BAIF, however, showing that other solutes also make a contribution to total bile flow (Ballatori and Truong, 1989, 1992; Nathanson and Boyer, 1991).

At low rates of bile acid uptake, periportal cells probably determine the bile acid flow with pericentral cells making an increasing contribution as bile acid input increases. All hepatocytes probably make a contribution to the BAIF, though the proportions between periportal and pericentral cells remain to be established (Yousef *et al.,* 1989).

16.5 *Experimental procedures for studying bile formation*

Bile can be obtained, under anaesthesia, from intact animals via the gall bladder or by cannulating the common bile duct (hepatic bile); hepatic bile lends itself more readily to kinetic and mechanistic studies of bile formation. Some of the problems which may have to be circumvented in whole-animal studies include: (a) the effects of the anaesthetic used; (b) potential mixing of bile with pancreatic juice; and (c) depletion of the enterohepatic circulation. It is now possible, using special procedures, to recycle bile in unanaesthetized animals and thereby avoid some of the above problems, (Coleman, 1987).

The provision of bile by isolated perfused livers allows improved presentation kinetics and concentrations, provides information only from the liver and allows metabolism and biliary output studies to be carried out on a single- or multiple-pass basis. Perfused livers have been much used for biliary studies in recent years (Coleman, 1987).

Bile formation is a property of polarized cells and since such polarity is lost on separating the cells, isolated hepatocytes have been of only limited use in studies of bile formation. In primary culture some isolated hepatocytes may form canaliculi, but the metabolic shortcomings of such cells for xenobiotic metabolism reduce the usefulness of such preparations; newer preparations supplemented to maintain biotransformation profiles may prove to be more useful (Coleman, 1987; Nathanson and Boyer, 1991; Berry *et al.,* 1992; Burwen *et al.,* 1992).

An increasingly useful preparation for hepatobiliary and hepatotoxicity studies is the hepatocyte couplet. Hepatocytes are isolated by a sufficiently mild collagenase digestion that a proportion of them do not separate properly and remain attached to a neighbour at the junctional complex, forming couplets (doublets) if two cells are involved and triplets or small multiples if more than two remain associated. If such cells are then incubated for a short period in a nutritive medium, they re-form tight junctions and seal a canalicular vacuole (rather than a canalicular tube) into which material destined for bile can be secreted. Such preparations have been used for a wide range of studies of uptake, transcellular movement, canalicular secretion, junctional integrity and cholestasis (Boyer *et al.,* 1988; Fentem *et al.,* 1990; Graf *et al.,* 1984; Graf and Boyer, 1990).

Couplets are used within about six hours of preparation thereby minimising the changes in biotransformation activities seen in cells in longer-term culture. This technique is under continuous development and has now been obtained virtually free of single cells (by centrifugal elutriation) and separated into couplets derived from perivenous and periportal regions; the latter preparation

should considerably facilitate zonal studies of the processes of bile formation (Wilton *et al.,* 1991, 1993a,b).

Another approach which has considerably enhanced our knowledge of the transport processes and driving-forces involved in bile formation is the use of sealed-vesicle preparations of known orientation derived from the canalicular membrane (CMV) or sinusoidal plasma membrane (SMV). Such vesicle preparations have recently demonstrated the involvement of ATP in the secretion of many cholephiles into the canaliculus (Meier *et al.,* 1984; Arias *et al.,* 1993).

16.6
Energetics of bile formation

For many materials excreted in bile the biliary concentration of a compound or its metabolites may exceed manifold that of the parent form in the plasma. Thus for bile acids and bilirubin, biliary concentrations are in the tens of millimolar or millimolar ranges respectively, but plasma concentrations are normally in the micromolar range. The same is true for many xenobiotics; infusion of sulphobromophthalein into rats results in a biliary excretion of 30% of the dose within 20 minutes from a plasma concentration of 25 μM to generate a biliary concentration of 25 mM. Such major concentration gradients (plasma to bile) suggest the presence of active transport systems operating at one or both poles of the hepatocyte (Klaasen and Watkins, 1984; Coleman, 1987; Elias *et al.,* 1983).

The hepatocyte plasma membrane has a substantial number of transport systems for ions including: (a) Na^+K^+ ATPase; (b) Ca^{++} ATPase; (c) Na^+/H^+ antiport; and (d) Na^+/HCO^-_3 symport. In the presence of metabolic energy (ATP), ion gradients and a membrane potential can be created across the plasma membrane and these, together with ATP, form the driving forces to accumulate materials into the cell and excrete them from it (Nathanson and Boyer, 1991; Boyer *et al.,* 1992; Arias *et al.,* 1993).

The overall process of biliary excretion can be broken up into a number of individual stages:

1. uptake into the hepatocyte across the sinusoidal membrane;
2. intracellular metabolism and transcellular movement across the hepatocyte to the canalicular membrane;
3. excretion (transport) across the bile canalicular membrane;
4. retention of materials within the canaliculus;
5. subsequent modification of canalicular bile in the ducts and gall bladder.

For some compounds more than one mechanism may operate at the above stages, the relative proportions of each mechanism being determined by the concentration of the cholephile or its metabolites.

16.7
Sinusoidal uptake

The best-characterized uptake process in the sinusoidal plasma membrane is a saturable Na^+-linked high affinity co-transport of bile acids, especially conjugated bile acids, e.g. taurocholate; photoaffinity labelling has shown that a 48kDa protein is involved. This system is competitively

inhibited by a number of xenobiotic compounds, e.g. phalloidin, bumetanide, iodipamide verapamil etc., which appear also to be transported in SMV preparations, but it is not yet clear whether the system is multispecific or whether a family of proteins is involved. The Na^+-dependent bile acid transporter has now, however, been expressed in frog oocytes, so a study of the specificity of this protein should help clarify whether it is a broad-specificity system, or whether there are separate proteins for bile acids and the xenobiotics (Meier, 1989; Frimmer and Ziegler, 1988; Nathanson and Boyer, 1991; Hagenbuch *et al.,* 1990; Burwen *et al.,* 1992; Boyer *et al.,* 1992).

Organic anions are also transported by a saturable Na^+-independent system which can be observed by omitting Na^+ from the medium; the driving force for the transport is probably a Cl- or OH- antiport from the suspending medium—photoaffinity-labelling has identified a molecule of 54kDa to be involved in this case. Once again it is not known whether a single protein or a family of proteins is involved. Amongst the substances transported by this system are sulphobromophthalein (BSP), bilirubin, indocyanine green and bile acids (unconjugated > conjugated) (Nathanson and Boyer, 1991; Berk and Stremmel, 1986; Burwen *et al.,* 1992; Boyer *et al.,* 1992; Berk *et al.,* 1987).

Plasma albumin is usually the vehicle which delivers organic anions and bile acids to the sinusoidal surface of the cell. The kinetics of uptake from albumin are often better than those of the anions in free solution. Albumin receptors have been suggested to account for this phenomenon. However, the evidence for such receptors is controversial and many of the phenomena can be accounted for in other ways (Berk and Stremmel, 1986; Nathanson and Boyer, 1992).

Weak organic anions and non-charged molecules are also taken up by a non-saturable Na^+-independent mechanism, i.e. simple diffusion of somewhat hydrophobic non-charged molecules. The driving force in this case will be the low intracellular concentration of the compound in the cytoplasm maintained due to binding, metabolic modification or excretion from the cell at the biliary pole (Nathanson and Boyer, 1992).

Organic cations are taken up by a saturable process, and show competitive inhibition of uptake, suggesting the involvement of a transport system or systems. For the compound N-methylnicotinamide the driving force appeared to be a H^+-antiport. It has also been suggested that some may be taken up by ion pairs with inorganic anions, using a further multispecific system (Nathanson and Boyer, 1992; Meijer, 1987).

16.8
Intracellular movements and metabolism

Some molecules entering the hepatocyte are excreted directly into the bile unchanged, e.g. indocyanine green, rose bengal, tartrazine. For such molecules the intracellular processing principally involves moving the molecules across the polarized cell to present them to the transport system that moves them on into the canalicular lumen (Coleman, 1987; Klaasen and Watkins, 1984).

For other molecules, however, the situation is more complicated since xenobiotics may be subject to metabolic modification (Phase I biotransformation), conjugation (Phase II biotransformation) or both; movement to the canaliculus clearly must take these processes into account. Most of the enzymes of Phase I biotransformations are found in the endoplasmic reticulum. Interaction with this organelle *en route* to the bile will feature in the transcellular movement. Likewise, some conjugation reactions are found in the membranes of the endoplasmic reticulum (e.g. glucuronidations) or in the

mitochondria (e.g. amino acid conjugation) so that the path of some conjugated compounds through the cell may be a complex one. Other Phase II reactions, e.g. glutathione conjugation, involve cytosolic proteins, so in these cases the pathway may remain in the cytosol but involve interacting with these proteins *en route* (Coleman, 1987; Nathanson and Boyer, 1992; Crawford and Gollan, 1991; Burwen *et al.,* 1992).

Glutathione conjugation often requires the involvement of one of a family of enzymes, the glutathione-S-transferases, all of which will interact with the bound substrates for glutathione conjugation. One of these proteins, glutathione-S-transferase B, binds a wide spectrum of organic anions, including some that are not conjugated with glutathione; this general binding activity is probably distinct from the catalytic site and has resulted in the protein being called ligandin or Y protein. The physiological role of ligandin is probably to maintain low cytosolic concentrations under normal conditions, thereby reducing sinusoidal efflux and general membrane uptake of the anions; ligandin will probably be involved in carrying cholephilic anions to the bile canaliculus. Other binding proteins are found in the liver cytosol, including one more specific for bile acids than other organic anions; this protein is termed the Y binding protein and has 3-αOH-dehydrogenase activity (Coleman, 1987; Klaasen and Watkins, 1984).

Although movement as single molecules, bound to intracellular proteins, towards the canalicular membrane is probably the normal transcellular route at low and normal levels of throughput, there is now an appreciable body of evidence suggesting a role for intracellular organelles in transcellular movement, especially at high excretion rates when the binding proteins would be saturated. Much of this evidence is drawn from work with bile acid transport, e.g. (a) bile acid secretion can be inhibited at high loading-levels by colchicine (an inhibitor of microtubular functions and vesicle movement); (b) bile acids have been observed associated with the Golgi apparatus at high loading-levels; and (c) a transport system for bile acids has been observed in Golgi vesicle preparations (Crawford and Gollan, 1991; Nathanson and Boyer, 1991; Coleman *et al.,* 1989; Meier, 1989).

The evidence for the participation of intracellular organelles in the transport of other organic anions is, however, much less. Clearly, some association of compounds with the endoplasmic reticulum is to be expected for those molecules undergoing metabolic modification or conjugation reactions therein. There are now, however, a few observations of a colchicine or vinblastine inhibition of anion secretion (i.e. bilirubin diglucuronide, diethylmaleate) in high loading-levels, as though this is a back-up mechanism. The involvement of vesicles in bile acid and organic ion transport is still a controversial area, but vesicle involvement is much better-characterized for some of the proteins destined for bile, e.g. pIgA, horseradish peroxidase (Crawford and Gollan, 1991; Coleman, 1987; Roma *et al.,* 1991).

An organized cytoskeleton is an important prerequisite for bile formation since major perturbations with phalloidin or cytochalasin B cause cholestasis; we have also demonstrated bile secretory inhibition following oxidative stress with menadione which affected the cytoskeleton (Stone *et al.,* 1994). The cytoskeleton is important for organizing the directed movement of organelles, for maintaining the spatial relationship of membrane processes; it is also responsible for canalicular contraction and helps to maintain the integrity of tight junctions. Whether the cytoskeleton is also involved in the polarized delivery of cholephiles is a subject for further study (Crawford and Gollan, 1991; Nathanson and Boyer, 1992).

Within the cell, the metabolic transformation of molecules by Phase I metabolism causes the original molecule to become more polar by hydroxyl addition, dealkylation, oxidation of N or S atoms etc. Conjugation of these new or exposed moieties with hydrophilic, negatively charged

groups increases the negative charge on the molecule, making the molecule a better substrate for the canalicular anion transport systems, and increasing the molecular weight, thus allowing the biliary excretion threshold (see 16.10) to be overcome (Klaasen and Watkins, 1984; Coleman, 1987).

16.9
Canalicular secretory processes

Rapid progress has been made in this area in recent years due to the availability of sealed canalicular membrane vesicles (CMV), giving access to the cytosolic face of the membrane and allowing the vectorial presentation of substrates and energy sources, and also allowing the imposition of membrane potentials of known sign and magnitude (Meier *et al.,* 1984; Arias *et al.,* 1993).

Early attention in such studies was focused on the bile acid transport system and it became clear that the characterization of the canalicular transporter differed greatly from the sinusoidal one. It showed somewhat different specificity and kinetics and most strikingly was Na^+-independent, drawing its energy from the membrane potential. The magnitude of this is however only of the order of 40 mV (cytosol negative), whereas to maintain the concentration gradients operating under physiological conditions a gradient of >100 mV would be required. A second system has now been identified which takes its energy from the hydrolysis of ATP. The contribution of the ATP system however still may not be enough to account for all the bile salt transport (Nathanson and Boyer, 1991; Arias *et al.,* 1993; Meier, 1989; Zimniak and Awasthi, 1993; Vore, 1993).

The identification of ATP-driven transport followed the discovery of an ATP-dependent organic cation transport system, involving the multidrug-resistance gene product (MDR), also termed P_{170} or P-glycoprotein. This protein has been identified in the plasma membranes of many cells, including erythrocytes and kidney, and is responsible for the ATP-driven removal from the cytosol of a number of hydrophobic cationic drugs, e.g. daunomycin, adriamycin and vinblastine, resulting in drug resistance in some cells. In the hepatocyte this transporter is localized on the bile canalicular membrane, therefore resulting in biliary rather than sinusoidal excretion. At present, the spectrum of substrates for the liver P-glycoprotein is not fully known nor what proportion it contributes to biliary cation secretion (Arias *et al.,* 1993; Zimniak and Awasthi, 1993; Vore, 1993).

The transport of organic anions across the canalicular membrane is a more distinct process from that of the bile acids than was the case at the sinusoidal membrane. Two organic anion systems have so far been identified. The first is driven by the membrane potential and has a wide specificity for endobiotic and xenobiotic anions, both conjugated and unconjugated. The membrane potential may not account for all the gradient and more recently an ATP-dependent system (or systems) has been identified. This also has a wide specificity, e.g. bilirubin conjugates, sulphates (including bile acid sulphates (Kuipers *et al.,* 1988)), BSP etc., but appears to be particularly important for the excretion of glutathione conjugates and oxidized glutathione (GSSG), though it does not appear to be responsible for the excretion of reduced glutathione (GSH). This ATP-dependent system is often termed MOAT (multispecific organic anion transporter) and may be similar to glutathione-conjugate pumps in other cells. Inhibitors of transported molecules of the MDR system, e.g. verapamil, have no effect upon anion secretion, but transport of particular anions can be reduced by the presence of high concentrations of other anions (e.g. glucuronides, glutathione conjugates) sharing the same transporter (Nathanson and Boyer, 1991; Arias *et al.,* 1993; Zimniak and Awasthi, 1993; Vore, 1993; Ishikawa, 1992; Kuipers *et al.,* 1988).

Genetic deficiency of the MOAT system occurs in TR- rats, which have hyper-bilirubinaemia (conjugated) and poorly excrete various anions. CMV obtained from such rats show defective ATP-dependent anion transport but there is no effect upon the transport of organic cations or bile salts, showing that the MOAT protein is distinct from these other ATP-dependent processes. Anion transport is also defective in humans in the Dubin-Johnson Syndrome and in Corriedale sheep, which both have normal bile acid transport; the relationship of the molecular defect in TR- rats to those in corresponding human and sheep diseases, however, remains to be established (Arias *et al.,* 1993; Zimniak and Awasthi, 1993; Vore, 1993).

Though glutathione conjugates and GSSG are actively excreted by the MOAT system their excretion is not inhibited by GSH; the presence in bile of GSH is probably due to the operation of the potential-driven organic ion transport system (Boyer *et al.,* 1992; Zimniak and Aswathi, 1993; Vore, 1993).

16.10
Retention of components in the biliary tree: biliary versus *renal excretion*

Canalicular bile flows in the direction of the ducts, probably aided by the pumping action of the microvilli. Vital to the maintenance of biliary composition is the sealing action of the tight junctions; these seal the canalicular lumen between hepatocytes and the ductular lumen surrounded by ductular cells. The junctions appear to restrict two-way movement of material between bile and blood, thereby preventing (a) material leaking from bile to blood; and (b) plasma constituents from unrestricted ingress into the bile. The tight junction appears to act as a molecular sieve that is permeable to small molecules; e.g. Na^+, Cl^- etc., allowing bile to be an iso-osmotic fluid, but it is increasingly restrictive to molecules of increasing molecular weight; hepatic tight junctions are of intermediate molecular permeability compared with the ‘loose’ ones in avian salt gland and the ‘tight’ ones in frog skin (Coleman, 1987; Burwen *et al.,* 1992).

Tight junctions are negatively charged structures and are therefore less permeable to organic anions; this may be an important factor contributing to the retention of bile acids and of negatively charged xenobiotic and endobiotic conjugates within the biliary tree. Significant biliary excretion of such organic anions appears to be dependent upon molecular weight; thus, small anions, <300Da, are predominantly excreted via the kidney, whereas above this molecular weight threshold biliary excretion becomes more important (Smith, 1973; Bradley and Herz, 1978; Levine, 1978; Klaasen and Watkins, 1984; Coleman, 1987).

The effect of conjugation of biliary-directed molecules with glucuronic acid (+176Da) sulphate (+96Da) glutathione (+306Da) by Phase II biotransformation reactions results in an increase in molecular weight of the parent compound. This enables the molecular weight threshold to be exceeded and may be an important pre-requisite for biliary excretion. The biliary excretion threshold shows variation within species, being approximately 325,400,475, 500Da for organic anions in rat, guinea pig, rabbit and man respectively. The different threshold for each species may be related to the characteristics of the negatively charged hepatobiliary tight junctions in each species (Hirom *et al.,* 1972; Levine, 1978; Elias et al., 1983; Klaasen and Watkins, 1984; Coleman, 1987).

Some agents bring about a reduction in bile flow (cholestasis) which may be due to an increase in junctional permeability and consequent loss in molecular retention of biliary constituents. Among these cholestasis-inducing compounds are phalloidin, oestradiol glucuronides and a-

naphthylisothiocyanate (ANIT); in the case of ANIT, progressive reduction of molecular sieving was observed, yielding increasing permeability to molecules of increasing molecular weight (Kan and Coleman, 1986; Coleman, 1987; Nathanson and Boyer, 1991).

16.11
Modifications to bile during passage through the biliary tree

A number of modifications may occur to the bile during onward passage down the biliary tree. In the canaliculus bile salts often exceed their critical micellar concentration and associate together forming micelles; in addition they also cause the release into the biliary tract of the biliary lipids (phospholipid and cholesterol) initially as vesicles, and, after processing, as mixed micelles. Some of the more hydrophobic constituents of the bile may also become associated with these vesicles and mixed micelles (Coleman, 1987; Coleman and Rahman, 1992).

A particularly important modification to the biliary constituent relates to the hydrolysis of glutathione conjugates, which occurs in the biliary tract, forming cysteinyl glycine and cysteine conjugates (Stein *et al.,* 1988; Nathanson and Boyer, 1991).

In the ductular lumen some bile acids, notably ursodeoxycholate, are reabsorbed in protonated form and re-excreted from hepatocytes, thereby generating further bile acid-dependent flow. In the gall bladder some bile acids, salts and water may be reabsorbed yielding a concentrated, but still iso-osmotic fluid. A number of xenobiotics may also be reabsorbed here yielding an 'enterohepatic' circulation based on the liver and gall bladder rather than the liver and intestines, and thus not involving the intestinal microflora (Hofman, 1989; Gregus and Klaasen, 1986, Nathanson and Boyer, 1991).

16.12
Conjugation of native or biotransformed molecules as a determinant of biliary secretion: biliary versus *renal excretion*

The distinction between whether a material is excreted in the bile or in the urine is often determined by the reactions that material has undergone in the liver. A few materials are excreted in the bile unchanged, e.g. some organic anions, but in many cases biotransformation and/or conjugation reactions appear to be a prerequisite for biliary excretion.

The molecules that result from Phase I biotransformation reactions, e.g. hydroxylations, demethylations etc., are rarely excreted in bile in unconjugated form but the metabolic modification may be behaving here as a prelude for the conjugation reaction. Changes in biotransformation activities, e.g. induction of individual cytochrome P_{450} species, may alter the rate of biliary excretion, e.g. of acetaminophen (Gregus *et al.,* 1990), though some inducers, e.g. methylcholanthrene, pregnenolone-16α-carbonitrile, may, in addition, bring about changes in the activities of conjugating enzymes (Madhu *et al.,* 1989; Gregus *et al.,* 1990).

Hepatic conjugation reactions play a dual role in determining biliary excretion. Conjugation with glutathione, glucuronic acid or sulphate will add to the original molecule a negative charge or charges and convert the molecule to a substrate, or a better substrate, for the organic anion transport systems in the canalicular membrane (Coleman, 1987). It will also increase the molecular weight of the compound by 306Da (glutathione), 176Da (glucuronic acid) or 96Da (sulphate). The increase in molecular weight may determine whether the biliary excretion threshold is exceeded; this

threshold is probably one of the most important determinants of biliary *versus* renal excretion (Hirom *et al.,* 1972; Klaasen and Watkins, 1984; Coleman, 1987; Firman *et al.,* 1990). From this information, it is clear that it is the physicochemical property of the excreted metabolite (not the parent compound) that should be considered when attempting to predict biliary excretion. Differences in the biliary excretion threshold contribute to differences in the extent of biliary excretion between species (see 16.10).

It is interesting that, where a compound can be conjugated by more than one reaction, it is usually the lower molecular mass forms which occur in the urine (Gregus *et al.,* 1988). Glutathione conjugation is therefore an especially important determinant for biliary excretion since the molecular weight gain here is most extensive (+306Da), and glutathione even brings about excretion of combined metals (Houwen *et al.,* 1990). The glutathione conjugate of acetaminophen can be used to indicate the extent of reactive metabolite formation from acetaminophen (Madhu *et al.,* 1989). If glutathione levels in the liver are reduced, the proportion of other (smaller) conjugates, e.g. sulphates, glucuronides, may rise in both blood and urine giving a diversion from bile to kidney (Gregus *et al.,* 1990). This may represent the smaller conjugates escaping through the junctions in the hepatobiliary system to be ultimately lost in the kidney.

Some mammalian species have appreciable τ-glutamyl transpeptidase activity in the biliary tract and thus can cleave glutathione conjugates to cysteinyl glycine or cysteinyl conjugates. In those species in which this occurs substantially, biliary excretion of total thiols and of methyl mercury is the lowest (Stein *et al.,* 1988). This could represent the escape of the lower molecular weight conjugates from the biliary tract via the tight junctions, as well as potential reabsorption of less polar species.

Species variability in biliary excretion is thus a complex set of interacting variables, being a combination of differences in the biotransformation and conjugation profiles, differences in biliary excretion threshold and differences in subsequent processing in the biliary tract.

16.13
Summary

Many xenobiotics are channelled through the route of biliary excretion via hepatocytes. Two organic anion transporters have been identified. Hepatic metabolism can be a major determinant in providing the physicochemical parameters necessary for biliary excretion such as sufficient polarity and molecular weight.

Species differences in biliary excretion are governed largely by the metabolic status, the molecular weight thresholds for biliary excretion and subsequent processing in the biliary tract.

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References

Arias, I.M., Che, M., Gatmaitan, Z., Leveille, C., Nishida, T. and St. Pierre, M., 1993, The biology of the bile canaliculus, *Hepatology,* **17,** 318–29.

Ballatori, N. and Truong, A.T., 1989, Relation between biliary glutathione excretion and bile acid independent bile flow, *Am. J. Physiol,* **256,** G22-G30.

Ballatori, N. and Truong, A.T., 1992, Glutathione as a primary osmotic driving force in hepatic bile formation, *Am. J. Physiol.,* **263,** G617–24.

Berk, P.D. and Stremmel, W., 1986, Hepatocellular uptake of organic anions, in Popper, H. and Schaffner, F. (Eds) *Progress in Liver Disease,* Vol. VIII, pp. 125–414, New York: Grune and Stratton.

Berk, P.D., Potter, B.J. and Stremmel, W., 1987, The sinusoidal surface of the hepatocyte: a dynamic plasma membrane specialized for high volume molecular transit, in Reutter, W. (Ed.) *Modulation of Liver Cell Expression,* pp. 107–25, Boston: HTP Press.

Berry, M.N., Halls, H.J. and Grivell, M.B., 1992, Techniques for pharmacological studies with isolated hepatocyte suspensions, *Life Sci.,* **51,** 1–16.

Boyer, J.L., Gautam, A, and Graf, J., 1988, Mechanisms of bile secretion: insights from the isolated rat hepatocyte couplet, *Sem. Liver Dis.,* **8,** 308–16.

Boyer, J.L., Graf, J. and Meier, P.J., 1992, Hepatic transport systems regulating pHi, cell volume and bile secretion, *Ann. Rev. Physiol.,* **54,** 415–38.

Bradley, S.E. and Herz, R., 1978, Permselectivity of bile canalicular membranes in rats: clearance probe analysis, *Am. J. Physiol.,* **235,** E570–6.

Burwen, S.J., Schmucker, D.L. and Jones, A.L., 1992, Subcellular and molecular mechanisms of bile secretion, *Int. Rev. Cytol.,* **135,** 269–313.

Coleman, R., 1987, Biochemistry of bile secretion, *Biochem. J.,* **244,** 249–61.

Coleman, R. and Rahman, K., 1992, Lipid flow in bile formation, *Biochim Biophys. Acta,* **1125,** 113–33.

Coleman, R., Rahman, K., Bellringer, M.E., Kan, K.S. and Hamlin, S.H., 1989, Secretion of biliary lipids and its control, in Paumgartner, G., Stiehl, A. and Gerok, W. (Eds) *Trends in Bile Acid Research,* pp. 161–76, London: Kluwer.

Crawford, J.H. and Gollan, J.L., 1991, Transcellular transport of organic anions in hepatocytes: still a long way to go, *Hepatol,* **14,** 192–97.

Dobrinska, M.R., 1989, Enterohepatic circulation of drugs, *J. Clin. Pharmacol.,* **29,** 577–80.

Elias, E., Iqbal, S., Knutton, S., Hickey, A. and Coleman, R., 1983, Increased tight junction permeability: a possible mechanism of oestrogen cholestasis, *Eur. J. Clin. Investig.,* **13,** 383–90.

Fentem, J.H., Foster, B., Mills, C.O., Coleman, R. and Chipman, J.K., 1990, Biliary excretion of fluorescent cholephiles in hepatocyte couplets: an *in vitro* model for hepatobiliary and hepatotoxicity studies, *Toxicol. In Vitro,* **4,** 452–57.

Firman, S., Leandersson, P., Tagesson, C. and Svanvik, J., 1990, Biliary excretion of different-sized polyethylene glycols in the cat, *J. Hepatol.,* **11,** 215–20.

Frimmer, M. and Ziegler, K., 1988, The transport of bile acids in liver cells, *Biochim. Biophys. Acta.,* **947,** 75–99.

Graf, J.A. and Boyer, J.L., 1990, The use of isolated rat hepatocyte couplets in hepatobiliary physiology, *J. Hepatol.,* **10,** 387–94.

Graf, J.A., Gautam, A. and Boyer, J.L., 1984, Isolated rat hepatocyte couplets: a primary secretory unit for electrophysiologic studies of bile secretory function, *Proc. Nat. Acad. Sci.,* **81,** 6516–20.

Gregus, Z. and Klaasen, C.D., 1986, Enterohepatic circulation of toxicants, in Rozman, K. and Hänninen, O. (Eds) *Gastrointestinal Toxicity,* pp. 57–116, Amsterdam: Elsevier.

Gregus, Z., Madhu, C. and Klaasen, C.D., 1988, Species variation in toxication and detoxication of acetaminophen *in vivo:* a comparative study of biliary and urinary excretion of acetaminophen metabolites, *J. Pharmacol. Exper. Therapeut.,* **244,** 91–98.

Gregus, Z., Madhu, C. and Klaasen, C.D., 1990, Effect of microsomal enzyme inducers on biliary and urinary excretion of acetaminophen metabolites in rats, *Drug Metab. Dispos.,* **18,** 10–19.

Hagenbuch, B., Lubbert, H., Stieger, B. and Meier, P.J., 1990, Expression of the hepatocyte Na+/bile acid cotransporter in *Xenopus laevis* oocytes, *J. Biol. Chem.,* **265,** 5357–60.

Hirom, P.C., Millburn, P., Smith, R.L. and Williams, R.T., 1972, Species variation in the threshold molecular weight factor for the biliary excretion of organic anions, *Biochem. J.,* **129,** 1071–77.

Hofmann, A.F., 1989, Current concepts of biliary secretion, *Dig. Dis. Sci.,* **34,** 16S-20S.

Houwen, R., Dijkstra, H., Kuipers, F., Smit, E.P., Havinga, R. and Vonk, R.J., 1990, Two pathways for biliary copper excretion in the rat: the role of glutathione, *Biochem. Pharmacol.,* **39,** 1039–44.

Ishikawa, T., 1992, The ATP-dependent glutathione S-conjugate export pump, *Trends Biochem. Sci.,* **17,** 463–68.

Kan, K.S. and Coleman, R., 1986, 1-Naphthylisothiocyanate-induced permeability of hepatic tight junctions to proteins, *Biochem. J.,* **238,** 323–28.

Klaasen, C.D. and Watkins, J.B., 1984, Mechanisms of bile formation, hepatic uptake and biliary secretion, *Pharmacol. Rev.,* **36,** 2–67.

Kuipers, F., Enserink, M., Havinga, R., van der Steen, Ad.B.H., Hardonk, M.J., Fevery, J. and Vonk, R., 1988, Separate transport systems for biliary secretion of sulphated and unsulphated bile acids in the rat, *J. Clin. Invest.,* **81,** 1593–99.

Levine, W.G., 1978, Biliary excretion of drugs and other xenobiotics, *Ann. Rev. Pharmacol. Toxicol.,* **18,** 81–96.

Madhu, C., Gregus, Z. and Klaasen, C.D., 1989, Biliary excretion of acetaminophen-glutathione as an index of toxic activation of acetaminophen: effect of chemicals that affect acetaminophen toxicity, *J. Pharmacol. Exper. Therapeut.,* **248,** 1069–79.

Meier, P.J., 1989, The bile salt polarity of hepatocytes, *J. Hepatol,* **9,** 124–29.

Meier, P.J. and Meier-Abt, A., Barret, C. and Boyer, J.L., 1984, Mechanisms of taurocholate transport in canalicular and basolateral rat liver plasma membrane vesicles, *J. Biol. Chem.,* **259,** 10614–22.

Meijer, D.K., 1987, Current concepts on hepatic transport of drugs, *J. Hepatol.,* **4,** 259–68.

Nathanson, M.H. and Boyer, J.L., 1991, Mechanisms and regulation of bile secretion, *Hepatology,* **14,** 551–66.

Roma, M., Marinelli, R.A. and Garay, E.A.R., 1991, Biliary excretion of polyethylene glycol molecular weight 900, *Biochem. Pharmacol,* **42,** 1775–81.

Smith, R.L., 1973, *The Excretory Function of Bile,* London: Chapman and Hall.

Stein, A.F., Gregus, Z. and Klaasen, C.D., 1988, Species variations in biliary excretion of glutathione-related thiols and methylmercury, *Toxicol. Appl. Pharmacol.,* **93,** 351–59.

Stone, V, Johnson, G.D., Wilton, J.C., Coleman, R. and Chipman, J.K., 1994, Effect of oxidative stress and disruption of calcium homeostasis on hepatocyte canalicular function in vitro, *Biochem. Pharmacol.*

Vore, M., 1993, Canalicular transport: discovery of ATP-dependent mechanisms, *Toxicol. Appl. Pharmacol.,* **118,** 2–7.

Wilton, J.C., Coleman, R., Lankester, D.J. and Chipman, J.K., 1993a, Stability and optimization of canalicular function in hepatocyte couplets, *Cell Biochem. Function,* **11,** 179–85.

Wilton, J.C., Chipman, J.K., Lawson, C.J. Strain, A.J. and Coleman, R., 1993b, Periportal and perivenous-enriched hepatocyte couplets: differences in canalicular activity and in response to oxidative stress, *Biochem. J.,* **292,** 773–79.

Wilton, J.C., Williams, D., Strain, A.J., Parslow, R.A, Chipman, J.K. and Coleman, R., 1991, Purification of hepatocyte couplets by centrifugal elutriation, *Hepatol.,* **14,** 180–83.

Yousef, I., Dionne, S., Plaa, G., 1989, Zonal diversity or zonal heterogeneity in bile secretion, *Gastroenterology,* **96,** 1230–31.

Zimniak, P. and Awasthi, Y., 1993, ATP-dependent transport systems for organic anions, *Hepatology,* **17,** 330–39.

Chapter 17
Mechanism and consequences of enterohepatic circulation

J.K.Chipman and R.Coleman

17.1
Introduction

As with much of the pioneering work on the biliary excretion of foreign compounds, considerable fundamental work on the enterohepatic circulation (EHC) of agents was performed at St. Mary's Hospital Medical School as reviewed by Smith (1973). The wide range of agents undergoing this process is reviewed by Gregus and Klaasen (1986). More recently, the process of EHC of drugs and some of its consequences regarding pharmacokinetics have been reviewed by Dobrinska (1989). It is not the purpose here to give an exhaustive list of examples, but to illustrate certain phenomena and to discuss the significance of the process, highlighting the important role of the microflora.

The wide variety of classes of compounds subject to EHC is indicated in Table 17.1.

17.2
Role of microfloral metabolism in EHC and species differences

A scheme for the involvement of the intestinal microflora in the process of EHC is indicated in Figure 17.1. Much of the xenobiotic metabolism catalyzed by the microflora is degradative, reductive and hydrolytic (see Chapter 2) in contrast to the generally oxidative and synthetic pathways catalyzed in the liver and other tissues. As a consequence, the influence of the microflora on biliary-excreted conjugates of xenobiotics is generally to reduce polarity and molecular weight. Reabsorption of the products may thus be enhanced (through increased lipid solubility) and the compound may subsequently be re-excreted either via the urine or via the bile to form an enterohepatic cycle (Figure 17.1). Of major importance is the hydrolysis of glucuronic acid conjugates to their respective aglycones by the β-glucuronidase of the microflora (Drasar and Hill, 1974).

The critical role of the microflora can be illustrated by the use of either antibiotics or by inhibition of β-glucuronidase activity. Thus the EHC of oestradiol and mestranol is markedly inhibited by neomycin and by the β-glucuronidase inhibitor, D-saccharic acid-1, 4-lactone in rats (Brewster *et al.*, 1977). Antibiotic suppression of the microflora has also been shown to decrease the EHC of norethisterone (Back *et al.*, 1980), morphine and phenolphthalein (Parker *et al.*, 1980). In germ-free rats with very low activity of faecal β-glucuronidase, the reabsorption of warfarin derived from its

Table 17.1 Examples of chemicals that undergo biliary excretion and enterohepatic circulation in animals or humans

Type of agent	*Example of chemical*
Neuroactive drugs	Chlorpromazine Diazepam Morphine
Hormonally active drugs	Testosterone Spironolactone Diethylestradiol
Circulatory system drugs	Digitoxin Digoxin Warfarin
Gastrointestinal active drugs	Phenolphthalein
Analgesic or anti-inflammatory drugs	Paracetamol (acetaminophen) Indomethacin
Antibiotic drugs	Griseofulvin Penicillins Chloramphenicol
Carcinogens	Benzo(a)pyrene Benzidine
Anti-tumour drugs	Aniline mustard Adriamycin
Vitamins	Vitamin A Vitamin D
Endogenous materials	Bile acids Bilirubin
Food additives	Amaranth
Pesticides or derivatives	Propachlor 3-Phenoxybenzoic acid
Metals	Methylmercury
Industrial chemicals	Hexachlorobutadiene

Taken from Gregus and Klaasen (1986) or other examples cited herein.

glucuronide in the bile is substantially lower than in rats with an enteral microfloral population (Remmel *et al.*, 1981).

In the case of ester glucuronides of carboxylic acids, a non-enzymatic, intramolecular rearrangement of the glucuronic acid moiety can render the molecule resistant to hydrolysis by *β*-glucuronidase (Sinclair and Caldwell, 1982). Consequently, conjugates of this type, such as of valproic acid, excreted in rat bile are less able to undergo EHC (Dickinson *et al.*, 1985). The acyl migration of the 1β–0-acyl glucuronide of 4–[2–(4-isopropylbenzamido)ethoxy]benzoic acid, either in the bile or within the small intestine, substantially decreases the EHC of this agent, based on measurement of pharmacokinetic parameters (Komura *et al.*, 1992).

Following the administration of [^{14}C]-phenolphthalein glucuronide to the rat duodenum, the aglycone was absorbed into the body and was re-excreted in the bile in the same form as the initial glucuronide (Hirom *et al.*, 1975). Fifty per cent of the re-excreted material appeared in the bile

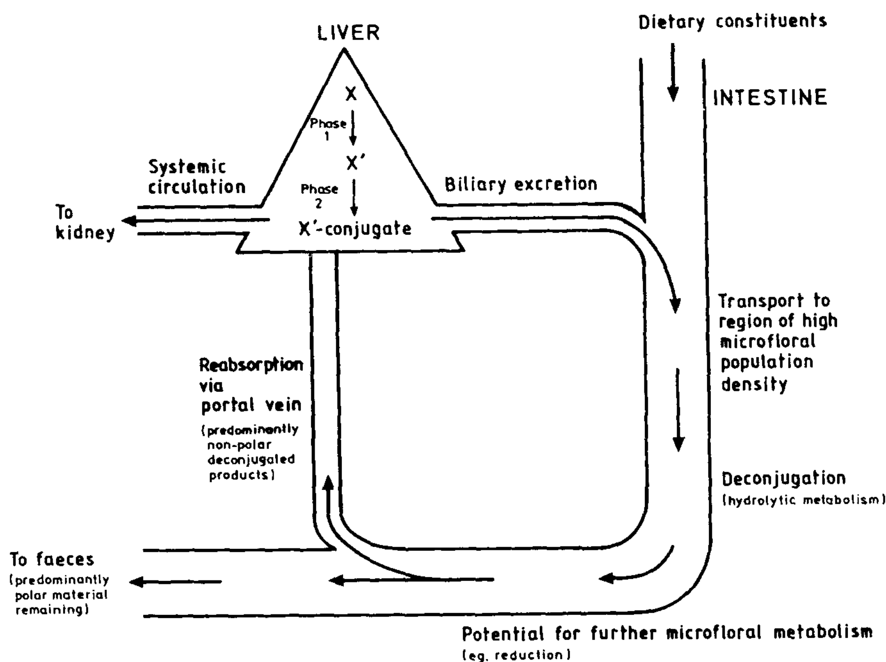

Figure 17.1 Diagram to follow the enterohepatic circulation of metabolites of compound X from the liver. Note the involvement of Phase 1 and Phase 2 (conjugation) metabolism. Following deconjugation in the intestine and subsequent intestinal absorption, there is the potential for a consequent increase in systemic availability in enterohepatorenal disposition.

approximately six hours after the administration of the material in the duodenum. The delay is largely due to the time required for deconjugation of the agent by the microflora; it may be influenced by the fact that β-glucuronidase is associated with genera such as *Bacteroides,* which are generally at a relatively high concentration in the lower regions of the intestinal tract.

The activity of β-glucuronidase in the small intestine is, however, much higher in the rat and mouse than in the guinea pig, rabbit and man (Drasar and Hill, 1974) and thus the time for an enterohepatic cycle to occur (and the extent of this occurrence) is likely to vary between species. Thus, as with the process of biliary excretion (see Chapter 16), the rabbit appears to be a better model for man regarding EHC than does the achlorhydric rat that also lacks a gall bladder. Great caution is therefore required in extrapolation from rats to humans. This is illustrated by the fate of the sedative lormetazepam (Hellstern *et al.,* 1990). The rat differs in this respect from other species in that approximately only 50% of biliary metabolites are reabsorbed from the intestine. In contrast there is almost complete urinary excretion in the rabbit despite an initial extensive secretion into bile. In humans only negligible amounts of lormetazepam appear in bile and hence extrahepatic cholestasis does not affect its clearance. It is difficult to study enterohepatic circulation in humans. In patients with bile duct stenosis, a nasobiliary drain may be introduced into the common bile duct for symptomatic treatment, overcoming some of the disadvantages of other techniques such as T-tube, multilumen duodenal tubes (mdt), and balloon-occludable mdt (as discussed by Hellstern *et al.,* 1990). Semmes and Shen (1990) highlight a number of methodological issues that have not been

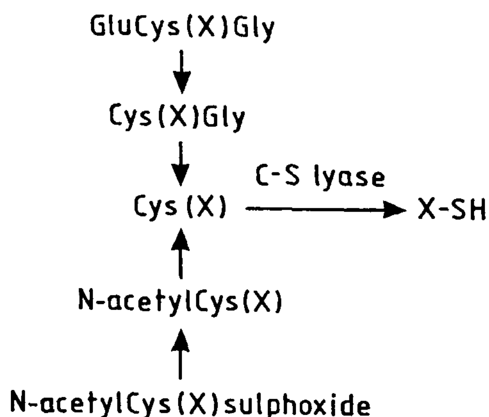

Figure 17.2 Outlines of intestinal metabolism of MAP and pre-MAP metabolites

resolved in the quantitation of the kinetics of EHC in humans and present a means by which the functional parameters for each step of the EHC can be determined experimentally.

β-Glucuronidase is not the only activity of the microflora that can influence EHC. Biliary thioether conjugates of the insecticide propachlor (2-chloro-N-isopropylacetanilide) in the rat are degraded in the intestine partly by the action of bacterial β-lyase, which leads to reabsorption of the product from the intestine and further metabolism (Bakke *et al.,* 1980; Larsen and Bakke, 1981). A major proportion of propachlor is excreted in rat bile as products of the mercapturic acid pathway (MAP; glutathione, cysteinylglycine, N-acetylcysteine and cysteine conjugates). The appearance of six methylsulphone metabolites in the urine was dependent on the intestinal microfloral action on the biliary metabolites. This involves the conversion of glutathione conjugates to cysteine conjugates by microbial and intestinal enzymes. Reabsorption into the body then follows degradation by β-lyase and possible methylation in the intestinal tissue (Bakke and Gustafsson, 1986). The pathway of intestinal metabolism is outlined in Figure 17.2.

A similar disposition occurs with thio-ether metabolites of pentachlorothioanisole (a metabolite of hexachlorobenzene and pentachloronitrobenzene). Methylthiocontaining metabolites are thought to be produced by the action of bacterial β-lyase and subsequent methylation in tissues. The products are then conjugated with glutathione and re-excreted in bile to appear in the faeces as 1, 4-bis(methylthio)tetrachlorobenzene. In the absence of the microflora, mercapturates appear in the faeces, probably due to the lack of N-deacetylase-catalyzed degradation to cycleable cysteine conjugates (Bakke *et al.,* 1990).

In species such as the pig and chicken that are poor biliary excretors of propachlor, metabolism and excretion across the intestinal wall provide an alternative route for the cysteine conjugate of propachlor to reach the intestine (Struble, 1991). This mode of delivery is not unique to propachlor and can be a major route of elimination of certain lipophilic compounds (Rozman, 1985).

The glutathione conjugates of benzo(a)pyrene-4, 5-epoxide (Elmhirst *et al.,* 1985) and naphthalene metabolites (Hirom *et al.,* 1983) also undergo considerable EHC in the rat, although the metabolic changes with these two agents differ. Naphthalene [S-(1, 2– dihydro-1-hydroxy-2-naphthyl) glutathione], is re-excreted in bile mainly unchanged within six hours of administration to the duodenum; the glutathione conjugate of benzo(a)pyrene-4, 5-epoxide however, is re-excreted only

after eight hours as an unidentified aryl-sulphatase-sensitive product. Using isolated *in situ* intestinal loops, evidence was obtained for the action of intestinal cell τ-glutamyltranspeptidase and aminopeptidase on benzo(a)pyrene-4, 5-epoxide glutathione conjugate, allowing the transport of mercapturic acid pathway metabolites. The critical role of thio-ether degradation of biliary metabolites of hexachlorobutadiene, leading to intestinal absorption and subsequent metabolism by *β*-lyase in the kidney is discussed later in this chapter (section 17.5) in relation to toxicity.

During the enterohepatic cycle, it is clear that a compound may be metabolized not only by the intestinal microflora and the liver but also by the tissue of the intestinal wall. In line with this, salicylamide administered to a closed intestinal loop in the rabbit was absorbed into the mesenteric venous blood largely (ca. 60%) in the form of its glucuronide (Barr and Riegelman, 1970). Glucuronidation by the intestinal mucosa also occurs during the reabsorption of aglycones produced by the microflora from biliary glucuronic acid conjugates of the carbamate insecticide 1-naphthyl-N-methylcarbamate (Pekas, 1983; Struble *et al.,* 1983). Sulphate conjugation in the intestinal tissue is particularly active (Powell *et al.,* 1974) and this conjugation step is likely to halt the EHC by producing a product relatively more suitable for elimination in the urine.

In contrast to the various cases described above, in some cases the EHC of a xenobiotic may be inhibited by the action of the intestinal microflora. This is particularly evident with alkyl metals that may be dealkylated to release the inorganic metal (e.g. mercury) which is less readily absorbed (Norseth, 1971). Interestingly, methyl mercury may even be absorbed from the gall bladder prior to reaching the intestinal tract (Dutczak *et al.,* 1991)

17.3
The influence of EHC on pharmacokinetics and persistence

The systemic blood levels of agents that undergo EHC can sometimes be shown to oscillate according to the time intervals between each cycle, gradually leading to the loss of the agent from the body through the fraction of the material that escapes the enterohepatic cycle. This is illustrated in the hypothetical model in Figure 17.3, for an agent that undergoes successive conjugation and hydrolysis to the parent compound during each cycle. In humans, where there is sporadic emptying of the gall bladder, the temporal relationships between each cycle are far more complex, and the secondary or multiple peaks of plasma concentrations often cannot be clearly detected. Mathematical modelling of this process can be achieved, e.g. from the following equation (Tse *et al.,* 1982):

Effective dose=intravenous bolus dose×$[1+FbFa+(FbFa)^2+(FbFa)n^{-1}]$, where Fb and Fa represent the fraction of the drug excreted in bile (Fb) and the fraction of excreted drug in bile that is subsequently reabsorbed (Fa).

This equation simplifies to:

$$\text{Effective dose} = \frac{\text{Dose}}{1-\text{FbFa}}$$

From these considerations it is clear that when a compound is well-absorbed from the intestine and where biliary excretion is a major clearance mechanism of both the parent compound and the recycled materials (in many cases identical to the parent compound), the ‘cumulative’ or ‘effective’ dose of the agent can greatly exceed the initial dose administered. In these cases, however, the ‘first-pass effect’ of the liver may limit the availability of some agents into the general circulation from the EHC (for example in the case of endogenous bile salts). With some other substances, interruption of

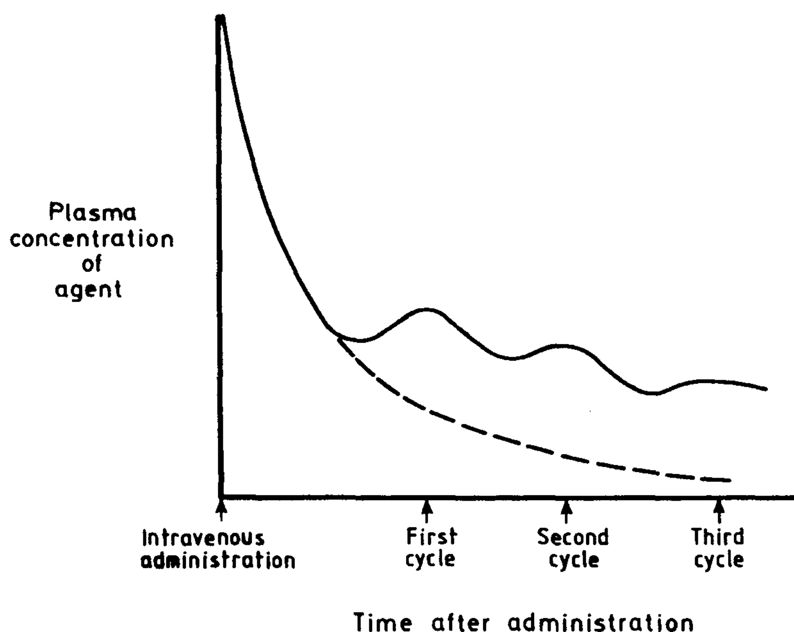

Figure 17.3 Illustration of plasma concentrations of a hypothetical drug with respect to time after intravenous injection. (Broken line indicates exponential decline in the absence of EHC; solid line is the decline including secondary plasma peaks due to EHC). In this case the parent molecule is successively conjugated and deconjugated during EHC. Note that clear secondary peaks are rarely discernible in humans but the influence on persistence (reduced clearance) is still present.

the EHC can decrease systemic availability dramatically. The pulmonary levels of methylsulfonyltrichlorobiphenyl, for example, are decreased by up to 99% in rats by bile duct cannulation (Bakke *et al.,* 1982). Therefore, without the knowledge of the process of EHC, the dosage regimen for a drug may not be optimal because of errors made in the calculation of the half-life.

By using ‘area under the concentration-time curve’ measurements in intact, bile ductcannulated and bile duct-cannulated/renal ligated rats, Horton and Pollack (1991) determined that approximately 16% of an intravenous dose of morphine was subject to EHC, which clearly contributed to systemic availability. As is often the case, the possible occurrence of such a phenomenon in humans is not fully understood. The confusion is evident in the letter by Hanks *et al.* (1988) concerning morphine.

Shepard *et al.* (1989) have developed a model of EHC which includes separate liver and gall bladder compartments, discontinuous gall bladder-emptying, and first-order kinetics of absorption. This approach is very useful for deriving a realistic mean residence time of a drug that undergoes EHC, although it is limited by the assumption of complete reabsorption from the gut. Particularly important in this study is the recognition that mean absorption times of a drug may be dominated by the time-course of recycling, and should be considered in decisions on *in vitro* release characteristics of oral drug formulations. Control of gall bladder emptying may also be a mechanism to reduce variability in pharmacokinetic studies on compounds that undergo EHC.

Interference of the EHC therefore can shorten the β-phase half-life of agents such as that of diazepam in humans (Sellman *et al.,* 1975) and can also decrease urinary excretion of various agents (e.g. nafenopin; Levine *et al.,* 1975). Marselos *et al.* (1975) suggest that the reduced biological activity of phenobarbitone and progesterone upon treatment with saccharolactone was through the inhibition of EHC. Treatment of rats with saccharolactone was, however, not effective in reducing EHC of T2-toxin glucuronide, which is a process thought to contribute to the protracted effects of T-2 toxicosis (Coddington *et al.,* 1989). Because of a relatively low activity of microfloral β-glucuronidase in neonates, limited EHC of agents such as diethylstilboestrol render them more persistent than in adults (Belknap *et al.,* 1981). From a clinical point of view, attempts to block EHC are more appropriately aimed at directly preventing reabsorption of a compound rather than inhibiting the function of microflora, thus cholestyramine has been particularly effective in inhibiting the EHC of a range of agents (Gregus *et al.,* 1980; Ballhorn *et al.,* 1981; Rosman *et al.,* 1982) including acetaminophen (Siegers *et al.,* 1983).

The influence of EHC on persistence of compounds is well-demonstrated by the insecticide DDT (2, 2-bis([-chlorophenyl)1, 1, 1-trichloroethane). The high lipid solubility and relatively slow metabolism (largely to DDA (2, 2-bis(p-chlorophenyl) acetic acid) are major determinants of the persistence of DDT. However, DDA is almost entirely excreted in the bile of rats, and when bile from rats given DDA was infused into the duodenum of recipient rats, 67% was re-absorbed and re-excreted in the bile (Gingell, 1975).

Various steroid hormones undergo an EHC which can be decreased by the suppression of the intestinal microflora. This has implications regarding the therapeutic level of contraceptive steroids, the efficacy of which may be markedly influenced by EHC. In situations where oral antibiotic treatments are given concurrent with contraceptive steroids, this may lead to failure of contraception (Back *et al.,* 1978). The plasma half-life of ethynodiol diacetate, for example, was approximately halved in Rhesus monkeys treated with rifampicin (Lewis *et al.,* 1980). However, the reduced efficacy of oral contraceptive steroids following antibiotic treatment has not been confirmed in controlled studies in humans. Despite the fact that *Clostridium* spp. are known to play a major role in the hydrolysis of sulphates and glucuronides of ethinyl oestradiol (Orme and Back, 1990), the blood levels of this steroid were not reduced by suppression of the microflora with oral antibiotics in human subjects; indeed the antibiotic cotrimoxazole actually enhanced the activity of ethinyl oestradiol.

Microfloral deconjugation and subsequent EHC play a critical role in maintaining the concentration of the active metabolites of phenolphthalein in the intestinal tract as a laxative (Sund *et al.,* 1981). This represents a dramatic dependence on EHC for efficacy.

Several lines of study have shown that various endogenous substances (in addition to obvious candidates such as the bile salts) follow an EHC. Rutgers *et al.* (1989) investigated the fate of glucuronide and sulphate conjugates of T_3 and found that biliary elimination occurred in the rat provided that deiodination of the latter is prevented. Reabsorption of T_3 into the circulation was greater in normal rats compared with those with reduced microflora.

Finally, in this section an important observation by Van-Eldere *et al.* (1988) should be highlighted, concerning the interpretation of data from germ-free animals. These authors demonstrated that, through the use of microflora that reduce the caecal volume in rats, the absorption and EHC of steroids was greatly influenced by the physiological modification of intestinal contents.

17.4
The influence of EHC on the ultimate excretory route

As well as affecting the persistence of xenobiotics, EHC can also be a determinant of the ultimate route of excretion and the nature of the ultimate metabolites (see also 17.2). Some biliary excreted compounds may eventually be excreted via the urine as different metabolites, partly due to the action of enteric microflora. The term 'enterohepatorenal disposition' describes this phenomenon, as, for example, with 3-phenoxybenzoic acid (a major metabolite of the photo-stable pyrethroid insecticides) (Huckle *et al.,* 1981). The process is indicated in Figure 17.1. Although almost one-half of an intraperitoneal dose (100 mg/kg) of 3-phenoxybenzoic acid was excreted in rat bile, only 7% appeared in the faeces. The reason for this is that the biliary glucuronic acid conjugates of 4'-OH-3-phenoxybenzoic acid are cleaved in the gastrointestinal tract and the aglycone is subsequently reabsorbed to be sulphated and eliminated via the urine. Suppression of the microflora with antibiotics inhibited this disposition. The urinary excretion of *p*-dichlorobenzene in rats is likewise via the biliary excretion of a glucuronide (of 2, 5-dichlorophenol) and EHC (Hawkins *et al.,* 1980). *In vivo* data solely on urinary and faecal metabolites, or *in vitro* data on hepatic metabolism alone, can therefore sometimes be very misleading.

Other metabolic changes by the microflora may influence the eventual route of excretion of agents. The azo-dye, amaranth, for example, is reduced by the intestinal microflora to yield sulphonated naphthylamines which are reabsorbed; these are not suitable candidates for biliary excretion and therefore appear in the urine (Radomski and Mellinger, 1962). The cycling of agents through the liver and intestine can therefore have a significant influence on the nature of the ultimate metabolites. This can be important with therapeutic agents; the extent of N-dealkylation of opiate analgesics, for example, increases with each enterohepatic cycle that occurs (Brewster *et al.,* 1981).

The influence of EHC can be a major factor determining differences between species in the route of excretion (see also Chapter 16.10). The monkey and dog show similar extents of biliary excretion of indomethacin. Although the biliary material is largely in the form of conjugates in the dog, the monkey differs by readily excreting the parent compound into bile in addition to conjugates. This leads to an overall greater proportion of the drug to be reabsorbed from the intestine, and thus to be eliminated ultimately via the urine in the monkey, since less escapes via the faeces (Yesair *et al.,* 1970).

17.5
Influence of EHC on toxicity

The preceding section indicates that EHC can have a marked influence on the kinetics and distribution within the body of various agents. Since the toxicity of chemicals is determined by the dose of the substance that reaches its target, EHC clearly can have a major influence on toxicity. Thus, in the same way that EHC prolongs the pharmacological effect of indomethacin (see 17.4) the ulcerogenic action of the compound is likewise enhanced. Bile duct ligation inhibits ulcer formation by indomethacin in dogs. Furthermore, bile from dogs treated with indomethacin produced ulcers when intubated into recipient animals. Relatively extensive EHC leads to a higher ulcerogenic effect than in species in which EHC occurs to a lesser extent (Walter and Diener, 1971; Duggan *et al.,* 1975).

The EHC can also significantly contribute to the toxicity of agents that escape the cycle to reach extrahepatic tissues. The heart and brain are target sites for the toxicity of digitoxin (Caldwell and Greenberger, 1971) and methylmercury (Rowland *et al.,* 1980) respectively, and the EHC can help to maintain the concentration of these agents at these sites. In the latter case, inhibition of the microflora with antibiotics can restrict demethylation and therefore can enhance EHC and the neurotoxicity of methylmercury. Both of these cases provide examples of the potential use of 'terminators' of EHC in the treatment of poisoning. Thus, polythiol resin given orally limited neurotoxicity in patients contaminated with methylmercury (Clarkson *et al.,* 1981) and cholestyramine protected against the cardiotoxicity of digitoxin (Caldwell and Greenberger, 1971). An interesting potential benefit of inhibition of EHC of amanitin following poisoning by *Amanita phalloides* has been recognized (Buchwald, 1989).

Hexachlorobenzene is toxic and carcinogenic to the kidney. Glutathione conjugates and cysteinyl glycine derivatives in rat bile appear to play an important role since nephrotoxicity was not seen in bile duct-cannulated rats (Nash *et al.,* 1984). The glutathione conjugates and/or the products of τ-glutamyl transpeptidase and dipeptidases present in the bile duct may be reabsorbed from the gut (if they escape degradation to the cysteine conjugate and subsequent cleavage by bacterial *β*-lyase) and are translocated to the kidney for excretion as the corresponding mercapturates. It is within the kidney that cysteine conjugate *β*-lyase activity leads to the formation of a reactive intermediate which is responsible for toxicity (Nash *et al.,* 1984; Dekant *et al.,* 1988). The results of Payan *et al.* (1991) using pairs of bile-duct-duodenum cannula-linked rats showed that, following a relatively high dose of hexachlorobutadione, about 40% of the metabolites excreted in urine was derived from the biliary excreted material, the reabsorption of which appeared to be saturable. Sinusoidal efflux of S-conjugates of hexachlorobutadiene from the liver appears also to contribute to directing these toxic precursors to the kidney (Payan *et al.,* 1993).

The influence of EHC on toxicity extends to natural toxins. Ochratoxin A appears to enter an EHC in mice (Roth *et al.,* 1988). This was inhibited by cholestyramine and (by an unknown mechanism) phenylalanine. The latter is of interest since it prevents acute poisoning and inhibition of protein synthesis by ochratoxin A in mice and is also protective against its teratogenic and nephrotoxic effects in rats.

Recent evidence has been provided in a prospective study to support an earlier suggestion that EHC may be important in some cases of neonatal jaundice in otherwise healthy full-term infants. The peak serum bilirubin level significantly correlated with faecal *β*-glucuronidase activity. Although *β*-glucuronidase was detected in breast milk, this appeared not to be the determinant of relatively high faecal activity of this enzyme in the neonates (Yau and Chen, 1992). Perhaps this is a prime candidate for considering interference of the enzyme activity.

So far we have considered the effects of the microflora on the material excreted in bile. The converse may also be important; some drugs produce gastrointestinal sideeffects in humans during therapy because of the effect of biliary metabolites on the microfloral population (e.g. the cephalosporin, ceftriazone, Arvidsson *et al.,* 1988).

17.6
Influence of EHC on carcinogenesis

We have been particularly interested in the influence of biliary excretion and enterohepatic circulation on the distribution of carcinogens in the body (Chipman, 1982). Carcinogenic aromatic

amines or amides, following their excretion in bile as O- or N-glucuronides (e.g. 2-acetylaminofluorene in the rat; Irving *et al.,* 1967) may be hydrolyzed by bacterial *β*-glucuronidase to liberate N-hydroxy derivates which, following subsequent uptake and esterification, can form reactive electrophiles capable of covalent binding and genetic toxicity. The various reactions leading to the ultimate carcinogenic products of aromatic amines are described by Bartsch *et al.* (1972). Reactive forms of this class of compound therefore have increased access particularly to the liver and intestinal cells, which are common target sites for their carcinogenicity. In accord with this route of action, 2, 3-dimethyl-4-aminobiphenyl was found not to be carcinogenic to the colon of germ-free animals in which the bacterial enzyme component is absent (Wynder *et al.,* 1969). Moreover, defunctionalized bowel segments of the rat were resistant to the carcinogenicity of this amine given subcutaneously (Cleveland *et al.,* 1967). However, caution is needed in interpretation of this finding since the loss of other components of the faecal stream such as bile acids may well have reduced susceptibility to carcinogenesis. Biliary metabolites of various aromatic amines (2-aminofluorene, 6-aminochrysene, 2-aminoanthracene and 2, 3-dimethyl-4-aminobiphenyl) are directly mutagenic to bacterial cells (Connor *et al.,* 1979; Moriya e*t al.,* 1979). This mutagenicity is enhanced considerably by mimicking the action of the intestinal microflora through the use of *β*-glucuronidase.

Metabolites of the carcinogenic aromatic amine benzidine (p, p'-diaminodiphenyl) are also excreted largely via the bile in rats. As a result of N-acetylation, N-hydroxylation and conjugation with glucuronic acid, a number of these metabolites are pro-mutagenic (Bos *et al.,* 1980; Lynn *et al.,* 1984; Chipman and Mohn, 1989). We have shown that rat biliary metabolites of benzidine, when re-infused into the duodena of a further group of rats, were reabsorbed and re-excreted as mutagens in the bile of recipients (Chipman and Mohn, 1989). Furthermore, in mouse host-mediated mutagenicity assays, intracaecal administration of rat biliary metabolites of benzidine produced a mutagenic response in *Salmonella typhimurium* isolated from the liver of the infected recipient mice. The biliary products of benzidine have also been implicated in nuclear anomaly formation in intestinal tissue of rats (Percy *et al.,* 1989). Clearly, EHC can add to the biological persistence of mutagenic metabolites and may contribute to the carcinogenicity of certain compounds in the liver and intestine.

Because of the activity of bacterial nitro- and azo-reductase (see Chapter 7), nitroaromatic- and azo-compounds may also be converted to potentially toxic and pro-mutagenic aromatic amines. The azo-dye, amaranth (Radomski and Mellinger, 1962) is directed into such a pathway. The genotoxicity of 2, 4-dinitrotoluene in liver is dependent on the action of the intestinal microflora (Mirsalis *et al.,* 1982), and the incidence of hepatocellular carcinoma induced by this substance is higher in male than in female rats (Chemical Industry Institute of Toxicology, 1979). This correlated with a relatively high rate of biliary excretion of conjugated metabolites in the male which enables a relatively high rate of EHC and hepatic exposure (Bond *et al.,* 1981). Glucuronic acid and sulphate-conjugate derivatives of this carcinogen are eliminated in rat bile and the highly mutagenic product 2, 4-dinitrobenzaldehyde, in particular, is involved in the EHC that ensues (Sayama *et al.,* 1989).

Pancreatic juice enzymes, in conjunction with sulphatase and *β*-glucuronidase of especially *Klebsiella* bacteria infecting the biliary tract, appear to co-operate in deconjugating and activating mutagens in the bile of patients with anomalous arrangement of the pancreaticobiliary duct, possibly involving biliary metabolites of nitro-aromatic compounds which are widespread environmental pollutants. This phenomenon may be a factor in the high incidence of biliary carcinoma in these patients (Qian *et al.,* 1993). Inhibition of the activity of intestinal microfloral

activities of β-lyase, β-glucuronidase and nitroreductase was associated with reduced urinary excretion of orally administered glutathione conjugates of 4, 5-epoxy-4,-dihydro-1-nitropyrene and 9, 10-epoxy-9, 10– dihydro-1- nitropyrene. The treatment also produced three distinct DNA adducts in the lower intestinal mucosa which were not found in antibiotic-treated mice. The microflora therefore are instrumental in the absorption of these compounds and their genotoxicity in the intestine (Kinouchi *et al.,* 1993).

Metabolites of various carcinogenic polycyclic aromatic hydrocarbons such as 7, 12-dimethylbenzanthracene (Levine, 1974) and benzo(a)pyrene (Boroujerdi *et al.,* 1981; Chipman *et al.*, 1981, 1982) are also excreted in bile of rats and rabbits. The biliary metabolites of benzo(a) pyrene have been partially characterized (Boroujerdi *et al.,* 1981; Chipman *et al.,* 1981) and include products that are pro-mutagenic (Connor *et al.,* 1979; Forti and Trieff, 1980; Chipman *et al.,* 1983). The classic deconjugation of glucuronic acid conjugates by the microflora occurs in addition to dehydroxylation of benzo(a)pyrene metabolites back to the parent molecule (Renwick and Drasar, 1976; Chipman *et al.,* 1981). Thus, extensive reabsorption of the potentially mutagenic products of benzo(a)pyrene occurs in both rats (Chipman *et al.,* 1981) and rabbits (Chipman *et al.,* 1982) leading to re-excretion of metabolites in the bile and urine, the latter indicating that EHC contributes to systemic availability of reabsorbed material.

Sometimes, as in the case of certain anti-cancer drugs, it is necessary to direct toxic agents to their target sites. Could the EHC be exploited for this purpose? Certainly there are drugs with anti-cancer activity that undergo EHC. Two compounds in particular (aniline mustard (N, N-di-2-chloroethyl aniline) and hycanthone methane sulphonate) are both excreted in rat bile as glucuronic acid conjugates to release highly-reactive aglycones on incubation with β-glucuronidase (Chipman, 1982). This suggests some potential for this approach.

17.7
Summary

The enteric microflora play a major role (largely through enzymic deconjugation) in allowing many compounds excreted in bile to be reabsorbed from the intestine and to be eliminated via the urine or to be recycled back into the bile. Enterohepatic cycling may be beneficial for some drugs in that it helps maintain useful therapeutic blood levels and, in the case of the bile acids, where intestinal transport systems play an important role, prevent the wastage of important endobiotic molecules from the body. For many xenobiotics, however, enterohepatic cycling has the effect of rendering the molecule or its metabolites more difficult to eliminate from the body, thus serving to contribute to, or prolong, the toxic insult.

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References

Arvidsson, A., Leijd, B., Nord, C.E. and Angelin, B., 1988, Interindividual variability in biliary excretion of ceftinaxone: effects on biliary lipid metabolism and on intestinal microflora, *Eur. J. Clin. Invest,* **18,** 261–66.

Back, D.J., Breckenridge, A.M., Challiner, M., Crawford, F.E., Orme, M.L.E., Rowe, P.H. and Smith, E., 1978, The effect of antibiotics on the enterohepatic circulation of ethinylestradiol and norethisterone in the rat, *J. Steroid. Biochem.,* **9,** 527–31.

Back, D.J., Breckenridge, A.M., Crawford, F.E., Cross, K.J., Orme, M.L.E., Percival, A. and Rowe, P.H., 1980, Reduction of the enterohepatic circulation of norethisterone by antibiotics in the rat: correlation with changes in the gut flora, *J. Steroid. Biochem.,* **13,** 95–100.

Bakke, J.E. and Gustafsson, J.A., 1986, Role of intestinal flora in metabolism of agrochemicals conjugated with glutathione, *Xenobiotica,* **16,** 1047–56.

Bakke, J.E., Bergman, A.L. and Larsen, G.L., 1982, Metabolism of 2, 4'5-trichlorobiphenyl by the mercapturic acid pathway, *Science,* **217,** 645–47.

Bakke, J.E., Feil, V.J. and Mulford, D.J., 1990, Biliary excretion and intestinal metabolism in the intermediary metabolism of pentachlorothioanisole, *Xenobiotica,* **20,** 601–5.

Bakke, J.E., Gustafsson, J.A and Gustafsson, B.E., 1980, Metabolism of propachlor by the germfree rat, *Science,* **210,** 433–35.

Ballhorn, L., Rozman, T., Rozman, K., Korte, F. and Greim, H., 1981, Cholestyramine enhances fecal elimination of pentachlorophenol in Rhesus monkeys, *Chemosphere,* **10,** 877–88.

Barr, W.H. and Riegelman, S., 1970, Intestinal drug absorption and metabolism 1. Comparison of methods and models to study physiological factors of *in vitro* and *in vivo* intestinal absorption, *J. Pharm. Sci.,* **59,** 154–63.

Bartsch, H., Dworkin, M., Miller, J.A. and Miller, E.G., 1972, Electrophilic N-acetoxyaminoarenes derived from carcinogenic N-hydroxy-N-acetylaminoarenes by enzymatic deacetylation and transacetylation in liver, *Biochim. Biophys. Acta,* **286,** 272–98.

Belknap, W.M., Balistreri, W.F., Suchy, F.J. and Miller, P.C., 1981, Physiologic cholestasis II: serum bile acid levels reflect the development of the enterohepatic circulation in rats, *Hepatology,* **1,** 613–16.

Bond, J.A., Medinsky, M.A., Dent, J.G. and Rickert, D.E., 1981, Sex-dependent metabolism and biliary excretion of [2, 4–^{14}C]-dinitrotoluene in isolated perfused rat liver, *J. Pharmacol. Exp. Ther.,* **219,** 598–603.

Boroujerdi, M., Kung, H.-C., Wilson, A.G.E. and Anderson, M.W., 1981, Metabolism and DNA binding of benzo(a)pyrene *in vivo* in the rat, *Cancer Res.,* **41,** 951–57.

Bos, R.P., Brouns, R.M.E., Van Doorn, R., Theuws, J.L.G. and Henderson, P.Th., 1980, The appearance of mutagens in urine of rats after the administration of benzidine and some other aromatic amines, *Toxicol.,* **16,** 113–22.

Brewster, D., Humphrey, M.J. and McLeavy, M.A., 1981, Biliary excretion, metabolism and enterohepatic circulation of buprenorphine, *Xenobiotica.,* **11,** 189–96.

Brewster, D., Jones, R.S. and Symons, A.M., 1977, Effects of neomycin on the biliary excretion and enterohepatic circulation of mestranol and 17 *β*-oestradiol, *Biochem. Pharmacol.,* **26,** 943–46.

Buchwald, A., 1989, Amanita poisoning, *Am. J. Med.,* **87,** 702.

Caldwell, J.H. and Greenberger, N.J., 1971, Interruption of the enterohepatic circulation of digitoxin by cholestyramine, *J. Clin. Invest.,* **50,** 2626–37.

Chemical Industry Institute of Toxicology, 1979, *Docket No. 327N8,* Research Triangle Park, NC 27709, USA.

Chipman, J.K., 1982, Bile as a source of potential reactive metabolites, *Toxicol.,* **25,** 99–111.

Chipman, J.K. and Mohn, G.R., 1989, Host-mediated bacterial mutagenesis and enterohepatic circulation of benzidine-derived mutagenic metabolites in rodents, *Xenobiotica,* **19,** 43–50.

Chipman, J.K., Millburn, P. and Brookes, T.M., 1983, Mutagenicity and *in vivo* disposition of biliary metabolites of benzo(a)pyrene, *Toxicol. Lett.,* **17,** 233–40.

Chipman, J.K., Bhave, N.A., Hirom, P.C. and Millburn, P., 1982, Metabolism and excretion of benzo(a)pyrene in the rabbit, *Xenobiotica,* **12,** 397–404.

Chipman, J.K., Hirom, P.C., Frost, G.S. and Millburn, P., 1981, The biliary excretion and enterohepatic circulation of benzo(a)pyrene and its metabolites in the rat, *Biochem. Pharmacol.,* **30,** 937–44.

Clarkson, T.W, Magos, L., Cox, C., Greenwood, M.R., Amin-Zaki, L., Majeed, M.A. and Al-Damlujii, 1981, Tests of efficacy of antidotes for removal of methylmercury in human poisoning during the Iraq outbreak, *J. Pharmacol. Exp. Ther.,* **218,** 74–83.

Cleveland, J.C., Litvak, S.F. and Cole, J.W, 1967, Identification of the route of action of the carcinogen 3, 2'-dimethyl-4-aminobiphenyl in the induction of intestinal neoplasia, *Cancer Res.,* **27,** 708–14.

Coddington, K.A., Swanson, S.P., Hassan, A.S. and Buck, W.B., 1989, Enterohepatic circulation of T-2 toxin metabolites in the rat, *Drug Metab. Dispos. Biol. Fate Chem.,* **17,** 600–5.

Connor, T.H., Forti, G.C., Sitra, P. and Legator, M.S., 1979, Bile as a source of mutagenic metabolites produced *in vivo,* and detected by *Salmonella typhimurium, Environ. Mutagen.,* **1,** 269–76.

Dobrinska, M.R., 1989, Enterohepatic circulation of drugs, *J. Clin. Pharmacol.,* **29,** 577–80.

Dekant, W., Schrenk, D., Vamvakas, S. and Henschler, D., 1988, Metabolism of hexachloro1, 3-butadiene in mice: *in vivo* and *in vitro* evidence for activation by glutathione conjugation, *Xenobiotica,* **18,** 803–16.

Dickinson, R.G., Kluck, R.M., Eadie, M.J. and Hooper, W.D., 1985, Disposition of β-glucuronidase-resistant "glucuronides" of valproic acid after intrabiliary administration in the rat: intact absorption, fecal excretion and intestinal hydrolysis, *J. Pharmacol. Exp. Ther.,* **233,** 214–21.

Drasar, B.S. and Hill, M.J., 1974, *Human Intestinal Flora,* Academic Press: London.

Duggan, D.E., Hooke, K.F., Noll, R. and Kwan, K.C., 1975, Enterohepatic circulation of indomethacin and its role in intestinal irritation, *Biochem. Pharmacol.,* **24,** 1749–54.

Dutczak, W.J., Clarkson, T.W. and Ballatori, N., 1991, Biliary-hepatic recycling of a xenobiotic: gallbladder absorption of methyl mercury, *Am. J. Physiol.,* **260,** G873–80.

Elmhirst, T.R.D., Chipman, J.K., Ribeiro, O., Hirom, P.C. and Millburn, P., 1985, Metabolism and enterohepatic circulation of benzo(a)pyrene-4, 5-epoxide in the rat, *Xenobiotica,* **15,** 899–906.

Forti, G.C. and Trieff, N.M., 1980, Kinetics of uptake and biliary excretion of benzo(a)pyrene and mutagenic metabolites in isolated perfused rat liver, *Teratogen. Carcinogen. Mutagen.,* **1,** 269–82.

Gingell, R., 1975, Enterohepatic circulation of bis(p-chlorophenyl) acetic acid in the rat, *Drug Metab. Dispos.,* **3,** 42–46.

Gregus, Z. and Klaasen, C.D., 1986, Enterohepatic circulation of toxicants, in Rozman, K. and Hänninen, O. (Eds) *Gastrointestinal Toxicity,* pp. 57–116, Amsterdam: Elsevier.

Gregus, Z., Earth, Z., Fischer, E., Zaumseil, J., Klinger, W. and Varga, F., 1980, Comparison of the effects of cholestyramine and aluminium hydroxide on the biliary bile acid excretion in rats. An experimental model for the depletion of bile acids in bile, *Acta Biol. Med. Ger.,* **39,** 705–9.

Hanks, G.W., Hoskin, P.J., Aherne, G.W., Chapman, D., Turner, P. and Poulain, P., 1988, Enterohepatic circulation of morphine, *Lancet,* **1,** 469.

Hawkins, D.R., Chasseaud, L.F., Woodhouse, R.N. and Gresswell, D.G., 1980, The distribution, excretion and biotransformation of p-dichloro [^{14}C] benzene in rats after repeated inhalation, oral and subcutaneous doses, *Xenobiotica,* **10,** 81–95.

Hellstern, A., Hildebrand, M, Humpel, M., Hellenbrecht, D., Saller, R. and Madetzki, C., 1990, Minimal biliary excretion and enterohepatic recirculation of lormetazepan in man as investigated by a new nasobiliary drainage technique, *Int. J. Clin. Pharmacol. Ther. Toxicol.,* **28,** 256–61.

Hirom, P.C., Chipman, J.K., Millburn, P. and Pue, M.A., 1983, Enterohepatic circulation of the aromatic hydrocarbons benzo(a)pyrene and naphthalene, in Rydstrom, J., Montelius, J. and Bengtsson, M. (Eds) *Extrahepatic Drug Metabolism and Chemical Carcinogenesis,* p. 275, Amsterdam: Elsevier.

Hirom, P.C., Millburn, P., Parker, R.J. and Williams, R.T., 1975, The enterohepatic circulation of ^{3}H-phenolphthalein in the rat, *Proc. Brit. Pharmacol. Soc.,* 335P.

Horton, T.L. and Pollack, G.M., 1991, Enterohepatic circulation and renal metabolism of morphine in the rat, *J. Pharm. Sci.,* **80,** 1147–52.

Huckle, K.R., Chipman, J.K., Hutson, D.H. and Millburn, P., 1981, Metabolism of 3-phenoxybenzoic acid and the enterohepatorenal disposition of its metabolites in the rat, *Drug Metab. Dispos.,* **9,** 360–68.

Irving, C.C., Wiseman, R.J. and Hill, J.T., 1967, Biliary excretion of the O-glucuronide of *N*-hydroxy-2-acetylaminofluorene by the rat and rabbit, *Cancer Res.,* **27,** 2309–17.

Kinouchi, T., Kataoka, K., Miyanishi, K., Akimoto, S. and Ohnishi, Y, 1993, Biological activities of the intestinal microflora in mice treated with antibiotics or untreated and the effects of the microflora on absorption and metabolic activation of orally administered glutathione conjugates of K-region epoxides of 1-nitropyrene, *Carcinogenesis,* **14,** 869–74.

Komura, H., Fukui, H., Sasaki, M. and Morino, A., 1992, Pharmacokinetic analysis of enterohepatic circulation of 4–(2–(4-isopropylbenzamido)ethoxy) benzoic acid. Effect of intramolecular rearrangement of its acyl glucuronide, *Drug Metab. Dispos. Biol. Fate Chem.,* **20,** 585–91.

Larsen, G.L. and Bakke, J.E., 1981, Enterohepatic circulation in formation of propachlor (2-chloro-N-isopropylacetanilide) metabolites in the rat, *Xenobiotica,* **11,** 473–80.

Levine, W.G., 1974, Hepatic uptake, metabolism and biliary excretion of 7, 12-dimethylbenzanthracene in the rat, *Drug Metab. Dispos.,* **2,** 169–77.

Levine, W.G., Braunstein, I.R. and Meijer, D.K.F., 1975, Effect of Nafenopin (SU-13, 437) on liver function. Mechanism of choleretic effect, *Naunyn-Schmied. Arch. Pharmacol.,* **290,** 221–34.

Lewis, C.J., Vose, C.W., Spalton, P.N., Ford, G.C., Haskins, N.J. and Palmer, R.F., 1980, Metabolism of ethynodiol diacetate in the Rhesus monkey before and after administration of rifampicin, *Xenobiotica,* **10,** 705–13.

Lynn, R.K., Garrie-Gould, C.T., Milam, D.F., Scott, K.F., Eastman, C.L., Ilias, A.M. and Rodgers, R.M., 1984, Disposition of the aromatic amine, benzidine in the rat: characterisation of mutagenic urinary and biliary metabolites, *Toxicol. Appl. Pharmacol.,* **72,** 1–14.

Marselos, M., Dutton, G. and Hänninen, O., 1975, Evidence that D-glucaro-1, 4-lactone shortens the pharmacological action of drugs being disposed via the bile as glucuronides, *Biochem. Pharmacol.,* **24,** 1855–58.

Mirsalis, J.C., Hamm, T.E., Sherrill, J.M. and Butterworth, R.E., 1982, Role of gut flora in the genotoxicity of dinitro-toluene, *Nature,* **295,** 322–23.

Moriya, M., Ohta, T., Sugiyama, F., Miyazawa, T. and Shirasu, Y., 1979, Assay for mutagenicity of bile in Sprague-Dawley rats treated subcutaneously with intestinal carcinogens, *J. Natl. Cancer Inst.,* **63,** 977–82.

Nash, J., King, L., Lock, E. and Green, T., 1984, The metabolism and disposition of hexachloro-1, 3-butadiene in the rat and its relevance to nephrotoxicity, *Toxicol. Appl. Pharmacol.,* **73,** 124–37.

Norseth, T., 1971, Biotransformation of methyl mercuric salts in the mouse studied by specific determination of inorganic mercury, *Acta Pharmacol. Toxicol.,* **29,** 375–84.

Orme, M.L. and Back, D.J., 1990, Factors affecting the enterohepatic circulation of oral contraceptive steroids, *Am. J. Obstet. Gynecol.,* **163,** 2146–52.

Parker, R.J., Hirom, P.C. and Millburn, P., 1980, Enterohepatic recycling of phenolphthalein, morphine, lysergic acid diethylamide (LSD) and diphenylacetic acid in the rat. Hydrolysis of glucuronic acid conjugates in the gut lumen, *Xenobiotica,* **10,** 689–703.

Payan, J.P., Fabry, J.P., Beydon, D. and de Ceaurriz, J., 1991, Biliary excretion of hexachloro-1, 3-butadiene and its relevance to tissue uptake and renal excretion in male rats, *J. Appl. Toxicol,* **11,** 437–42.

Payan, J.P., Beydon, D., Fabry, J.P., Morel, G., Brondeau, M.T., Ban, M. and de Ceaurriz, J., 1993, Partial contribution of biliary metabolites to nephrotoxicity, renal content and excretion of (^{14}C) hexachloro-1, 3-butadiene in rats, *J. Appl. Toxicol.,* **13,** 19–24.

Pekas, J.C., 1983, Intestinal metabolism and absorption of carbaryl studied with a portal fistula, *Pest. Biochem. Physiol,* **19,** 36–43.

Percy, A.J., Moore, N. and Chipman, J.K., 1989, Formation of nuclear anomalies in rat intestine by benzidine and its biliary metabolites, *Toxicology,* **57,** 217–23.

Powell, G.M., Miller, J.J., Olavesen, A.H. and Curtis, C.G., 1974, Liver as a major organ of phenol detoxication? *Nature,* **252,** 234–35.

Qian, D., Kinouchi, T, Kunitomo, K, Kataoka, K, Matin, M.A., Akimoto, S., Komi, N. and Ohnishi, Y., 1993, Mutagenicity of the bile of dogs with an experimental model of an anomalous arrangement of the pancreaticobiliary duct, *Carcinogenesis,* **14,** 743–47.

Radomski, J.L. and Mellinger, T.J., 1962, The absorption, fate and excretion in rats of the water-soluble azo dyes, FD+C Red No. 2, FD+C Red No. 4, and FD+C Yellow No. 6, *J. Pharmacol. Exp. Ther.,* **136,** 259–66.

Remmel, R.P., Pohl, L.R. and Elmer, G.W., 1981, Influence of the intestinal microflora on the elimination of warfarin in the rat, *Drug Metab. Dispos.,* **9,** 410–14.

Renwick, A.G. and Drasar, B.S., 1976, Environmental carcinogens and large bowel cancer, *Nature,* **263,** 234–35.

Roth, A., Chakor, K., Creppy, E.E., Kane, A., Raschenthaler, R., Dirheimer, G., 1988, Evidence for an enterohepatic circulation of ochratoxin A in mice, *Toxicology,* **48,** 293–308.

Rowland, I.R., Davies, M.J. and Evans, J.G., 1980, Tissue content of mercury in rats given methylmercuric chloride orally: influence of intestinal flora, *Arch. Environ. Health,* **35,** 155–60.

Rozman, K., 1985, Intestinal excretion of toxic substances, *Arch Toxicol. Suppl.,* **8,** 87–93.

Rosman, T., Ballhorn, L., Rozman, K., Klaassen, C.D. and Greim, H., 1982, Effect of cholestyramine on the disposition of pentachlorophenol in rhesus monkeys, *J. Toxicol. Environ. Health,* **10,** 277–83.

Rutgers, M., Heusdens, F.A., Bonthuis, F, de Herder, W.W., Hazenberg, M.P. and Visser, T.J., 1989, Enterohepatic circulation of triiodothyronine (T3) in rats: importance of the microflora for the liberation and reabsorption of T3 from biliary T3 conjugates, *Endocrinology,* **125,** 2822–30.

Sayama, M., Mori, M., Ishida, M., Okumura, K. and Kozuka, H., 1989, Enterohepatic circulation of 2, 4-dinitrobenzaldehyde, a mutagenic metabolite of 2, 4-dinitrotoluene, in male Wistar *rats, Xenobiotica,* **19,** 83–92.

Sellman, R., Kanto, J. and Pekkarinin, J., 1975, Biliary excretion of diazepam and its metabolites in man, *Acta Pharmacol. Toxicol.,* **37,** 242–49.

Semmes, R.L. and Shen, D.D., 1990, A reversible clearance model for the enterohepatic circulation of drug and conjugate metabolite pair, *Drug. Metab. Dispos. Biol. Fate Chem.,* **18,** 80–87.

Shepard, T.A., Lockwood, G.F., Aarons, L.J. and Abrahams, I.D., 1989, Mean residence time for drugs subject to enterohepatic cycling, *J. Pharmacokinet. Biopharm.,* **17,** 327–45.

Siegers, C.P., Rozman, K. and Klaassen, C.D., 1983, Biliary excretion and enterohepatic circulation of paracetamol in the rat, *Xenobiotica,* **13,** 591–96.

Sinclair, K.A. and Caldwell, J., 1982, The formation of β-glucuronidase-resistant glucuronides by the intramolecular rearrangement of glucuronic acid conjugates at mild alkaline pH, *Biochem. Pharmacol.,* **31,** 953–57.

Smith, R.L., 1973, *The Excretory Function of Bile,* London: Chapman and Hall.

Struble, C.B., 1991, *In situ* intestinal absorption of 2-chloro-N-isopropylacetanilide (propachlor) and non-biliary excretion of metabolites into the intestinal tract of rats, pigs and chickens, *Xenobiotica,* **21,** 85–95.

Struble, C.B., Pekas, J.C. and Gerst, J.W., 1983, Enterohepatic circulation and biliary secretion of 5, 6-dihydro-5, 6-dihydroxy [^{14}C] carbaryl glucuronide in the rat, *Pest. Biochem. Physiol.,* **19,** 95–103.

Sund, R.B., Songedal, K., Harestad, T., Salvesen, B. and Kristiansen, S., 1981, Enterohepatic circulation, urinary excretion and laxative action of some bisacodyl derivatives after intragastric administration in the rat, *Acta Pharmacol. Toxicol.,* **48,** 73–80.

Tse, F.L.S., Ballard, F. and Skinn, J., 1982, Estimating the fraction reabsorbed in drugs undergoing enterohepatic circulation, *J. Pharmacokin. Biopharm.,* **10,** 455–61.

Van-Eldere, J., Robben, J., Caenepeel, P. and Eyssen, H., 1988, Influence of a cecal volume-reducing intestinal microflora on the excretion and entero-hepatic circulation of steroids and bile acids, *J. Steroid Biochem.,* **19,** 33–39.

Walter, J.E. and Diener, R.M., 1971, Mechanisms of enteric ulcer formation in rats and dogs treated with non-steroid anti-inflammatory agents, *Toxicol. Appl. Pharmacol.,* **19,** 376.

Wynder, E.L., Kajitani, T., Ishikawa, S., Dado, H. and Takano, A., 1969, Environmental factors of cancer of the colon and rectum. II Japanese epidemiological data, *Cancer,* **23,** 1210–20.

Yau, K.I. and Chen, C.L., 1992, Factors affecting the severity of neonatal jaundice of unknown etiology: the role of enterohepatic circulation, *Acta Paediatr. Sin.,* **33,** 20–28.

Yesair, D.W., Callahan, M., Remington, L. and Kensler, C.J., 1970, Role of the enterohepatic cycle of indomethacin on its metabolism, distribution in tissues and its excretion by rats, dogs and monkeys, *Biochem. Pharmacol.,* **19,** 1579–90.

Section 8

Probiotics

Chapter 18
Influence of the probiotics, lactobacilli and bifidobacteria on gastrointestinal disorders in adults

K.Orrhage, A.Lidbeck and J.Rafter

18.1
Introduction

Probiotics, in the form of dairy foods with lactic acid bacteria, have been consumed for centuries by humans. Over the last decade there has been increased interest in bacterial food supplements, what we now call probiotics. The term 'probiotic' was first used for growth-promoting animal feeds in the 1970s. Fuller (1989) has now defined the term 'probiotic' as: 'a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance'.

The probiotic preparations currently on the market are in the main based on lactic acid bacteria (lactobacilli, bifidobacteria and streptococci). There are also other microorganisms used as probiotics. Effects and modes of action of probiotics can differ. The most evident effects involve changes in viable counts of micro-organisms in the intestinal flora after ingestion. These effects can, according to Fuller (1991), be caused by competition for adhesion sites and nutrients between the ingested micro-organisms and potential pathogens. Another mode of action for the probiotic can be production of antibacterial substances. However, the influence of a probiotic supplement is not always that pronounced. There can also be an alteration of microbial metabolism in the gut, which can be detected as for instance altered bacterial enzyme activities, changed pH or influence on levels of cholesterol. There are several characteristics that are of importance for organisms that are to be used as probiotics (Kim, 1988). These include that the organisms should be normal inhabitants of the intestinal tract; should survive the upper digestive tract; should be capable of surviving and growing in the intestine; should produce beneficial effects when in the intestinal tract; and should maintain viability and activity in the carrier food before consumption. It is also important that the organism is non-pathogenic and non-toxic. Most common media for probiotics in lyophilized form include tablets and capsules and in live form fermented and non-fermented milk. Even products like candy bars and ice-cream have been used as vehicles. In the present chapter, rather than trying to cover the whole field of probiotics, the authors have limited themselves to a thorough discussion of lactobacilli and bifidobacteria. For further information, the reader is referred to some excellent recent reviews on the subject (Fuller, 1989, 1991; Salminen and Deighton, 1992).

18.2
Lactobacilli

Lactobacilli in the stomach are found at 10^3–10^4 CFU/g together with streptococci and bifidobacteria. Due to the acidic environment in the stomach, the multiplication of most micro-organisms is retarded and mainly lactic acid bacteria are recovered. Small bowel motility also prevents overgrowth of micro-organisms, but in the distal part of the ileum there is a significant increase in bacterial counts and lactobacilli are recovered in numbers of 10^3–10^7 CFU/g. *Escherichia coli* dominates among the aerobic micro-organisms and is isolated in numbers of 10^7–10^8 CFU/g. In the colon the bacterial counts increase further to 10^{10}–10^{12} CFU/g and the anaerobic bacteria outnumber the aerobic bacteria by a factor of 1000:1. Lactobacilli are recovered in numbers of 10^4–10^8 CFU/g, while *Bacteroides fragilis* is the dominating anaerobic micro-organism (Nord and Kager, 1984; Goldin, 1986). In elderly persons the numbers of lactobacilli and clostridia are significantly higher and the number of bifidobacteria lower than those seen in younger adults (Speck, 1976). In the Wadsworth study (Finegold *et al.,* 1983), *Lactobacillus* sp. were found in 73% of individuals eating a 'Western' diet, with a mean count of 10^{93} organisms per gram dry weight faeces (range $10^{3.6}$–$10^{12.5}$). In strict vegetarians, lactobacilli were recovered in 85% with a mean value of $10^{11.1}$ (range $10^{8.6}$–$10^{12.1}$).

Lactobacillus acidophilus was the most frequently isolated species and was recovered in 44.7% of these subjects. The establishment of certain lactobacilli in the gastrointestinal tract is believed to contribute to the stabilization of the microflora of healthy subjects and to exert beneficial effects on the host (Sandine, 1979; Fernandes *et al.,* 1987). Because of these proposed healthful properties, several *Lactobacillus* sp., especially *Lact. acidophilus,* have been used both as dietary supplements and in attempts to prevent gastrointestinal disturbances.

Lactobacilli belong to the Gram-positive non-sporing facultative or anaerobic rods. These micro-organisms utilize carbohydrates as the main nutritional source and are found in fermenting animal and plant products. The main end-product of glucose fermentation is lactic acid, resulting in a decrease of pH in the medium. Besides lactic acid, lactobacilli also produce acetic acid and hydrogen peroxide. These metabolites make the environment less favourable for the *in vitro* growth of potentially pathogenic microorganisms, such as staphylococci, *Pseudomonas* and *Salmonella* (Mehta *et al.,* 1983; Speck, 1983).

Lact. acidophilus, Lactobacillus bulgaricus, Lactobacillus casei and *Lactobacillus plantarum* belong to the homofermentative lactobacilli, producing more than 85% lactic acid from glucose. *Lact. acidophilus* produces DL-lactic acid from lactose, glucose, maltose, saccharose and other carbohydrates. The heterofermentative lactobacilli produce at least 50% lactic acid together with acetic acid, ethanol and carbon dioxide (Kandler and Weiss, 1986). Several other substances with antimicrobial properties are also produced (Axelsson *et al.,* 1989; Muriana and Klaenhammer, 1991).

18.3
Bifidobacteria

Bifidobacteria constitute a major part of the normal intestinal microflora in humans throughout life (Mitsuoka and Kaneuchi, 1977). They appear in the stools a few days after birth and increase in number. The number of bifidobacteria in the colon of adult people is 10^9–10^{11} CFU/g. The numbers

of bifidobacteria decrease during ageing (Mitsuoka *et al.,* 1974). Demographic differences in the numbers and species of bifidobacteria have been reported (Benno *et al.,* 1986).

Bifidobacteria were first found at the end of the last century in faeces from breastfed infants (Tissier, 1899). They were thought to be beneficial for the health of newborns. Later on they were also discovered in healthy adults. Investigations performed at this time dealt with the importance of the bifidobacteria for the normal digestion of food and their inhibition of 'putrefactive bacteria'. The proliferation of bifidobacteria in the gut was considered as a 'self cleanage process'.

Bifidobacteria are nonmotile, nonsporulating Gram-positive rods with varying appearance (Scardovi, 1986). Most strains are strictly anaerobic. Acetic and lactic acids are produced in a theoretical molar ratio of 3:2 from glucose, through the characteristic fructose 6-phosphate shunt. Most of the lactic acid produced has an L-configuration, which is easily metabolized in the body. The morphology of the cells is influenced by the nutritional circumstances, but their shape is usually elongated with a slight bend or Y- or V-branch. Ten human species of bifidobacteria are now identified of which the most common are *Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, Bifidobacterium adolescentis* and *Bifidobacterium breve.*

18.4
Lactic acid bacteria in fermented milk products

Fermentation with lactic acid bacteria is a very old method of food preservation and lactobacilli have been used together with streptococci in the manufacture of dairy products. Since enzymes of lactic acid cultures degrade proteins, lipids and lactose of milk, these nutritional components are partially predigested (Alm, 1982). Almost every civilization has consumed cultured milks and these products have been, and still are, of great importance in the nutrition of people throughout the world. Many lactic cultures require vitamins for growth, but they are on the other hand also capable of synthesizing B-vitamins during fermentation. Folic acid, in particular, is found in higher levels in cultured dairy products.

In the preparation of probiotic fermented milk products, it is crucial to choose suitable strains.

18.5
Survival of ingested probiotics in the gastrointestinal tract

Animal studies have shown that the capacity of the micro-organisms to colonize the epithelial surface may be specified as much by a capacity to bind to the epithelium as by nutritional and environmental conditions. Animal studies have indicated that lactobacilli adhere to squamous epithelial cells via acidic polysaccharides. However, it has been reported that macromolecules other than polysaccharides may be involved in the adhesion (Savage, 1984).

In a study by Conway *et al.* (1987), the ability of different strains of lactobacilli to survive in gastric juice was tested in healthy subjects. Three subjects received *Lactobacillus acidophilus* strain N2 and two subjects *Lact. acidophilus* strain ADH and *Lactobacillus bulgaricus,* respectively. The survival of the lactobacilli was related to pH and the strain showing the best survival in gastric juice was *Lact. acidophilus* strain ADH. This strain had also the best adhesion capacity to human ileal cells. The addition of milk to the supplement caused a rise in pH and increased survival times for all lactobacilli in gastric juice.

Pochart *et al.* (1992) investigated the survival of a strain of *Bifidobacterium* sp., ingested in fermented milk, after passage through the upper gastrointestinal tract. Intake of 1×10^{10} CFU of bifidobacteria in 400 g fermented milk led to an increased ileal flow, from 1×10^{4} to 6×10^{8} CFU of bifidobacteria/hour within two hours. The results indicate that *Bifidobacterium* sp. in fermented milk can survive transit through the stomach and small intestine in healthy adults. To study the further fate of an ingested *Bifidobacterium* sp. the same group of investigators administered an antibiotic-resistant *Bifidobacterium* strain for eight days (Bouhnik *et al.,* 1992). The exogenous bifidobacteria appeared in the stools and reached a mean level of 6.3×10^{8} CFU/g faeces. When the ingestion stopped there was a gradual decrease in number of detected cells, so obviously the exogenously administered *Bifidobacterium* sp. did not colonize the human colon.

18.6
Protecting effects of the gut microflora

There are several lines of evidence which strongly indicate that the intestinal microflora provides protection against various diseases. It has been shown that germ-free animals are more susceptible to disease than their conventional counterparts (Collins and Carter, 1978; Moberg and Sugiyama, 1979). Antibiotic-treated animals including humans can become more susceptible to disease (Bartlett *et al.,* 1978). Rectal instillations with faecal suspensions have been shown to prevent infection in the gut. For example, in humans it has been shown that *Clostridium difficile* infection can be reversed by administering faecal enemas derived from a healthy human adult (Eiseman *et al.,* 1958; Schwan *et al.,* 1984). Moving to *in vitro* studies, it has been shown that isolates of intestinal bacteria can inhibit pathogenic bacteria. For example, gut isolates of lactobacilli, bifidobacteria, propionibacteria and enterococci inhibited *Clostridium botulinum* (Sullivan *et al.,* 1988) and *Clostridium difficile* was inhibited by a variety of intestinal bacteria (Borriello, 1988).

18.6.1
Disturbances in the protective flora

The normal gastrointestinal microflora is relatively constant during lifetime, but several factors may affect this equilibrium: dietary and environmental conditions can influence this ecosystem (Tannock, 1983). As a result of emotional stress in humans, the secretion of hydrochloric acid in the stomach may decrease. Normally low numbers of bacteria, mostly lactobacilli and streptococci are found in the stomach, but subjects with gastric achlorhydria may harbour high numbers of coliforms and *Bacteroides* (*Drasar et al.,* 1969). In children suffering from protein-calorie malnutrition, lactobacilli are found in lower counts and coliforms in higher counts compared with normal subjects (Tannock, 1983). Disturbances in the intestinal microflora can also be caused by different diseases and some types of therapy. Decreased intestinal motility, constipation, liver-cirrhosis and disturbances in the immune system are conditions that can have an impact on the intestinal microflora. Treatment with antibiotic agents and radiation therapy can also cause changes in the flora. Such disturbances have been associated with decreases in lactobacilli and bifidobacteria in the intestinal flora (Poupard *et al.,* 1973). These conditions, where the balance of the gut microflora is adversely affected, are all situations where probiotics are of potential value. The restoration of the gut flora will enable the host animal to return to normal.

18.7
Beneficial effects of probiotics

It should be mentioned that in many cases the effects of probiotics have been investigated only in animal experiments. However, the human studies available indicate that the results from animal experiments may well be applicable to the human situation.

18.7.1
Effects on composition of normal intestinal microflora

Several investigations have been performed in order to study the effects of oral supplementation of bifidobacteria and lactobacilli on the composition and metabolic activities of the normal intestinal microflora. Usually the changes in numbers of different bacterial groups are small, if there is an ecological balance in the flora at the start of supplementation. The numbers of lactobacilli increased about two log cycles or more in most healthy volunteers when *Lact. acidophilus* supplements were given (Lidbeck *et al.,* 1987, 1988). This increase occurred within one week when the amount of fermented milk given was 500 ml per day. The daily ingested dose thus corresponded to at least 2. 5×10^{11} CFU of *Lact. acidophilus*. About one week after the supplementation was stopped, the numbers of lactobacilli had decreased to almost the same levels as before the administration, indicating that *Lact. acidophilus*-fermented milk had to be ingested continuously to keep the attained higher levels of lactobacilli in the intestine. In a Finnish study, the same doses of lactobacilli were found to be necessary for colonizing all volunteers with *L. casei* strain GG (Saxelin *et al.,* 1991). However, when *Lactobacillus acidophilus* was given in the fermented milk to colon cancer patients for six weeks at a somewhat lower dose, 300 ml daily, a similar increase in *Lactobacillus* spp. counts was noted (Lidbeck *et al.,* 1991). In a study by Gilliland *et al.* (1984), it was shown that a strain of *Lact. acidophilus* with a higher level of bile resistance gave higher numbers of lactobacilli in the intestinal tract compared with a strain with lower bile resistance. Antagonistic effects of lactobacilli against *E. coli in vitro* and *in vivo* have been reported by a number of investigators (Ayebo *et al.,* 1980; McGroarty and Reid, 1988). *In vivo* studies have demonstrated that during the administration of *Lact. acidophilus,* lower levels of *E. coli* were detected in several healthy volunteers as well as in colon cancer patients (Lidbeck *et al.,* 1987, 1991).

Oral supplementation of healthy volunteers with *Bifido. longum* during three and five weeks respectively resulted in a significant decrease in faecal pH, but no major changes in dominating intestinal bacterial groups (Orrhage *et al.,* 1991; Benno and Mitsuoka, 1992). Besides, Benno and Mitsuoka (1992) found a decrease in lecithinase-negative clostridia and a lower concentration of ammonia in faeces during the supplementation.

18.7.2
Attempts to re-establish the microflora, after treatment with antimicrobial agents

Therapy with antimicrobial agents may cause pronounced disturbances in the normal microflora (Nord *et al.,* 1986). Antibiotics are important in the treatment and prophylaxis of infections. However, it should be considered that some of these agents have a harmful effect on the human microflora, leading to undesired effects such as overgrowth and superinfections with commensal micro-organisms. Suppression of the normal microflora lowers the colonization resistance and

potentially pathogenic microorganisms can be established. These pathogens are often resistant to the antimicrobial agents used and may cause stomatitis, diarrhoea or colitis. Furthermore, in immunocompromised patients *Candida* may cause systemic infections. Overgrowth by toxinproducing *Clostridium difficile* can give rise to diarrhoea, colitis and pseudomembranous colitis. Attempts have been made to re-establish the balance of the flora with supplements of lactobacilli and bifidobacteria.

In many cases, antimicrobial therapy is accompanied by gastrointestinal disturbances and either a reduction or an elimination of lactobacilli and bifidobacteria in the intestinal microflora (Daikos *et al.,* 1968; Heimdahl and Nord, 1979; Allen *et al.,* 1980; Finegold *et al.,* 1983; Ambrose *et al.,* 1985; Knothe *et al.,* 1985; Lidbeck *et al.,* 1988). In several studies, lactobacilli have been used in attempts to prevent diarrhoea associated with microbial agents (Beck and Necheles, 1961; Pearce and Hamilton, 1974). In order to prevent ampicillin-associated diarrhoea, Gotz *et al.* (1979) gave a mixture of *L. acidophilus* and *L. bulgaricus* (Lactinex) four times daily for the first five days of ampicillin therapy to one group of patients, while the other group received a placebo. When the patients with diarrhoea unrelated to ampicillin were excluded, the incidence of ampicillin-related diarrhoea in the placebo group was significantly higher compared with the *Lactobacillus* group. The effect of the same *Lactobacillus* mixture was studied for its possible efficacy to prevent neomycin-associated diarrhoea. Two different batches were given to healthy young adults. Each 1-ounce packet contained between 1.2×10^8–9.2×10^8 viable lactobacilli. The preparation was given four times a day, beginning three hours after the first dose of neomycin and continuing throughout the five-day period of neomycin administration. One batch of the preparation reduced the frequency and severity of diarrhoea in volunteers, while no protective effect could be detected with the other batch (Clements *et al.,* 1983). This is perhaps dependent on variations from one batch to another. Zoppi *et al.* (1982) administered a preparation of *Lact. acidophilus andBifidobacterium bifidum* to infants treated with ampicillin. The administration resulted in a significant increase in aerobic and anaerobic lactobacilli and cocci. Capsules with *Lact. acidophilus* and *B. bifidum* have also been given to a group of ten healthy adults during ampicillin administration and 14 days thereafter (Black *et al.,* 1991). There were only minor effects on the intestinal microflora.

Pronounced changes have been observed in the intestinal microflora in patients receiving clindamycin (Nord *et al.,* 1984). When clindamycin was given to ten healthy volunteers, the anaerobic flora was strongly suppressed (Lidbeck *et al.,* 1988). In two subjects lactobacilli disappeared and a decrease occurred in five subjects. Administration of *Lact. acidophilus*-fermented milk for seven days to five subjects after clindamycin treatment resulted in a significant increase in numbers of lactobacilli in all subjects. Most other anaerobic bacteria returned to the same levels as before clindamycin treatment one week later. *Candida albicans* was detected in eight subjects, four in each group, during clindamycin administration. During *Lact. acidophilus* supplementation *Candida albicans* disappeared in three of four subjects in the lactobacillus group, while no similar decrease occurred in the other group. These findings indicate that *Lact. acidophilus* administration might lower the risk of *Candida* infections in compromised patients. Thirty healthy volunteers in three groups participated in a study of the influence of yoghurt with *Bifido. longum* and *Lact. acidophilus* on the intestinal microflora during administration of clindamycin (Orrhage *et al.,* 1994). The reduction in anaerobic microorganisms, especially *Bifidobacterium* and *Bacteroides* spp., was smallest in the group receiving *Bifido. longum* and *Lact. acidophilus,* larger in the group who had only *Bifido. longum* and largest in the placebo yoghurt group. There was also a smaller incidence of gastrointestinal discomfort in the first-mentioned group. In another study, the efficacy

of *Lactobacillus* GG preparation in preventing antibiotic-associated diarrhoea was investigated (Siitonen *et al.,* 1990). Healthy human volunteers receiving erythromycin had less diarrhoea if they took *Lactobacillus* GG yoghurt, compared with a control group taking erythromycin and pasteurized yoghurts. Other side-effects of erythromycin such as abdominal distress, stomach cramps and flatulence were also less common in the *Lactobacillus* group than in the group taking pasteurized yoghurt. Faecal counts of *Lactobacillus* GG indicated that these organisms colonized the bowel, in spite of erythromycin treatment. Another study was conducted on 200 patients with pulmonary tuberculosis being treated with antitubercular drugs (Borgia *et al.,* 1982). During the long-term antibiotic treatment of such patients, gastrointestinal side-effects are common. Treatment with an *Enterococcus faecium* preparation significantly reduced the incidence of diarrhoea and appeared to have some normalizing effect on biochemical parameters.

18.7.3
Clostridium difficile diarrhoea and pseudomembranous colitis

The risk of developing *Clostridium difficile* diarrhoeal disease, most seriously pseudomembraneous colitis, in connection with the use of antimicrobial agents is well-established (Aronsson *et al.,* 1985). The frequency of recurrence of *Clost. difficile c*olitis after an initial antibiotic treatment is high. Erythromycin was given with simultaneous intake of yoghurts containing *Bifido. longum* or placebo yoghurt to ten healthy volunteers for a period of three days (Colombel *et al.,* 1987). Faecal weight, frequency of defecations and abdominal disturbances increased during intake of erythromycin with placebo yogurt but not with *Bifido. longum.* The number of individuals with counts of clostridial spores also decreased from eight to one after the *Bifidobacterium* yoghurt. Gorbach *et al.* (1987) showed that *Lactobacillus* strain GG was effective in preventing antibiotic-treated patients from relapses of pseudomembranous colitis.

Six patients with chronic relapsing diarrhoea caused by *Clost. difficile,* previously treated with metronidazole, were treated with rectal installations of a suspension of faeces (one patient) or a mixture of ten different facultative aerobic and anaerobic bacteria diluted in sterile saline (five patients) (Tvede and Rask-Madsen, 1989). They were also initially treated with vancomycin to eradicate *Clost. difficile.* All patients lost *Clost. difficile* and its toxin from the stools and had a restoration of normal bowel function within 24 hours. *Bacteroides* spp., normally one of the dominating species, had been absent during the illness and during the vancomycin therapy, but was present after recovery. Other authors have successfully treated patients with recurrent diarrhoea due to *Clost. difficile* with rectal infusions of homologous faeces (Bowden *et al.,* 1981; Schwan *et al.,* 1984).

A nonpathogenic yeast, *Saccharomyces boulardii,* has been used to prevent or cure antibiotic-associated diarrhoea. A group of 13 patients with recurring *Clost. difficile* diarrhoea was treated with an oral intake of approximately 30 days of *Sacch. boulardii* (Surawicz *et al.,* 1989a). The administration was initiated by ten days' intake of vancomycin. Eighty-five per cent of the patients reported no further recurrences after cessation of *Sacch. boulardii.* In a prospective double-blind study *Sacch. boulardii* was given in capsules concurrently with antibiotics to 180 hospitalized patients (Surawicz *et al.,* 1989b). Of the patients receiving placebo, 22% experienced diarrhoea compared with 9.5% of patients receiving *Sacch. boulardii.* Attempts have also been made to re-establish the intestinal microflora in patients with diarrhoea by administration *of Enterococcus* (formerly *Streptococcus*) *faecium* 68 (Lewenstein *et al.,* 1979). Another approach that has shown some success

is the administration of non-pathogenic strains of *Clost. difficile* (Borriello, 1988), which presumably occupy the niche that the pathogen would normally expect to find available. The use of probiotics for the treatment of pseudomembranous colitis looks promising because 'at risk' patients can be readily identified and treatment can be started before onset of the disease.

18.7.4
Intestinal disorders

The composition of the intestinal microflora is often changed in intestinal disorders. The rectal microflora of 16 patients with inflammatory bowel disease, colon cancer or some other disorder was compared with healthy controls (Neut *et al.,* 1989). The total bacterial counts in the patients showed only small decreases, but strict anaerobes, mainly *Eubacterium* and *Bifidobacterium* spp. were reduced. Tamura *et al.* (1983) treated a group of 14 patients suffering from different disturbances in the intestinal system. The group was heterogeneous with diseases like acute enteritis, diverticulae in the colon, liver cirrhosis, ulcerative colitis and chronic hepatitis. A preparation of $>10^9$ CFU/g of *Bifidobacterium breve* and *Bifidobacterium bifidum* was administered three or six times daily for two or three weeks. Almost all patients experienced a subjective improvement and pH, urease activity and ammonia in faeces showed tendencies to decrease. An increase in the number of bifidobacteria in the faecal flora was seen, while the coliform bacteria decreased. Recently, two studies with elderly patients with nonspecific bowel disorders, treated with three or six capsules respectively of *Lact. acidophilus* and *Bifido. bifidum* during ten days, revealed a restoration of the duodenal anaerobic flora, function of the muciparous glands and normalization of the duodenal mucosa (Motta *et al.,* 1991; Pecorella *et al.,* 1992). A subsidence of the clinical symptoms was also observed.

18.7.5
Enteritis

Attempts have been made to treat patients suffering from gastritis and duodenitis, related to the presence of *Helicobacter* (formerly *Campylobacter*) *pylori,* with probiotic supplements. In an Italian study 15 patients were treated with Infloran (*Bifido. bifidum* and *Lact. acidophilus*) together with traditional therapy with ampicillin and bismuth (Gismondo *et al.,* 1990). Endoscopic observations revealed an improvement in this group compared with the traditionally treated control group. Another recent study reported *in vitro* inhibition of *Helico. pylori* by *Lact. acidophilus* (Bhatia *et al.,* 1989). *Helico. pylori* has been implicated as the cause of antral gastritis. Because lactobacilli are acid-tolerant and able to persist in the stomach longer than other bacteria, it was suggested that *Lact. acidophilus* preparations may be useful for the treatment of gastritis.

Children with enteritis caused by *Campylobacter jejuni* were given doses of *Bifido. breve* orally until *Camp, jejuni* was eradicated from stool specimens (Tojo *et al.,* 1987). They were compared with patients treated with erythromycin and untreated patients. The number of bifidobacteria in the stools of the enteritis patients was decreased before start of the therapy. Diarrhoea duration was not significantly different between the three groups. *Camp, jejuni* was eradicated faster from the *Bifido.* breve-supplemented patients than from the untreated, but most effectively from erythromycin-treated patients. A significant increase of total bacteria and bifidobacteria was observed after the administration of *Bifido. breve.*

18.7.6
Irritable bowel syndrome

Ent. faecium preparations have been evaluated for treatment of patients with irritable bowel syndrome whose symptoms had been present for an average of seven years. Patient-recorded symptoms did not differ significantly in the placebo and *Ent. faecium* groups. However, it was claimed that the physicians' subjective clinical evaluation of symptoms revealed a significant improvement in the treated group (Gate and Thorn, 1989).

18.7.7
Constipation

Bifidobacteria and lactulose have been used in the treatment of chronic constipation. Lactulose is a synthetic disaccharide which is not digested by the human. The undigested lactulose can then go unaffected to the colon and serve there as a substrate for the bacteria. Doerbeck and Tobiasch (1973) gave 100 constipated patients Eugalan Töpfer, a preparation with bifidobacteria and lactulose. After two weeks of treatment most of the patients reported subjective improvement with regular defecations. Seki *et al.* (1978) treated a group of constipated elderly with fermented milk with bifidobacteria. The frequency of defecations and the numbers of bifidobacteria in faeces increased. More recently, Alm *et al.* (1983) and Graf (1983) have also had encouraging results in the use of *acidophilus* milk for the treatment of constipation.

18.7.8
Radiation therapy

Therapeutic use of irradiation in the treatment of tumours usually causes acute gastrointestinal side-effects like enterocolitis and diarrhoea. It has been shown that radiation causes changes in the colonic bacterial flora, permeability of the mucosal cells and intestinal motility (Friberg, 1980). Recently, Cuzzolin *et al.* (1992) observed a significant decrease of the intestinal microflora after post-operative radiation therapy of patients with carcinoma of the uterine cervix or endometrium. At the end of the therapy all bacteria increased to basal values except *Enterococcus faecium,* lactobacilli and total anaerobes. Mettler et al. (1973) studied a group of women with colon carcinoma who were treated with irradiation. Fifty per cent of the patients were supplemented with a peroral bifidobacterium supplement (>9×10^6 CFU/day). Thirty per cent of the patients treated with bifidobacteria showed diarrhoea, compared with 65% in the control group. The numbers of bifidobacteria, *E. coli* and enterococci were also higher in the treated group than in the control group and the number of bifidobacteria in the intestinal flora was shown to be important for the preservation of other bacterial strains of the intestinal flora. In a pilot study, the influence of a test yoghurt containing *Lact. acidophilus* (NDCO 1748) on the side-effects of radiotherapy was investigated (Salminen *et al.,* 1988). Twentyfour female patients suffering from gynaecological malignancies and scheduled for irradiation of the pelvic area were selected for the study. The patients were randomized into two groups. Both groups received dietary counselling recommending a low-fat, low-residue diet during radiotherapy. The control group received dietary counselling only, while the test group received both dietary counselling and a daily dose of at least 2×10^9 live *Lact. acidophilus* in a yoghurt-type product. The test group received the product daily for five days

before, during and for ten days after the therapy regimen. In the treatment group, gastrointestinal side-effects after radiotherapy were less frequent and less severe than among patients receiving dietary counselling only.

18.7.9
Traveller's diarrhoea

Lactobacilli have also been tested for the prevention of traveller's diarrhoea (Clements *et al.*, 1981). The volunteers were challenged with 10^8–10^9 CFU of enterotoxigenic *E. coli* (ETEC) and given either a *Lactobacillus* preparation or placebo. The preparation contained 1.4×10^8–6.8×10^8 lactobacilli and was taken together with 240 ml of skim milk at six-hour intervals beginning 36 hours before and continuing for 96 hours after challenge with virulent ETEC. There was no significant difference in clinical symptoms between the two groups. Possibly, the challenge dose of ETEC given to the volunteers might have overwhelmed any protection offered by the lactobacilli. Recently, Black *et al.* (1989) showed that capsules containing a mixture of four different bacterial species, *Lact. acidophilus* plus *Bifido. bifidum* (90%), *Lact. bulgaricus* and *Streptococcus thermophilus,* significantly reduced the frequency of diarrhoea from 71% to 43% in tourists visiting Egypt. Each lactobacillus capsule contained approximately 3×10^9 live lyophilized organisms and one capsule was given three times daily, starting three days prior to departure, ending on the last day of travel. In a double-blind study, *Lactobacillus* GG was given orally in a daily dose of 2×10^9 CFU in order to investigate a possible effect of lactobacilli against traveller's diarrhoea. Altogether, 820 persons travelling to two destinations in Turkey received either *Lactobacillus* GG or a placebo. In the placebo group, the total incidence of diarrhoea was 46.5% compared with 41% in the *Lactobacillus* GG group, indicating an overall protection of $46.5-41.0=5.5/46.5=11.8\%$ (Oksanen *et al.,* 1990). The protection rates varied between the two different destinations with a maximum protection rate reported as 39.5%. *Lactobacillus* GG significantly reduced traveller's diarrhoea in one of the two destinations. Another series of randomized double-blind studies (Kollaritsch and Wiedermann, 1990) among Austrian tourists used one preparation containing *Lact. acidophilus* and another containing *Ent. faecium* SF68. Neither preparation offered significant protection against traveller's diarrhoea.

18.7.10
Salmonella infections

It has been reported that, when consumed at an early stage in the carrier state, *Lact. acidophilus* given in milk shortened the period of carriage for individuals infected with *Salmonella* (Alm, 1983). Daily consumption of at least 500 ml of milk containing 6×10^9 CFU/ml *Lact. acidophilus* was necessary to produce this effect.

18.7.11
Lactose intolerance

Congenital lactose intolerance is caused by a deficiency in the enzyme β-galactosidase (lactase), resulting in an inability to digest the disaccharide lactose. The prevalence of lactose intolerance varies depending on the ethnic origin. It is common in Japan, China, Africa and amongst Australian

Aborigines but less common in North European and North American countries. Temporary or permanent lactase deficiency may follow pelvic radiotherapy or infection with rotavirus which preferentially infects and destroys lactase-producing cells at the sides and tips of the villi (Christensen, 1989).

It has been known for some time that lactase-deficient subjects could tolerate lactose in yoghurt better than the same amount of lactose in milk (Kolars *et al.,* 1984). Although the mechanism remains unclear, it has been suggested that the yoghurt is supplying either preformed lactase or bacteria which produce lactase when they get into the small intestine. It is possible to show increased lactase activity in the small intestine of rats fed yoghurt; that this is of bacterial origin is shown by the large increase in activity in the gut contents compared with the gut wall (Garvie *et al.,* 1984). Early experiments with *Lact. acidophilus* improved lactose intolerance, but the results of subsequent trials have been variable (Kim and Gilliland, 1983). This variation may be due to strain differences in the *Lact. acidophilus* used. Intestinal infection can produce lactase deficiency. Yoghurt has been used to restore lactase activity in the intestine of children with *Giardia lamblia* infection (Pettoello *et al.,* 1989). Martini *et al.* (1991) tested the ability of various strains and species of lactic acid bacteria to digest lactose *in vivo*. Yoghurts with mixtures of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckeii* subsp. *bulgaricus,* and fermented milks containing species of *Strep, thermophilus, Lact. bulgaricus, Lact. acidophilus* or *Bifidobacterium bifidus* (probably *bifidum*) were fed to healthy non-lactose digesters. All yoghurts improved lactose digestion, regardless of their *β*-galactosidase activity, but the effects of fermented milks varied from marginal improvement with *Bifido. bifidus* to almost complete digestion with *Lact. bulgaricus* milk.

18.7.12
Antimutagenic and anticarcinogenic effects

Renner and Münztner (1991) showed the antimutagenic effects of a lyophilized preparation of *Bifido. longum,Lactobacillus gasseri* and *E. coli* (Omniflora), *Lactobacillus casei* only or a commercial yoghurt 'with living bifidobacteria'. In the Ames' test *Lact. casei* showed high antimutagenic activity on mutagenicity induced by nitrosated beef extract and Omniflora exhibited antimutagenic action on beef extract. *Lact. casei,* Omniflora and yoghurt orally given to small rodents with the mutagen busulfan showed strong anticlastrogenic action in the chromosome aberration and the micronucleus tests.

Intestinal bacteria (both Gram-positive and Gram-negative) have been shown to bind potent mutagenic pyrolyzates *in vitro* (Morotomi and Mutai, 1986). When *Lact. casei* YIT 9018 was added to the reaction mixture, the mutagenic activity of 3-amino-1-methyl-5*H*-pyrido[2, 3-b]indole (Trp—P-2) was inhibited. The binding was pH-dependent, occurred instantaneously and was inhibited by the addition of metal salts. Fermented milk products and lactic acid bacteria have been shown to inhibit mutagenic activity *in vitro* (Hosono *et al.,* 1986a,b). In a recent study by Zhang and Ohta (1991) it was shown that freeze-dried cells of *Lactococcus (Streptococcus) cremoris* Z-25 had the ability to simultaneously bind several mutagenic pyrolyzates. This binding was found to be pH-dependent and less of the mutagen was bound in gastric juice at low pH (below pH 2) than at higher pH values. In addition, two intestinal lactic acid bacteria, *Lact. acidophilus* IFO *and Bifido. bifidum* IFO were tested together with *Lactoc. (Streptococcus) cremoris* for their ability to bind the food mutagen Trp-P-2. *Lact. acidophilus* IFO was found to have the best survival in gastric juice and bound about 72% of the Trp-P-2, while *Bifido. bifidum* IFO bound about 67% of the mutagen

and *Lactoc. (Streptococcus) cremoris* had a binding of 64%. The same mutagen-binding capacity was found whether these bacterial strains were viable or had been killed by gastric juice (pH 1.5). In an *in vitro* study with *Lactoc. (Streptococcus) cremoris* and *Lactoc. (Streptococcus) lactis*, the polysaccharide of the cell wall was found to be responsible for the binding of mutagens via an adsorption mechanism (Zhang *et al.*, 1990).

When *Lact. acidophilus* was given to healthy volunteers on a fried meat diet known to increase faecal mutagenicity, on day three a 28% lower value in faecal .mutagen activity was noted compared with volunteers who had been given fried meat and *Lactococcus* milk (Lidbeck *et al.*, 1992). High levels of mutagenicity appeared in urine on days two and three of the fried meat and Lactococcus-fermented milk dietary regimen. During *Lact. acidophilus* administration, the mutagenic activity on day two was 72% lower compared with the *Lactococcus* milk period (P<0. 01), and on day three 55% lower value was observed (*P*<0.05). In most cases, however, an increase in the number of faecal lactobacilli corresponded to a lower mutagen excretion, particularly in urine (Lidbeck *et al.*, 1992).

In view of the *in vitro* results described above, it is possible that the *Lact. acidophilus* supplements are influencing excretion of mutagens by simply binding them in the intestine. Lactobacilli are one of the dominant species in the small intestine, and these microorganisms presumably affect metabolic reactions occurring in this part of the gastrointestinal tract. The ileal mucosa (Venitt, 1988) as well as the colonic mucosa (Fang and Stobel, 1978) has the capacity to absorb mutagenic compounds from the intestinal lumen, whereafter the compounds are passed into the bloodstream, either unchanged or as metabolites. Baker *et al.*, (1982) have shown that high levels of mutagenicity were detected in human urine already two to four hours after intake of fried meat. Therefore, it is likely that a main part of the mutagen absorption occurs in the upper small intestine, where presumably large amounts of the supplemented *Lact. acidophilus* could be found in the above-mentioned study (Lidbeck *et al.*, 1992). The *Lact. acidophilus strain* NCFB (formerly NCDO) 1748 which was given has been shown to have a survival rate of 1.3% through the stomach and small intestine (Pettersson *et al.*, 1983). Thus there were about 3×10^9 CFU/ml of *Lact. acidophilus* left to possibly affect Gram-negative microorganisms such as *E. coli* (Lidbeck *et al.*, 1987; Lidbeck *et al.*, 1991). Hawksworth et *al.* (1971) have reported *that E. coli* strains produced high levels of *β*-glucuronidase *in vitro*, while lactobacilli produced very low amounts of this enzyme. If the number of lactobacilli in the small intestine increase and *E. coli* decreases, it is conceivable that the levels of *β*-glucuronidase are decreased, thereby enabling an increased excretion of non-mutagenic glucuronide conjugates (Turesky *et al.*, 1988).

Many compounds are detoxified by glucuronide formation in the liver before entering the intestine via the bile. The bacterial enzyme *β*-glucuronidase has the ability to hydrolyze many glucuronides due to its wide substrate-specificity, and thus may liberate carcinogenic aglycones in the intestinal lumen. Other bacterial enzymes such as 7α-reductase, nitroreductase and azoreductase have also been shown to be implicated in the carcinogenic process, releasing carcinogens in the intestinal tract. *When Lact. acidophilus* was fed to rats, either on a grain or beef diet, the levels of *β*-glucuronidase, azoreductase and nitroreductase were significantly reduced in the beef-fed rats. The grain-fed rats already had low levels of these enzymes. Rats challenged with the carcinogen 1, 2-dimethylhydrazine (DMH) and fed a beef diet had a cancer incidence of 77%, but when given beef and *Lact. acidophilus*, the incidence was only 40% (Goldin and Gorbach, 1980). Rats associated with human faecal flora were fed with *Lact. acidophilus* strain NCFM or *Bifidobacterium adolescentis* 2204 for three days (Cole *et al.*, 1989). This resulted in significantly decreased activities of both *β*-

glucosidase and *β*-glucuronidase in the *Lact. acidophilus-treated* rats but no significant effect in the *Bifidobacterium* group.

Koo and Rao (1991) studied the effect of bifidobacteria on precursor lesions of colonic cancer in CF1 mice. The mice were treated with 1, 2-dimethylhydrazine and fed bifidobacteria with a bifidogenic factor, Neosugar, to promote the growth. The incidence of aberrant crypts and foci was significantly lower in animals fed bifidobacteria than in controls 38 weeks after the last injection of the carcinogen.

In healthy human volunteers the effect on faecal enzymes of giving milk and *Lact. acidophilus* has been investigated (Goldin and Gorbach, 1984). Only *Lact. acidophilus* feeding decreased the levels of *β*-glucuronidase. The results from a recent study in colon cancer patients partially support these findings (Lidbeck *et al.,* 1991). The mean faecal *β*-glucuronidase activity was reduced by 14% after two weeks of *Lact. acidophilus* supplementation and 9% after four and six weeks. Interestingly, the trend towards a reduction in this enzyme activity coincided with an increase in numbers of lactobacilli in most patients and a decrease in the number of *E. coli.* In another study, Marteau *et al.,* (1990) observed no changes in faecal *β*-glucuronidase, *β*-galactosidase and azoreductase after three weeks' administration of a fermented dairy product containing *Lact. acidophilus and Bifido. bifidum* to nine healthy volunteers. The activity of nitroreductase decreased and *β*-glucosidase increased significantly. The increase in *β*-glucosidase activity was suggested to be caused by colonic fermentation of cellulose by *Bifido. bifidum.*

Dietary fat has been considered a risk factor for colon cancer, and it has been suggested that this phenomenon may be mediated by increased levels of bile acids in the colon (Weisburger and Wynder, 1987). One hypothesis regarding colon carcinogenesis involves a cytotoxic effect on colonic epithelium exerted by bile acids in the aqueous phase of faeces (soluble bile acids), followed by an increased proliferation of cells in the intestine (Bruce, 1987).

In a study by Lidbeck *et al.* (1992) soluble faecal bile acids were characterized in 12 colon cancer patients. The mean value of the concentration of total soluble bile acids (deoxycholic acid and its 5-α-isomer, chenodeoxycholic acid and cholic acid) decreased from 147±108 mM to 125±113 mM after six weeks of *Lact. acidophilus* administration. The concentration of soluble deoxycholic acid in faeces was reduced from 92± 68 mM to 75±70 mM. Although the decrease in the concentration of bile acids in this fraction of faeces was not significant (perhaps due to a low number of patients or a limited supplementation period), it was of interest that a definite trend towards decreased levels of soluble bile acids was observed in the colon cancer patients receiving *Lact. acidophilus*-fermented milk supplements.

When *Lact. acidophilus* supplements were given, there were several changes in the intestinal microflora, which could have resulted in a decrease in the amount of bile acids in the aqueous phase of faeces. In this regard, the findings of Salvioli *et al.* (1982) are interesting in that another lactic acid-producing micro-organism, *Enterococcus* (formerly *Streptococcus*) *faecium,* given to healthy volunteers resulted in a slower formation of secondary bile acids from primary bile acids. Furthermore, the concentration of deoxycholic acid in bile decreased, while lithocholic acid was reduced in both bile and faeces.

Recently, 20 patients with colonic adenomas participated in a three-month study, where *Lact. acidophilus* was administered together with *Bifido. bifidum* (Biasco *et al.,* 1991). During this period, the faecal pH was reduced significantly from 7.46±0.12 to 7.01±0.08. In addition, eight patients having a higher proliferative activity in the upper colonic crypts than that calculated for subjects at low risk for colon cancer, showed a significant decrease after therapy with the lactic acid

bacteria. In view of the results in the above-mentioned study (Lidbeck *et al.,* 1991), it is interesting to speculate that this latter effect was in part due to decreased levels of bile acids in the aqueous phase of faeces.

In conclusion, the mechanisms underlying the antimutagenic and anticarcinogenic properties of lactobacilli and other lactic acid bacteria can be classified into different categories (Fernandes *et al.,* 1987). One mechanism would involve the above-mentioned effect on faecal enzymes thought to be involved in colon carcinogenesis. Another mechanism might be cellular uptake of potentially toxic compounds by lactobacilli, e.g. nitrites (Dodds and Collins-Thompson, 1984). A third mechanism could possibly involve cellular uptake of mutagenic compounds, as demonstrated in a number of studies (Zhang *et al.,* 1990; Zhang and Ohta, 1991). A fourth mechanism might involve suppression of tumours by an immune response mechanism (De Simone *et al.,* 1989; Perdigon *et al.,* 1990). The insoluble fraction of sonicated cells of *Lact. bulgaricus* has been shown to exert tumour-suppressing activity (Friend *et al.,* 1982).

18.7.13
Liver cirrhosis and hepatic encephalopathy

Chronic hepatic encephalopathy is a condition affecting the central nervous system as a complication to advanced hepatic cirrhosis. Blood with nitrogen compounds created from the breakdown of proteins by certain bacteria in the intestine pass the damaged liver without being detoxified. These toxic compounds then circulate to the brain and affect its function. This condition was originally treated with protein restriction in the diet, laxative and long-term broad-spectrum antibiotics. Treatment with lactulose (β-galactosido-fructose), a synthetic, non-absorbable disaccharide extensively used in the treatment of hepatic encephalopathy, has improved the condition in cirrhotic patients (Vince *et al.,* 1974; Riggio *et al.,* 1990). A significant increase in bifidobacteria and decrease in lecithinase-positive clostridia, after two weeks' supplementation with lactulose, was seen in a group of eight healthy volunteers (Terada *et al.,* 1992). Two early studies (Macbeth *et al.,* 1965; Read *et al.,* 1966) demonstrated a decrease in faecal urease, a lowering of blood ammonia and associated clinical improvement in patients treated with a *Lact. acidophilus* preparation. A more recent study demonstrated that a preparation of *Ent. faecium* SF68 was as effective as lactulose, if not more so, in the treatment of this disease (Loguercio *et al.,* 1987).

Müting *et al.* (1968) treated a group of patients with liver cirrhosis or chronic hepatitis with Eugalan Forte containing *'Bacterium bifidum'* during two weeks to 18 months. This resulted in a decrease in blood ammonia, free serum phenols and free amino nitrogen. Also, the excretion of free phenols and free amino acids in the urine decreased significantly. The content of *'Bacterium bifidum'* in the faecal flora increased. More recently, Müting *et al.* (1986) also reported a clinical study of *Bifidobacterium bifidum* administration to liver cirrhotic patients. *Bifido. bifidum* was orally administered during 14 days. A significant reduction in faecal pH and hyperammoniaemia was observed. Hepatic protein and urea synthesis were improved and there was a tendency towards an increase in faecal *Lact. acidophilus* and *Bifido. bifidum* counts.

18.7.14 Enhancement of immune response

In the past few years the interactions between orally-administered probiotics and immunocompetence have been studied. Some of these organisms seem to increase their host's specific and nonspecific immune mechanisms. This is presumably mediated either by absorption of soluble antigens or by translocation of the bacteria through the intestinal wall into the blood. The effect on the peripheral and intestinal immune system of fifteen elderly individuals supplemented with capsules with lyophilized *Bifido. bifidum* and *Lact. acidophilus* for 28 days, was investigated (De Simone *et al.,* 1992). The supplement significantly reduced the colonic inflammatory infiltration, without altering T, B and Leu7+ cell percentage. A significant increase of B-cell frequency in the peripheral blood was also noted. Oral administration of *Lact. casei* to mice has been shown to increase phagocytic activity (Perdigon *et al.,* 1986). Yasui *et al.* (1992) used an *in vitro* screening test with murine Peyer's patch cell cultures to detect strains of bifidobacteria inducing large quantities of IgA. Two human strains of *Bifido. breve* and one of *Bifido. longum* were selected. When one of these *Bifido. breve* strains was administered orally with cholera toxin to mice, the amount of anti-cholera toxin IgA production and the proliferation in Peyer's patch cells were significantly increased.

18.8 Conclusions

In conclusion, the field of the use of probiotics in human medicine is a rapidly expanding one. However, it is still not certain that the organisms currently being used as probiotics are those which are responsible for the beneficial effects of the normal intestinal flora. More trials are needed to establish the efficacy of those probiotics which are currently on the market. The possible use of probiotics for decreasing risk for colonic malignancies is an exciting area, but there is still a great deal of work to be done in this field. When more is known regarding the mechanisms by which probiotics function, a more rational approach to the selection of strains used in probiotic preparations will be possible. It may then become possible to genetically manipulate organisms to combine a capacity to establish itself in the gut with an ability to produce the factors responsible for the probiotics beneficial effect.

References

Allen, S.D., Siders, J.A., Cromer, M.D., Fischer, J.A., Smith, J.W. and Israel, K.S., 1980, Effect of LY 127935 (6059-S) on human fecal flora, in Nelson, J.D. and Grassi, C. (Eds) *Current Chemotherapy and Infectious Disease,* Vol. 1, pp. 101–3, Washington: The American Society for Microbiology.

Alm, L., 1982, *The Effect of Fermentation on Nutrients in Milk and Some Properties of Fermented Liquid Milk Products,* Thesis, Stockholm, Karolinska Institute.

Alm, L., 1983, The effect of *Lactobacillus acidophilus* administration upon the survival *of Salmonella* in randomly selected human carriers, *Prog. Food Nutr. Sci.,* **7,** 13–17.

Alm, L., Humble, D., Ryd-Kjellen, E. and Setterberg, G., 1983, The effect of *acidophilus* milk in the treatment of constipation in hospitalised geriatric patients, *Symposia of Swedish Nutrition Foundation,* **XV,** 131–38.

Ambrose, N.S., Johnson, M., Burdon, B.W. and Keighley, M.R., 1985, The influence of single-dose intravenous antibiotics on faecal flora and emergence of *Clostridium difficile, J.Antimicrob. Chemother.,* **15,** 319–26.

Aronsson, B., Möllby, R. and Nord, C.E., 1985, Antimicrobial agents and *Clostridium difficile* in acute enteric disease: epidemiological data from Sweden 1980–1982, *J. Infect. Dis.,* **151,** 476–81.

Axelsson, L.T., Chung, T.C., Dobrogosz, W.J. and Lindgren, S.E., 1989, Production of a broadspectrum antimicrobial substance by *Lactobacillus reuteri, Microb. Ecol. Health Dis.,* **2,** 131–36.

Ayebo, A.D., Angelo, I.A. and Shahani, K.M., 1980, Effect of ingesting *Lactobacillus acidophilus* milk upon fecal flora and enzyme activity in humans, *Milchwissenschaft,* **35,** 730–33.

Baker, R., Arlauskas, A., Bonin, A. and Angus, D., 1982, Detection of mutagenic activity in human urine following fried pork or bacon meals, *Cancer Lett.,* **16,** 81–89.

Bartlett, J.G., Chang, T.W., Gurwith, M., Gorbach, S.L. and Onderdonk, A.B., 1978, Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia, *N. Engl. J. Med.,* **298,** 531–34.

Beck, C. and Necheles, H., 1961, Beneficial effects of administration *of Lactobacillus acidophilus* in diarrhoeal and other intestinal disorders, *Am. J. Gastroenterol.,* **35,** 522–30.

Benno, Y. and Mitsuoka, T., 1992, *Impact of Biftdobacterium longum* on human fecal microflora, *Microbiol. Immunol.,* **36,** 683.

Benno, Y., Suzuki, K., Suzuki, K., Narisawa, K., Bruce, W.R. and Mitsuoka, T., 1986, Comparison of the fecal microflora in rural Japanese and urban Canadians, *Microbiol. Immunol.,* **30,** 521–32.

Bhatia, S.J., Kochar, N., Abraham, P., Nair, N.G. and Mehta, A.P., 1989, *Lactobacillus acidophilus* inhibits growth of *Campylobacter pylori in vitro, J.Clin. Microbiol.,* **27,** 2328–30.

Biasco, G., Paganelli, G.M., Brandi, G., Brillanti, S., Lami, F., Callegari, C. and Gizzi, G., 1991, Effect *of Lactobacillus acidophilus* and *Bifidobacterium bifidum* on rectal cell kinetics and fecal pH, *Ital. J. Gastroenterol,* **23,** 142.

Black, F., Einarsson, K., Lidbeck, A., Orrhage, K. and Nord, C.E., 1991, Effect of lactic acid-producing bacteria on the human intestinal microflora during ampicillin treatment, *Scand. J. Infect. Dis.,* **23,** 247–54.

Black, F.T., Andersen, P.L., Orskov, J., Orskov, F., Gaarslev, K. and Laulund, S., 1989, Prophylactic efficacy of lactobacilli on traveller's diarrhea, in Steffen, R. (Ed.) *Travel Medicine. Conference on International Travel Medicine 1,* Zürich, Switzerland, 1988, pp. 333–35, Berlin: Springer-Verlag.

Borgia, M., Sepe, N., Brancato, V. and Borgia, R., 1982, A controlled clinical study on *Streptococcus faecium* preparation for the prevention of side-reactions during long term antibiotic therapy, *Curr. Ther. Res.,* **6,** 352–56.

Borriello, S.P., 1988, The application of bacterial antagonism in the prevention and treatment of *Clostridium difficile* infection of the gut, in Hardie, J.M. and Borriello, S.P. (Eds) *Anaerobes Today,* pp. 195–202, London: John Wiley and Sons.

Bouhnik, Y., Pochart, P., Marteau, P., Arlet, G., Goderel, I. and Rambaud, J.C., 1992, Fecal recovery in humans of viable *Bifidobacterium* sp. ingested in fermented milk, *Gastroenterol.,* **102,** 875.

Bowden, T.A., Mansberger, A.R. and Lykins, L.E., 1981, Pseudomembranous enterocolitis: mechanism of restoring floral homeostasis, *Amer. Surg.,* **47,** 178–83.

Bruce, W.R., 1987, Recent hypotheses for the origin of colon cancer, *Cancer Res.,* **47,** 4237–42.

Christensen, M.L., 1989, Human viral gastroenteritis, *Clin. Microbiol. Rev.,* 51–89.

Clements, M.L., Levine M.M., Black, R.E., Robins-Browne, R.M., Cisneros, L.A., Drusano, G.L., Lanata, C.F. and Saah, A.J., 1981, *Lactobacillus* prophylaxis for diarrhea due to enterotox*igenic Escherichia coli, Antimicrob. Agents Chemother.,* **20,** 104–8.

Clements, M.L., Levine, M.M., Ristaino, P.A., Daya, V.E. and Hughes, T.P., 1983, Exogenous lactobacilli fed to man—their fate and ability to prevent diarrheal disease, *Prog. Food Nutr. Sci.,* **7,** 29–37.

Cole, C.B., Fuller, R. and Carter, S.M., 1989, Effect of probiotic supplements of *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* 2204 on *β*-glucosidase and *β*-glucuronidase activity in the lower gut of rats associated with a human faecal flora, *Microbial. Ecol. Health Dis.,* **2,** 223–25.

Collins, F.M. and Carter, P.B., 1978, Growth of salmonellae in orally infected germfree mice, *Infect. Immun.,* **21,** 41–47.

Colombel, J.F., Cortot, A., Neut, C. and Romond, C., 1987, Yoghurt with *Bifidobacterium longum* reduces erythromycin-induced gastrointestinal effects , *Lancet,* **ii,** 43.

Conway, P.L., Gorbach, S.L. and Goldin, B.R., 1987, Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells, *J. Dairy Sci.,* **70,** 1–12.

Cuzzolin, L., Zambreri, D., Donini, M., Griso, C. and Benoni, G., 1992, Influence of radiotherapy on intestinal microflora in cancer patients, *J. Chemother.,* **4,** 176–79.

Daikos, G.K., Kontomichalou, P., Bilalis, D. and Pimendiou, L., 1968, Intestinal flora ecology after oral use of antibiotics, *Chemother.,* **13,** 146–60.

De Simone, C., Bianchi Salvatori, B., Jirillo, E., Baldinelli, L., Di Fabio, S. and Vesely, R., 1989, Yoghurt and the immune response, *in Les Laits Fermentés Actualité de la Recherche,* pp. 63–67, London, Paris: John Libbey Eurotext.

De Simone, C., Ciardi, A,, Grassi, A., Lambert Gardini, S., Tzantzoglou, S., Trinchieri, V, Moretti, S. and Jirillo, E., 1992, Effect of *Bifidobacterium bifidum and Lactobacillus acidophilus* on gut mucosa and peripheral blood B-lymphocytes, *Immunopharmacol. Immunotoxicol.,* **14,** 331–40.

Dodds, K.L. and Collins-Thompson, D.L., 1984, Incidence of nitrite-depleting lactic acid bacteria in cured meats and in meat starter cultures, *J. Food Protect.,* **47,** 7–10.

Doerbeck, F. and Tobiasch, V., 1973, Zur der Therapie der chronischen Obstipation (The therapy of chronic obstipation), *Medizinische Monatsschrift,* **27,** 81–84.

Drasar, B.S., Shiner, M. and McLeold, G.M., 1969, Studies in the intestina flora I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons, *Gastroenterol.,* **56,** 71–79.

Eiseman, B., Silem, W., Boscomb, W.S. and Kanov, A.J., 1958, Faecal enema as an adjunct in the treatment of pseudomembranous enterocolitis, *Surgery,* **44,** 854–58.

Fang, W.-F. and Strobel, H.W., 1978, Activation of carcinogens and mutagens by rat colon mucosa, *Cancer Res.,* **38,** 2939–44.

Fernandes, C.F., Shahani, K.M. and Amer, M.A., 1987, Therapeutic role of dietary lactobacilli and lactobacillic-fermented dairy products, *FEMS Mirobiol. Rev.,* **46,** 343–56.

Finegold, S.M., Sutter, V.L. and Mathisen, G.E., 1983, Normal indigenous intestinal flora, in Hentges, D.J. (Ed.) *Human Intestinal Micro flora in Health and Disease,* pp. 3–31, New York: Academic Press.

Friberg, H., 1980, Effects of irradiation on the small intestine of the rat. A SEM study, Thesis, p. 235, Sweden: University of Lund.

Friend, B.A., Farmer, R.E. and Shahani, K.M., 1982, Effect of feeding and intraperitoneal implantation of yoghurt culture cells on Ehrlich ascites tumor cells, *Milchwissenschaft,* **37,** 708–10.

Fuller, R., 1989, Probiotics in man and animals, *J. Appl. Bacterial.,* **66,** 365–78.

Fuller, R., 1991, Probiotics in human medicine, *Gut,* **32,** 439–42.

Gade, J. and Thorn, P., 1989, Paraghurt for patients with irritable bowel syndrome. A controlled clinical investigation from general practice, *Scand. J. Prim. Health Care,* **7,** 23–26.

Garvie, E.I., Cole, C.B., Fuller, R. and Hewitt, D., 1984, The effect of yoghurt on some components of the gut microflora and on the metabolism of lactose in the rat, *J. Appl. Bacterial.* **56,** 237–45.

Gilliland, S.E., Staley, T.E. and Bush, L.J., 1984, Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct, *J. Dairy Sci.,* **67,** 3045–51.

Gismondo, M.R., Lo Bue, A.M., Chisari, G., Pecorella, G., Malandrino, G. and Petralito, E., 1990, Studio dellàttività competitiva di un preparato batterico sulla colonizzazione e patogenicità di *C. pylori, Clin. Terapeutica,* **134,** 41–46.

Goldin, B.R., 1986, *In situ* bacterial metabolism and colon mutagens, *Ann. Rev. Microbiol.,* **40,** 367–93.

Goldin, B.R. and Gorbach, S.L., 1980, Effect of *Lactobacillus acidophilus* dietary supplements on 1, 2-dimethylhydrazine dihydrochloride-induced intestinal cancer in rats, *J. Natl. Cancer Inst.,* **64,** 263–65.

Goldin, B.R. and Gorbach, S.L., 1984, The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity , *Am. J. Clin. Nutr.,* **39,** 756–61.

Gorbach, S.L., Chang, T.W. and Goldin, B., 1987, Successful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG, *Lancet,* **ii,** 1519.

Gotz, V., Romankiewics, J.A., Moss, J. and Murray, H.W., 1979, Prophylaxis against ampicillin-associated diarrhea with a lactobacillus preparation, *Am. J. Hosp. Pharm.,* **36,** 754–57.

Graf, W., 1983, Studies on the therapeutic properties of acidophilus milk, *Symposia of Swedish Nutrition Foundation,* **XV,** 119–21.

Hawksworth, G., Drasar, B.S. and Hill, M.J., 1971, Intestinal bacteria and the hydrolysis of glycosidic bonds, *J. Med. Microbiol.,* **4,** 451–59.

Heimdahl, A. and Nord, C.E., 1979, Effect of phenoxymethylpenicillin and clindamycin on the oral, throat and faecal microflora of man, *Scand. J. Infect. Dis.,* 233–42.

Hosono, A., Kashina, T. and Kada, T., 1986a, Antimutagenic properties of lactic acid-cultured milk on chemical and fecal mutagens, *J. Dairy Sci.,* **69,** 2237–42.

Hosono, A., Sagae, S. and Tokita, F., 1986b, Desmutagenic effect of cultured milk on chemically induced mutagenesis in *Escherichia coli* B/r WP 2 trp- her-, *Milchwissenschaft,* **41**(3), 142–45.

Kandler, O. and Weiss, N., 1986, Regular, nonsporing Gram-positive rods, in Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (Eds) *Bergey's Manual of Systematic Bacteriology,* Vol. 2, pp. 1208–34, Baltimore: Williams and Wilkins.

Kim, H.S., 1988, Characterization of lactobacilli and bifidobacteria as applied to dietary adjuncts, *Cult. Dairy Prod. J.,* **23,** 6–9.

Kim, H.S. and Gilliland S.E., 1983, *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans, *J. Dairy Sci.,* **66,** 959–66.

Knothe, H., Dette, G.A. and Shah, P.M., 1985, Impact of injectable cephalosporins on the gastrointestinal microflora: observations in healthy volunteers and hospitalised patients, *Infection,* **13,** S129–33.

Kolars, J.C., Levitt, M.D., Aouji, M. and Savaiano, D.A., 1984, Yoghurt—an autodigesting source of lactose, *N. Engl. J. Med.,* **310,** 1–3.

Kollaritsch, H. and Wiedermann, G., 1990, Traveller's diarrhoea among Austrian tourists: epidemiology, clinical features and attempts at nonantibiotic drug prophylaxis, in Pasini, W. (Ed.) *Proceedings of the Second International Conference on Tourist Health,* pp. 74–82, Rimini: WHO.

Koo, M. and Rao, A.V., 1991, Long-term effect of bifidobacteria and neosugar on precursor lesions of colonic cancer in CF1 mice, *Nutr. Cancer,* **16,** 249–57.

Lewenstein, A., Frigerio, G. and Moroni, M., 1979, Biological properties of SF 68, a new approach for the treatment of diarrhoeal diseases, *Current Therapeutic Res.* **26,** 967–81.

Lidbeck, A., Gustafsson, J.-Å. and Nord, C.E., 1987, Impact of *Lactobacillus acidophilus* supplements on the human oropharyngeal and intestinal microflora, *Scand. J. Infect. Dis.,* **19,** 531–37.

Lidbeck, A., Edlund, C., Gustafsson, J.-Å., Kager, L. and Nord, C.E., 1988, Impact of *Lactobacillus acidophilus* on the normal intestinal microflora after administration of two antimicrobial agents, *Infection,* **16,** 329–36.

Lidbeck, A., Övervik, E., Rafter, J., Nord, C.E. and Gustafsson, J.-Å., 1992, Effect of *Lactobacillus acidophilus* supplements on mutagen excretion in faeces and urine in humans, *Microbial. Ecol. Health Dis.,* **5,** 59–67.

Lidbeck, A., Geltner Allinger, U., Orrhage, K.M., Ottova, L., Brismar, B., Gustafsson, J.-Å., Rafter, J.J. and Nord, C.E., 1991, Impact of *Lactobacillus acidophilus* supplements on the faecal microflora and soluble faecal bile acids in colon cancer patients, *Microb. Ecol. Health Dis.,* **4,** 81–88.

Loguercio, C., Del Vecchio, B. and Coltori, M., 1987, *Enterococcus* lactic acid bacteria strain SF 68 and lactulose in hepatic encephalopathy: a controlled study, *J. Int. Med. Res.,* **15,** 335–43.

McGroarty, J.A. and Reid, G., 1988, Detection of a lactobacillus substance that inhibits *Escherichia coli, Can. J. Microbiol.,* **34,** 974–78.

Macbeth, W.A., Kass, E.H. and McDermott, W.V., 1965, Treatment of hepatic encephalopathy by alteration of intestinal flora with *Lactobacillus acidophilus, Lancet,* **i,** 399–403.

Marteau, P., Pochart, P., Flourié, B., Pellier, P., Santos, L., Desjeux, J.-F. and Rambaud, J.-C., 1990, Effect of chronic ingestion of a fermented dairy product *containing Lactobacillus acidophilus* and *Bifidobacterium bifidum* on metabolic activities of the colonic flora in humans, *Amer. J. Nutr.,* **52,** 685–88.

Martini, M., Lerebours, E., Lin, W., Harlander, S., Berrada, N., Antoine, J. and Savaioni, D., 1991, Strains and species of lactic acid bacteria in fermented milks (yoghurts): effect on *in vivo* lactose digestion, *Am. J. Clin. Nutr.,* **54,** 1041–46.

Mehta, A.M., Patel, K.A. and Dave, P.J., 1983, Isolation and purification of an inhibitory protein from *Lactobacillus acidophilus* AC 1, *Microbios,* **37,** 37–43.

Mettler, L., Romeyke, A. and Brieler, G., 1973, Zur Beeinflussung der para- und postradiologishen Dysbakterie und Strahlenreaktion des Darmes durch *Bakterium Bifidum* Substitutionstherapie, *Strahlentherapie,* **145,** 588–99.

Mitsuoka, T., Hayakawa, K. and Kimura, N., 1974, Die Faekalflora bei Menschen. II. Mitteilung: die zusammensetzung der bifidobacterienflora der verschiedenen altersgruppen, Zentralbl. *Bakteriol. Hyg. I. Abt. Orig.,* **A226,** 469–78.

Mitsuoka, T. and Kaneuchi, C., 1977, Ecology of the bifidobacteria, *Am. J. Clin. Nutr.,* **30,** 1799–810.

Moberg, L.J. and Sugiyama, H., 1979, Microbial ecological basis of infant botulism as studied with germ-free mice, *Infect. Immun.,* **25,** 653–57.

Morotomi, M. and Mutai, M., 1986, *In vitro* binding of potent mutagenic pyrolyzates to intestinal bacteria, *J. Natl. Cancer Inst.,* **77,** 195–201.

Motta, L., Blancato, G., Scornavacca, G., De Luca, M., Vasquez, E., Gismondo, M.R., LoBue, A. and Chisari, G., 1991, Studio sull' attività di un associazione batterioterapica nei disturbi dell alvo dell' anziano, *Clin. Terapeutica,* **138,** 27–35.

Muriana, P.M. and Klaenhammer, T.R., 1991, Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088, *Appl. Environ. Microb.,* **57,** 114–21.

Müting, D., Eschrich, W. and Mayer, J.B., 1968, The effect *of Bacterium bifidum* on the intestinal bacterial flora and toxic protein metabolites in chronic liver disease , *Am. J. Proctol.,* **19,** 336–42.

Müting, D., Leimbeck, R., Flasshoff, H.J. and Rusch, V, 1986, *Bifidobacterium bifidum* administration in humans: a controlled clinical study in liver cirrhosis, Abstract of a poster published in *Microecol Therapy,* **16,** 271.

Neut, C., Colombel, J.F., Guillemot, F., Cortot, A., Gower, P., Quandelle, P., Ribet, M., Romond, C. and Paris, J.C., 1989, Impaired bacterial flora in human excluded colon, *Gut,* **30,** 1094–98.

Nord, C.E. and Kager, L., 1984, The normal flora of the gastrointestinal *tract, Netherlands J. Med.,* **27,** 249–52.

Nord, C.E., Heimdahl, A. and Kager, L., 1986, Antimicrobial induced alterations of the human oropharyngeal and intestinal microflora, *Scand. J. Infect. Dis.,* **49** (**Suppl.**)**,** 64–72.

Nord, C.E., Heimdahl, A., Kager, L. and Malmborg, A.S., 1984, The impact of different antimicrobial agents on the normal gastrointestinal microflora of humans, *Rev. Infect. Dis.,* **6** (**Suppl.**), 270–75.

Oksanen, P.J., Salminen, S., Saxelin, M., Hämäläinen, P., Ihantola-Vormisto, A., Muurasniemi-Isoviita, L., Nikkari, S., Oksanen, T., Pörsti, I., Salminen, E., Siitonen, S., Stuckey, H., Toppila, A. and Vapaatalo, H., 1990, Prevention of traveller's diarrhoea *by Lactobacillus GG,Ann. Med.,* **22,** 53–56

Orrhage, K., Brismar, B., and Nord, C.E., 1994, Effect of supplements with *Bifidobacterium longum* and *Lactobacillus acidophilus* on the intestinal *microbiota* during administration of clindamycin, *Microbial. Ecol Health Dis.,* **7,** 17–25.

Orrhage, K., Lidbeck, A. and Nord, C.E., 1991, Effect of *Bifidobacterium longum* supplements on the human faecal microflora, *Microbial. Ecol. Health Dis.,* **4,** 265–70.

Pearce, J.L. and Hamilton, J.R., 1974, Controlled trial of orally administered lactobacilli in acute infantile diarrhea, *J. Pediatr.,* **84,** 261–62.

Pecorella, G., Vasquez, E., Gismondo, M.R., Lo Bue, A.M. and Chisari, G., 1992, *Lactobacillus acidophilus* e *Bifidobacterium bifidum* sull' ecosistema intestinale dell' anziano, *Clin. Terapeutica,* **140,** 3–10.

Perdigon, G., De Macias, M.E.N., Alvarez, S., Oliver, G. and DeRuiz Holgada, A.A.P., 1986, Effect of perorally administered lactobacilli on macrophage activation in mice, *Infect. Immun.,* **53,** 404–10.

Perdigon, G., Nader de Macias, M.E., Alvarez, S., Oliver, G. and Pesce de Ruiz Holgado, A.A., 1990, Prevention of gastrointestinal infection using immunobiological methods with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus, J. Dairy Res.,* **57,** 255–64.

Pettersson, L., Graf, W. and Sewelin, U., 1983, Survival of L. *acidophilus* NCDO 1748 in the human gastrointestinal tract. 2. Ability to pass the stomach and intestine *in vivo,* in Hallgren, B. (Ed.) *Nutrition and the Intestinal Flora. XV Symp. Swed. Nutr. Found.,* pp. 127–30, Uppsala: Almqvist and Wiksell.

Pettoello Mantovani, M., Guandalini, S., Ecuba, P., Corvino, C, and di Martino, L., 1989, Lactose malabsorption in children with symptomatic *Giardia lamblia* infection: feasibility of yoghurt supplementation , *J. Ped. Gastroenterol.,* **9,** 295–300.

Pochart, P., Marteau, P., Bouhnik, Y., Goderel, I., Bourlioux, P. and Rambaud, J.-C., 1992, Survival of bifidobacteria ingested via fermented milk during their passage through the human small intestine: an *in vivo* study using intestinal perfusion, *Am. J. Nutr.,* **55,** 78–80.

Poupard, J.A., Husain, I. and Norris, R.F., 1973, Biology of the bifidobacteria, *Bacterial. Rev.,* **37,** 136–65.

Read, A.E., McCarthy, C.F., Heaton, H.W. and Laidlaw, I., 1966, *Lactobacilus acidophilus* (Enpac) in treatment of hepatic encephalopathy, *Brit. Med. J.,* **i,** 1267–69.

Renner, H.W. and Müntzner, R., 1991, The possible role of probiotics as dietary antimutagens, *Mut. Res.,* **262,** 239–45.

Riggio, O., Varriale, M., Testore, G.P., Di Rosa, R., Di Rosa, E., Merli, M., Candiani, C. and Capocaccia, L., 1990, Effect of lactitol and lactulose administration on the fecal flora in cirrhotic patients , *J. Clin. Gastroenterol.,* **12,** 433–36.

Salminen, S. and Deighton, M., 1992, Lactic acid bacteria in the gut in normal and disordered states, *Dig. Dis.,* **10,** 227–38.

Salminen, E., Elomaa, I., Minkkinen, J., Vapaatalo, H. and Salminen, S., 1988, Preservation of intestinal integrity using *live Lactobacillus acidophilus* cultures, *Clin. Radiol.,* **34,** 435–37.

Salvioli, G., Salati, R., Bondi, M., Fratalocchi, A., Sala, B.M., and Gibertini, A., 1982, Bile acid transformation by the intestinal flora and cholesterol saturation in bile. Effects of *Streptococcus faecium* administration, *Digestion,* **23,** 80–88.

Sandine, W.E., 1979, Roles of lactobacillus in the intestinal tract, *J. Food Prot.,* **42,** 259–62.

Savage, D., 1984, Adherence of the normal flora, in Boedeker, E.G. (Ed) *Attachment of Organisms to the Gut Mucosa,* pp. 4–10, Florida: CRC Press.

Saxelin, M., Elo, S., Salminen, S. and Vapaatalo, H., 1991, Dose-response colonization of faeces after oral administration *of Lactobacillus casei* strain GG, *Microb. Ecol. Health Dis.,* **4,** 209–14.

Scardovi, V., 1986, Genus *Bifidobacterium,* in Sneath, P.H.A., Mair, N.S. and Sharpe, E. (Eds) *Bergey's Manual of Systematic Bacteriology,* Vol. 2, pp. 1418–34, Baltimore: Williams and Wilkins.

Schwan, A., Sjölin, S., Trottestam, U. and Aronsson, B., 1984, Relapsing *Clostridium difficile* enterocolitis cured by rectal infusion of normal faeces, *Scand. J. Infect., Dis.,* **16,** 211–15.

Seki, M., Igarashi, M., Fukuda, Y., Shiamura, S., Kawashima, T. and Ogasa, K., 1978, The effect of *Bifidobacterium* cultured milk on the 'regularity' among an aged group, *J. Jpn. Soc. Nutr. Food Sci.,* **31,** 379–87.

Siitonen, S., Vapaatalo, H., Salminen, S., Gordin, A., Saxelin, M., Wikberg, R., Kirkkola, A.-M., 1990, Effect of *Lactobacillus* GG yoghurt in prevention of antibiotic associated diarrhea, *Ann. Med.,* **22,** 57–60.

Speck, M.L., 1976, Interactions among lactobacilli and man, *J. Dairy Sci.,* **59,** 338–43.

Speck, M.L., 1983, Lactobacilli as dietary supplements and manifestations of their functions in the intestine, in Hallgren, B. (Ed.) *Nutrition and the Intestinal Flora. XV Symp. Swed. Nutr. Found.,* pp. 93–98, Uppsala: Almqvist and Wiksell.

Sullivan, N.M., Mills, D.C., Riemann, H.P. and Arnon, S.S., 1988, Inhibition of growth of *Clostridium botulinum* by intestinal microflora isolated from healthy infants, *Microb. Ecol. Health Dis.,* **1,** 179–92.

Surawicz, C.M., McFarland, L.V., Elmer, G. and Chinn, J., 1989a, Treatment of recurrent *Clostridium difficile* colitis with vancomycin and *Saccharomyces bonlardii,Am. J. Gastroenterol.,* **84,** 1285–87.

Surawicz, C.M., Elmer, G., Speelman, P., Lynne, V., McFarland, L.V., Chinn, J. and van Belle, G., 1989b, Prevention of antibiotic-associated diarrhea by *Saccharomyces bonlardii:* a prospective study, *Gastroenterol.,* **96,** 981–88.

Tamura, N., Norimoto, M., Yoshida, K., Hirayama, C, Nakai, R. and Takagi, A., 1983, Alteration of fecal bacterial flora following oral administration of bifidobacterial preparation, *Gastroenterologia Japonica,* **18,** 47–55.

Tannock, G.W., 1983, Effect of dietary and environmental stress on the gastrointestinal microbiota, in Hentges, D.J. (Ed.) *Human Intestinal Microflora in Health and Disease,* pp. 517–39, New York: Academic Press.

Terada, A., Hara, H., Kataoka, M. and Mitsuoka, T., 1992, Effect of lactulose on the composition and metabolic activity of the human faecal flora, *Microb. Ecol. Health Dis.,* **5,** 43–50.

Tissier, H., 1899, Recherches sur la flore intestinale des nourrissons. (Etat normal et pathologique). Thesis, University of Paris (Med) Carré, G., Naud, C. (Eds), Paris, France.

Tojo, M., Oikawa, T., Morikawa, Y., Yamashita, N., Iwata, S., Satoh, Y., Hanada, J. and Tanaka, R., 1987, The effects of *Bifidobacterium breve* administration on Campylobacter enteritis, *Acta Paediatr. Jpn.,* **29,** 160–67.

Turesky, R.J., Aeschbacher, H.U., Würzner, H.P., Skipper, P.L. and Tannenbaum, S.R., 1988, Major routes of metabolism of the food-borne carcinogen 2-amino-3, 8-dimethylimidazo[4, 5–f] quinoxaline in the rat, *Carcinogenesis,* **9,** 1043–48.

Tvede, M. and Rask-Madsen, J., 1989, Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients, *Lancet,* **i,** 1156–60.

Venitt, S., 1988, Mutagens in human faeces and cancer of the large bowel, in Rowland, I.R. (Ed.) *Role of the Gut Flora in Toxicity and Cancer,* pp. 399–460, London: Academic Press.

Vince, A., Zeegen, R., Drinkwater, J.E., O'Grady, F. and Dawson, A.M., 1974, The effect of lactulose on the faecal flora of patients with hepatic encephalopathy, *J. Med. Microbiol.,* **7,** 163.

Weisburger, J.H. and Wynder, E.L., 1987, Etiology of colorectal cancer with emphasis on mechanism of action and prevention, in De Vita, V.T., Hellman, S. and Rosenberg, S.A. (Eds) *Important Advances in Oncology,* pp. 197–220, Philadelphia: J.B.Lippincott.

Yasui, H., Nagaoka, N., Mike, A., Hayakawa, K. and Ohwaki, M., 1992, Detection of *Bifidobacterium* strains that induce large quantities of IgA, *Microb. Ecol. Health Dis.,* **5,** 155–62.

Zhang, X.B., Ohta, Y. and Hosono, A., 1990, Antimutagenicity and binding of lactic acid bacteria from a Chinese cheese to mutagenic pyrolyzates, *J. Dairy Sci.,* **73,** 2702–10.

Zhang, X.B. and Ohta, Y., 1991, *In vitro* binding of mutagenic pyrolyzates to lactic acid bacterial cells in human gastric juice, *J. Dairy Sci.,* **74,** 752–57.

Zoppi, G., Deganello, A., Benoni, G. and Saccomani, F., 1982, Oral bacteriotherapy in clinical practice, *Eur. J. Pediat.,* **139,** 18–21.

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