THE EFFECT OF RAW AND PROCESSED VEGETABLES ON COLONOCYTE DNA DAMAGE

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ABSTRACT

Cruciferous vegetables and their bioactive constituents have been shown to inhibit chemically induced colon cancer (IARC, 2004). However, the results of epidemiological studies have been inconsistent (IARC, 2004). This may reflect a lack of sensitivity of such studies. One factor that is often overlooked by epidemiological studies is the effect of processing. Processing may alter the content of the bioactive compounds present in cruciferous vegetables or their bioavailability. Cruciferous vegetables contain numerous bioactive compounds, but their anticarcinogenic properties have been mainly attributed to their content of glucosinolates (GLS). The breakdown of GLS to their bioactive products is largely dependent on the plant enzyme myrosinase, whereas the profile of products formed, depends on the parent GLS, conditions of hydrolysis and the presence of a cofactor, epithiospecifier protein (ESP). Thermal processing may inactivate myrosinase and ESP thereby altering the location and extent of GLS breakdown within the GI tract and the profile of breakdown products formed. This may in turn, determine whether cruciferous vegetables exert beneficial or detrimental effects. A pig feeding trial was conducted to investigate the effect of blanch-freezing on the ability of broccoli (600 g/d; 12 d) to influence putative intermediary biomarkers of colon cancer, including DNA damage in colonocytes, the xenobiotic metabolising enzyme (XME) system, the colonic microflora and SCFA concentrations. The consumption of raw broccoli (cv. Marathon) caused a significant 27% increase in DNA strand breakage (measured by the 'comet assay') in colonocytes (P=0.025), whereas blanched-frozen broccoli had no significant effect. Both broccoli diets had no significant effect on XME or the concentration of SCFA, but they caused an increase in the ratio of lactobacilli to coliforms of borderline significance (P=0.065). A second trial was conducted to further investigate the effect of raw broccoli consumption. Pigs were fed a different cultivar of raw broccoli (cv. Monaco) or raw carrots (cv. Nairobi). Carrots were fed to explore whether a raw vegetable high in antioxidants but devoid of GLS would influence colonocyte DNA damage. Results were similar to the first experiment, raw broccoli caused a significant 54% increase in DNA strand breakage (P<0.001), whereas raw carrots had no significant effect; both raw vegetables caused a significant increase in the ratio of lactobacilli to coliforms (P<0.001, broccoli; P=0.002, carrots), but had no effect on other measures. These studies appear to be the first to report that raw broccoli consumption causes an increase in DNA strand breakage in colonocytes. Collectively, they suggest that the consumption of high intakes of raw broccoli may not be advisable.

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Abbreviations

A	amp
ACF	aberrant crypt foci
AITC	allyl isothiocyanate
ANOVA	analysis of variance
AOM	azoxymethane
ARE	antioxidant responsive element
Apaf1	apoptotic protease activating factor
APC	adenomatous polyposis coli
BAX	Bcl-2 associated X protein
Bcl-2	B cell leukaemia/lymphoma 2
BEITC	benzyl isothiocyanate
BHT	butylhydroxytoluene
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BSA	bovine serum albumin
BW	bodyweight
^{o}C	degrees centigrade
CC	colon cancer
cdc	cell division cycle
CDNB	1-chloro-2, 4-dinitrobenzene
CDKs	cyclin dependent kinases
CKIs	cyclin dependent kinase inhibitor
CHO	carbohydrate
CI	confidence interval
CIMP	CpG island mutator phenotype
CIN	chromosomal instability
c-MYC	myelocytomatosis cellular oncogene
CpG	cytosine-phosphate-diester-guanine
CO	carbon monoxide
CRC	colorectal cancer
$CuSO_4 \cdot 5H_2O$	copper(II) sulfate pentahydrate
cv.	cultivar
cv	coefficient of variation

CYP450	cytochrome P450
d	day
DAPI	4′, 6-diamidine-2-phenylindole dihydrochloride
DCC	deleted in colorectal cancer gene
DEA	1, 4-dioxan-ethanol-acetonitrile
di.H_20	distilled water
DIM	diindolylmethane
DM	dry matter
DMH	dimethylhydrazine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPIP	2, 6-dichlorophenolindophenol
DTT	dithiothreitol
ECOD	ethoxycoumarin *O*-deethylase
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EPIC	The European Prospective Investigation into Cancer
EROD	ethoxyresorufin *O*-dealkylation
ESP	epithiospecifier protein
F344	Fisher 344
FAD	flavin adenine dinucleotide
FAP	familial adenomatous polyposis (FAP)
FCS	fetal calf serum
FFQs	food frequency questionnaires
g	grams
µg	micrograms
mg	milligram
g	gravitational force
G	gap
GAP	GTPase activating proteins
GDP	guanosine diphosphate
GI	gastrointestinal
GLO	1-gulono-gamma-lactone oxidase
GLS	glucosinolate(s)
GST	glutathione S-transferases
GTP	guanine triphosphate
h	hours

H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HCT116	human colon carcinoma cell line
Hepa1c1c7	mouse hepatoma cells - clone 7 of clone 1 hepatoma line 1
HepG2	human hepatocellular liver carcinoma cell line
HFA	human flora associated
HMP	high melting point agarose
HNPCC	hereditary non-polyposis colon cancer
hMLH1	human mutL homolog1 gene
hMSH2	human mutS homolog 2 gene
HBSS	hanks balanced salt solution
HPLC	high performance liquid chromatography
HR	hazard ratio
HT29	human Caucasian colon adenocarcinoma grade II cell line
I3C	indole-3-carbinol
IGFIIR	insulin-like growth facto-II-receptor
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
ITC	isothiocyanates
kcal	kilocalorie
KCl	Potassium chloride
kg	kilogram
K_2HPO_4	Potassium phosphate dibasic
KH_2PO_4	Potassium phosphate monobasic
$KNaC_4O_64H_2O$	Potassium sodium tartrate
KRAS	Kirstan-ras gene
l	litre
µl	microlitre
ml	millilitre
LMP	low melting point agarose
LOH	loss of heterozygosity
m	milli
M	molar
µM	micromolar
mM	millimolar
$MgSO_4$	magnesium sulphate
min	minute
MMR	mismatch repair

MPA	metaphosphoric acid
MROD	methoxyresorufin *O*-dealkylation
MRS	De Man Rogosa Sharpe
MSI	microsatellite instability
MSI-H	microsatellite instability-high
N_2CO_3	sodium carbonate
NAC	*N*-acetylcysteine
NaCl	Sodium chloride
Na_2EDTA	Disodium ethylenediamine tetraacetate
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced
NaOH	sodium hydroxide
NI3C	*N*-methoxyindole-3-carbinol
NIH-AARP	National Institute of Health-American Association of Retired Persons
nm	nanometre
NMR	nuclear magnetic resonance
no.	number
NOC	N-nitroso compounds
OR	odds ratio
8-oxo-dG	8-oxo-7,8-dihydro-2'-deoxyguanosine
p	petite arm, the shorter arm of a chromosome
P	probability
PBS	phosphate buffered saline
PEITC	phenethylisothiocyanate
pH	hydrogen ion concentration
PhiP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
pKa	acid dissociation constant
PLCO	The Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial
q	queue arm, the longer arm of a chromosome
QR	quinone reductase
r	correlation coefficient
R^2	square of the correlation coefficient
RAS	rat sarcoma gene
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute media
RR	relative risk

SCFA	short chain fatty acids
SD	standard deviation
sec	second
SF-MA	sulforaphane mercapturic acid
SMAD	Sma and mad related proteins
SMCSO	*S*-methyl cysteine sulfoxide
spp.	species
TGF-β	Transforming growth factor beta
TP53	tumour protein p53
Tris	Tris(hydroxymethyl)aminomethane
Triton X	4-(1, 1, 3, 3-Tetramethylbutyl)phenyl-polyethylene glycol
UGT	Uridine diphosphate-glucuronosyltransferase
v	versus
v/v	volume for volume
V	volts
W	watts
WCA	Wilkins-Chalgren Anaerobe
wnt	wingless type
w/w	weight for weight
w/v	weight for volume
XME	xenobiotic metabolising enzymes
XRE	xenobiotic responsive element

Chapter 1

Literature Review

1.0 Introduction

Colorectal cancer (CRC) is a major public health burden in westernised societies. It is the third most common cancer in the UK, with approximately 36,000 new cases and 17,000 deaths registered each year (Ferlay *et al.* 2004). The incidence of CRC increases with age in both sexes, but age standardised incidence rates are higher for males than females, with a ratio of approximately 1.4:1 (Ferlay *et al.* 2004). Inherited cancer syndromes, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC, also called Lynch syndrome) account for 5-10% of CRCs, but most appear to arise sporadically (Lynch & de la Chapelle 2003). A number of environmental factors appear to influence risk such as alcohol intake, smoking, physical activity, non-steroidal anti-inflammatory drugs, and diet (Giovannucci *et al.* 1994; WCRF/AICR 1997; Colditz *et al.* 1997; DoH, 1998; Giovannucci *et al.* 2001; WCRF/AICR 2007).

1.1 The biology of the colon and pathogenesis of CRC

The colon, in common with the rest of the gastrointestinal tract, consists of four layers: the mucosa, submucosa, muscularis, and serosa (Tortora & Derrickson 2009). The luminal surface of the mucosa is lined with epithelial cells, which fold inwards to form a number of crypts (Booth & Potten 2000). The three principal differentiated epithelial cell types are absorptive colonocytes, goblet cells and enteroendocrine cells (Cohn *et al.* 2009). These appear to develop from common epithelial stem cell precursors believed to be located at the base of each crypt (Cohn *et al.* 2009).

Stem cells populate each crypt by a process of asymmetrical division that results in the production of one stem cell and one daughter cell. The daughter cell is thought to undergo several further symmetrical divisions as it moves up the lower part of the crypt (Potten & Morris 1988). As each daughter cell continues its upward migration it undergoes terminal differentiation. At the surface epithelium, senescent cells are lost into the lumen probably by a combination of shedding and apoptosis (Hall *et al.*1994). Goblet cells and absorptive colonocytes take from 3-6 d from mitotic daughter cell to loss at the surface epithelium, whereas some enteroendocrine subtypes seem to have a longer lifespan (Tsubouchi & Leblond, 1979).

Normally, cell numbers in each crypt are tightly controlled and the rate of production of new cells in the proliferative zone at the crypt base and the rate of cell loss at the surface epithelium is matched (Kinzler & Vogelstein 2002). During carcinogenesis there appears to be an expansion of the proliferative zone and a dysregulation of crypt homeostasis that leads to an accumulation of abnormal cells (Chapkin *et al.* 2000; Kinzler & Vogelstein 2002).

The first morphologically recognisable change during colorectal carcinogenesis is the appearance of aberrant crypt foci (ACF), which can be either dysplastic or heteroplastic (Mäkinen, 2007). Dysplastic ACF may progress to form adenomatous polyps, whereas heteroplastic ACF may progress to form hyperplastic polyps (Mäkinen, 2007). The majority of CRCs appear to arise from adenomatous polyps, however, some CRCs seemingly develop from a neoplastic subset of the hyperplastic polyps, termed 'sessile serrated adenomas' (Goldstein *et al.* 2003; Torlakovic *et al.* 2003).

Fearon and Vogelstein (1990) proposed a model of common genetic alterations to explain the transition from normal mucosa through the adenoma-carcinoma sequence (Fig 1.1). The model described loss of function of 3 tumour suppressor genes (*APC*, 5q21; *DCC*, 18q; *TP53*,

17q), gain of function of 1 oncogene (*KRAS*, 12p) and global DNA hypomethylation as being common key events driving colorectal tumourigenesis (Fearon & Vogelstein 1990). The loss of function of the *APC* gene was identified as the initiating event in most CRCs. The *APC* gene was originally identified as the gene responsible for the inherited syndrome, FAP, which is characterised by the appearance of multiple adenomas in early adulthood, some of which progress to cancer (Bodmer *et al.* 1987; Kinzler & Vogelstein 2002). The main mechanism through which the loss of function of *APC* is thought to initiate carcinogenesis is by promoting aberrant Wnt signalling (Fodde, 2002). The wild type *APC* protein negatively regulates Wnt signalling by promoting the degradation of a signalling protein, called β-catenin (Logan & Nusse 2004). The loss of *APC* function seemingly prevents the degradation of β-catenin. This results in the aberrant activation of Wnt signalling and the expression of a number of genes such as cyclin D1 and c-MYC, which promote cellular proliferation (He *et al.* 1998; Logan & Nusse 2004). Other mechanisms through which *APC* mutation may contribute to colorectal tumourigenesis include disturbing the orderly upward migration of cells in the colonic crypts and possibly contributing to the development of genetic instability (Fodde, 2002).

Fearon & Vogelstein (1990) also identified global DNA hypomethylation as an early event in colorectal tumourigenesis. They proposed that hypomethylation, may lead to the expression of genes that are normally silent and also contribute to genetic instability, thereby increasing the rate of acquisition of allelic losses of tumour suppressor genes. More recently it has become apparent that in addition to global hypomethylation, tumours often contain genes that have been inactivated by hypermethylation of their gene promoters (see section on CpG island hypermethylation, pg 7; Esteller, 2005).

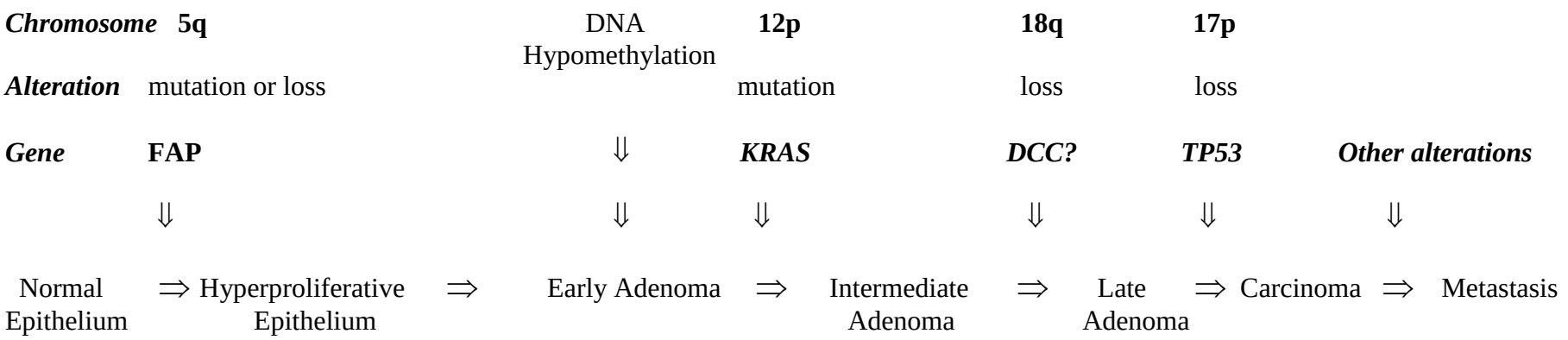


Fig.1.1 A genetic model for colorectal tumourigenesis.

(Source: Fearon & Vogelstein 1990)

Mutation of *KRAS* was suggested to occur in approximately 50% of tumours at an intermediate stage of CRC development, promoting further growth and division (Vogelstein *et al.* 1988). The *KRAS* gene encodes for a plasma membrane associated GTP binding protein, which functions as an on/off switch for a diverse array of signalling cascades (Campbell, 1998). The *RAS* protein becomes active when extracellular ligands bind to receptors on cell surfaces and stimulate the *RAS* protein to bind to GTP. Activity of the normal *RAS* protein is terminated by the hydrolysis of GTP to GDP, catalysed by GTPase activating proteins (GAPs). However, mutant *RAS* proteins become insensitive to GAPs, and therefore remain in a GTP bound active state, promoting proliferation and survival of transformation prone colon cells (Haubruck & McCormick 1991; Kranenberg, 2005).

Loss of all, or part of the long arm of chromosome 18 was identified as a common event occurring relatively late during tumour progression. Such abnormalities are rarely found in early adenomas, but Vogelstein *et al.* (1988) reported a frequency of approximately 50% and 70% in late stage adenomas and carcinomas respectively. Initially, the DCC (Deleted in Colorectal Cancer) tumour suppressor gene was proposed to be the critical gene loss on 18q (Fearon & Vogelstein 1990). Subsequently, however, two further candidate tumour suppressor genes, SMAD2 and SMAD4 were identified near to DCC (Takagi *et al.* 1996; Takagi *et al.* 1998). Currently, it is uncertain which if any of these genes are the most important target of loss of heterozygosity (LOH).

The transformation from large adenoma to carcinoma is often accompanied by the loss of function of the *TP53* gene located on chromosome 17p. Generally, loss of *TP53* function results from deletion of a large part of chromosome 17p that eliminates one *TP53* allele, and a point mutation that inactivates the remaining *TP53* gene (Baker *et al.* 1990). There is evidence, however, that some *TP53* gene mutations can affect cell growth even in the presence of one wild type allele. The mutant *TP*53 gene product may be able to bind to the

normal gene product and prevent it from binding to DNA within the promoter regions of its target genes (Kern *et al.*1992).

The *TP53* gene plays an important role in maintaining the integrity of the genome and its loss seems to be a critical event driving tumour progression. In response to DNA damage, the *TP53* protein product accumulates in the nucleus and increases the transcription of a number of growth inhibitory genes (Kinzler & Vogelstein 2002). These include $p21^{waf1/cip1}$, which arrests cells in the G1 phase and 14-3-3σ, which in combination with $p21^{waf1/cip1}$ can cause a prolonged G2 arrest (el-Deiry *et al.* 1993; Waldman *et al.* 1995; Bunz *et al.* 1998). Cell cycle arrest provides cells with time to repair damaged DNA. In addition, *TP53* also assists with repairing damage by increasing the transcription of some DNA repair enzymes (Oren, 2003). If the damaged DNA cannot be repaired, *TP53* activates an apoptotic pathway probably involving the increased transcription of a number of pro-apoptotic proteins, such as *BAX* (Oren, 2003). So, loss of *TP53* function eliminates an important mechanism for inhibiting the replication of cells with damaged DNA.

The accumulation of genetic alterations occurs slowly in normal cells and it appears that tumour cells develop some type of genetic instability that accelerates the process (Terdiman, 2000). The most common type of genetic instability present in colorectal tumours appears to be chromosomal instability (CIN; Terdiman, 2000). CIN is characterised by the loss or gain of whole chromosomes or segments of chromosomes during cell division (Lengauer *et al.* 1997). The exact cause of CIN remains largely elusive, although, recent studies suggest that the mutation of genes involved in sister chromatid cohesion may occur in greater than 20% of colorectal tumours (Barber *et al.* 2008).

In the early 1990s, investigations aimed at identifying the loci of tumour suppressor genes and oncogenes within the cancer genome led to the discovery of an alternate pathway to CRC characterised by another form of genetic instability called microsatellite instability (MSI;

Aaltonen *et al.* 1993; Ionov *et al.* 1993; Thibodeau *et al.* 1993). Researchers randomly amplifying sections of DNA in colon tumours discovered small insertions or deletions of nucleotides, present in short repetitive sequences called microsatellites (Aaltonen *et al.* 1993; Ionov *et al.* 1993; Thibodeau *et al.* 1993). Shortly after, it was discovered that MSI was caused by loss of DNA mismatch repair activity (Fishel *et al.* 1993; Leach *et al.* 1993; Bronner *et al.* 1994; Papadopoulos *et al.* 1994).

Subsequently, MSI was found to be present in the tumours of all individuals with the inherited cancer syndrome, HNPCC (Aaltonen *et al.* 1993). Individuals with HNPCC inherit a mutation in a DNA mismatch repair gene, most commonly hMLH1 or hMSH2 (Cappell, 2008). Somatic mutation of the corresponding wild type allele leads the cell and its progeny to gain mutations at an increased rate. Approximately 15% of sporadic CRCs are also thought to exhibit high levels of MSI (termed MSI-H; Grady, 2004). However, in these sporadic tumours, the DNA mismatch repair gene, hMLH1 is inactivated by the epigenetic mechanism of methylation, rather than by mutation (Kane *et al.* 1997; Herman *et al.* 1998). Although, tumours from individuals with HNPCC often contain mutations in *APC*, *KRAS* and *TP53*, these mutations appear to occur infrequently in sporadic MSI-H tumours (Jass *et al.* 1999; Salahshor *et al.* 1999; Jass *et al.* 2003). Instead, sporadic MSI-H tumours tend to exhibit mutations to tumour suppressor genes such as transforming growth factor beta (TGF-β) type II receptor, insulin-like growth factor-II-receptor (IGFIIR) and *BAX*, which all contain microsatellite repeats in their encoding regions (Markowitz *et al.* 1995; Souza *et al.* 1996; Rampino *et al.* 1997; Salahshor *et al.* 1999). Recently, it has been proposed that rather than adenomatous polyps, sessile serrated adenomas are the precursor lesions of most, if not all sporadic tumours with MSI-H (Jass, 2007; Mäkinen, 2007).

Some colorectal tumours are characterised by a high level of methylation of CpG islands (Goel *et al.* 2003). Tumours exhibiting such hypermethylation have been described as being CIMP (CpG island methylator phenotype) positive (Toyota & Issa 1999). CpG islands are

present in the promoter regions of approximately 50-60% of human genes (Boland & Goel 2005). Hypermethylation of CpG islands in the promoter region of genes results in their silencing (Esteller *et al.* 2005). As discussed earlier, hypermethylation appears to be the mechanism by which the DNA mismatch repair gene, hMLH-1 is inactivated leading to MSI in sporadic MSI-H CRC (Kane *et al.* 1997; Herman *et al.* 1998). CIMP is present in some CIN tumours and also appears to be present in a subset of colorectal tumours that exhibit neither, CIN nor MSI (Goel *et al.* 2003). It has been suggested that such tumours evolve through a distinct third pathway where CIMP is the driving force that favours tumour growth (Boland & Goel 2005). These tumours appear to exhibit a high rate of *BRAF* and *KRAS* mutations, but rarely contain mutations to *TP53* (Toyota *et al.* 2000; Kambara *et al.* 2004).

The model of Fearon and Vogelstein was widely accepted in the period following its publication. However, it has become increasingly apparent that it explains the genetic pathology of only a small proportion of sporadic CRCs (reviewed by Jass, 2007, see also Smith *et al.* 2002; Samowitz 2008). In response to expanding knowledge, a number of researchers have suggested new classification systems for CRC. These are based on the type of genetic alterations present in tumours and tend to group tumours according to their degree of MSI and presence or absence of CIMP (Boland & Goel 2005; Jass, 2007 see Table 1.1 for the classification system suggested by Jass, 2007).

There is a suggestion that different environmental factors may increase the risk of only certain subsets of CRC. For example, smoking may only increase the risk of tumours exhibiting CIMP and/or mutation to the *BRAF* gene (Samowitz *et al.* 2006). It is possible that some of the inconsistencies in the nutritional epidemiology of CRC may be explained by the fact that studies have traditionally viewed CRC as a single entity rather than a disease comprising a number of subgroups with distinct pathology and possibly different environmental risk factors.

Table 1.1 A classification system for CRC proposed by Jass (2007)

Feature	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
MSI status	High	Stable/low	Stable/low	Stable	High
Methylation	+++	+++	++	+/-	+/-
Ploidy	Diploid	Diploid	Aneuploid	Aneuploid	Diploid
APC	+/-	+/-	+	+++	++
KRAS	-	+	+++	++	++
BRAF	+++	++	-	-	-
TP53	-	+	++	+++	+
Location	R>L	R>L	L>R	L>R	R>L
Gender	F>M	F>M	M>F	M>F	M>F
Approximate frequency (%)	12%	8%	20%	57%	3%
Precursor lesion	Serrated polyps	Serrated polyps	Adenomas or serrated polyps	Adenomas	Adenomas

Location, R= right colon, L=left colon

Source: Jass (2007)

1.2 Environmental Risk Factors for CRC

Differences in occurrence rates across countries, migrant studies, and temporal changes in cancer incidence in countries such as Italy and Japan provide some of the most compelling evidence that CRC is influenced by the environment (Haenszel & Kurihara 1968; Tamura *et al.* 1996; Parkin *et al.* 2005). Classic studies on Japanese immigrants to the USA found that the rates of CRC progressively increased until they matched, or in some cases even surpassed the incidence rates within the US population (Smith, 1956; Haenszel & Kurihara 1968). More recently, a similar increase in CRC has been repeated on a larger scale in Japan, with age-adjusted incidence rates more than trebling between 1974 and 1991 (Tamura *et al.* 1996). Such rapid increases can only be the result of a change in environmental risk factors, because an alteration in genetic susceptibility would take many generations to develop. The westernisation of Japanese diets has been proposed as the causative factor, although other factors such as a reduction in the level of physical activity could also be responsible (Tamura *et al.* 1996).

1.3 Dietary risk factors

It has been estimated that up to 80% of sporadic CRCs may be attributable to diet (Bingham, 2000). Epidemiological and experimental studies have identified a number of putative risk factors and protective factors in the human diet. For example, some studies indicate that high intakes of red meat and alcohol may increase risk, whereas diets high in dietary fibre, milk and calcium may be protective (WCRF/AICR 1997; IARC, 2003; Bingham *et al.* 2003; Cho *et al.* 2004; Norat *et al.* 2005; WCRF/AICR 2007).

1.3.1 Fruits and Vegetables

Among other dietary components, fruits and vegetables have received much interest as possible cancer-protective components of the human diet (Steinmetz & Potter, 1991; WCRF/AICR 1997; IARC, 2003; WCRF/AICR 2007). Fruits and vegetables contain many

components that may exert anti-carcinogenic effects. These include, dietary fibre, nutrients such as, vitamin C, vitamin E, folate, selenium, and carotenoids with pro-vitamin A activity, and non-nutrient bioactive compounds such as other carotenoids, dithiolthiones, flavonoids, allium compounds, plant sterols, coumarins, protease inhibitors, isothiocyanates, salicylates, and indoles (Steinmetz & Potter 1991). Experimental studies have identified numerous mechanisms through which many of these bioactive compounds may influence the cancer process. These include modulation of the xenobiotic metabolising enzyme (XME) system, stimulation of the immune system, inhibition of the cell cycle, induction of apoptosis, modulation of the gut microflora, antioxidant actions and anti-inflammatory effects (Lampe 1999; Dragsted *et al.* 2006; Johnson, 2007).

Early case-control studies generally supported the hypothesis that high intakes of vegetables reduce the risk of CRC, whereas the findings for fruit consumption were less convincing (see reviews by Steinmetz & Potter 1991 & 1996). Based largely on the results of these studies, the first comprehensive review of diet and cancer published by the WCRF/AICR (WCRF/AICR 1997) concluded 'Evidence that diets rich in vegetables protect against cancers of the colon and rectum is convincing'. It also stated that no judgement could be made regarding fruit consumption, because of 'more limited and inconsistent' evidence. Since then, a number of large prospective cohort studies have been published. These have reported null or only weak inverse associations between fruit and vegetable intake and CRC (Pietinen *et al.* 1999; Voorips *et al.* 2000; Michels *et al.* 2000; Terry *et al.* 2001; Bueno de Mesquita *et al.* 2002; Flood *et al.* 2002; McCullough *et al.* 2003; Lin *et al.* 2005; Tsubono *et al.* 2005; Park *et al.* 2007; Nomura *et al.* 2008; van Duijnhoven *et al. et al.* 2009).

As the number studies reporting null or weak inverse associations began to accumulate, it was suggested that that a minimum threshold of fruit and vegetable intake may exist, below which, risk of CRC increases, but above which, further increases in fruit and vegetable intake have no effect (Terry *et al.* 2001). In a study of 61,463 Swedish women (aged 40-74 years, 9.6 years

follow up) Terry *et al.* (2001) observed an increased risk of CRC among women consuming less than 1.5 servings of fruit and vegetables a day in comparison to those consuming more than 2.5 servings per day (RR 1.65; 95% CI 1.23, 2.20). However, further increases in consumption appeared to confer no additional benefit. Similar threshold effects have subsequently been reported in some other studies, but in contrast to the study of Terry *et al.* (2001), these have only noted a threshold effect in men (McCullough *et al.* 2003; Park *et al.* 2007).

Considering the results from cohort studies published up to the end of 2005, the second expert report by the WCRF/AICR recently downgraded its conclusions regarding the protective effects of vegetable consumption, stating 'there is limited evidence that vegetables protect against CRC' (WCRF/AICR, 2007). They reached the same conclusion regarding fruit consumption but did note that a meta–analysis of 8 cohort studies found a significant inverse association between fruit consumption and risk of CRC in women, but not men. The panel suggested that this could reflect a hormone dependent mechanism of action, or simply indicate more accurate reporting of fruit intake by women (WCRF/ACIR, 2007). Studies have previously indicated that women are more likely than men to over-report their intake of foods perceived to be healthy, so it seems doubtful that more accurate reporting of fruit intake by women explains the gender difference (Bogers *et al.* 2004; Park *et al.* 2007).

It is possible that some of the cohort studies failed to detect a significant inverse association between fruit and vegetable intake and CRC because they lacked statistical power to detect modest inverse associations, especially by tumour site. Over the last several decades there has been a trend towards an increase in proximal colon cancers and a decrease in distal colon cancers in western populations. Tumours in each site may have different environmental risk factors (Iacopetta *et al.* 2002). The Pooling Project of Prospective Studies of Diet and Cancer addressed this issue in a pooled analysis of 14 North American and European cohort studies (756, 217 subjects, 5838 cases of CC, 6 to 20 years follow up; Koushik *et al.* 2007). The large

number of cases provided the Pooling Project with sufficient statistical power to conduct analyses by tumour site. Multivariate analysis revealed only a weak non-significant inverse association between fruit and vegetable intake and risk of colon cancer (RR 0.91; 95% CI 0.82, 1.01, P_{trend}=0.19; (Multivariate analysis is a statistical technique that enables epidemiologists to control for the effect of factors that are associated with both the dietary exposure of interest and the disease outcome. Controlling for these "confounding" factors provides a clearer indication of the true association between the dietary exposure of interest and risk of disease (Cramer & Lifford, 2007)). However, when proximal and distal colon cancers were investigated separately, fruit and vegetable intake was significantly inversely associated with distal colon cancer (total fruit and vegetable intake RR 0.74; 95% CI 0.57, 0.95, P_{trend}=0.02; total vegetables RR 0.82; 95% CI 0.68, 0.98, P_{trend}=0.01; total fruits RR 0.77; 95% CI 0.64, 0.93, P_{trend}=0.06), but not with proximal colon cancer. The authors suggested that if fruits and vegetables only protect against distal colon cancer this could partially explain the disagreement between case-control and cohort studies. In many cohort studies reporting null or weak inverse associations, the study population included more proximal than distal colon cancers, whereas in many of the case-control studies that reported strong inverse associations, distal colon cancers predominated (Koushik *et al.* 2007). As discussed in the pathogenesis section of this review, CRC is a disease consisting of a number of different subgroups that appear to follow largely separate genetic pathways. Segregating tumours by colon site would be expected to result in the distal group containing relatively few tumours with high levels of MSI (see Table 1.1). So, the results of The Pooling Project may indicate that fruits and vegetables are ineffective at protecting against tumours with MSI-H (Koushik *et al.* 2007). However, this requires further confirmation by studies designed specifically to investigate this hypothesis.

Since the publication of the second WCRF/ACIR report and the Pooling Project, at least 3 large prospective studies have reported inverse associations between fruit and/or vegetable intake and CRC however, in two of these studies, protection was only seen in men (Park *et al.*

2007; Nomura *et al.* 2008; van Duijnhoven *et al.* 2009). The NIH-AARP Diet and Health Study of 488, 043 men and women aged 50-71 y found a significant inverse association between total vegetable intake and CRC in men (RR 0.82; 95% CI 0.74, 0.94), but not women (RR 1.12; 95% CI 0.90, 1.38). Analysis by colon subsite found a stronger protective effect for vegetables against distal CC (RR 0.76; 95% CI 0.59, 0.98) than proximal CC in men (RR 0.95; 95% CI 0.72, 1.14). In contrast, fruit consumption was associated with an increased risk of rectal cancer in women (RR 1.59; 95% CI 1.04, 2.44; Park *et al.* 2007). The Multi-Ethnic Cohort Study of 191, 011 subjects from 5 ethnic groups (African-Americans, Japanese Americans, Latinos, Native Hawaiians, and whites) recruited in Hawaii and California also reported a significant inverse association between combined fruit and vegetable intake and CRC in men (adjusted RR 0.74; 95% CI 0.59, 0.93), but not women (adjusted RR 1.04; 95% CI 0.81, 1.33; Nomura *et al.* 2008). Gender specific error in reporting intakes of fruit and vegetables, may explain why some studies have found that evidence of protection in men, but not women (Park *et al.* 2007). As discussed above, women appear more likely than men to over-report their intake of healthy foods (Bogers *et al.* 2004; Park *et al.* 2007). This would lead to misclassification of exposure and attenuate any association between fruit and vegetable intake and CRC (Park *et al.* 2007).

Most recently, the EPIC study reported a weak inverse association between combined fruit and vegetable intake and CRC (HR 0.86; 95% CI 0.75, 1.00 P_{trend}= 0.04) in a cohort of 452,755 male and female subjects, mainly aged 35-70 y (van Duijnhoven *et al.* 2009). Separating CRC into colon cancer and rectal cancer revealed a stronger inverse association with colon cancer (HR 0.76; 95% CI 0.63, 0.91, P_{trend}<0.01) than CRC, and no association with rectal cancer (HR 1.09; 95% CI 0.85, 1.40, P_{trend}=0.67). Interestingly, the inverse association between fruit and vegetable intake and CRC appeared to be influenced by smoking status. Fruit and vegetable intake was only associated with reduced risk in never and former smokers whereas in current smokers there was a non-significant trend towards increased risk. The authors suggested the need for further research to clarify the interaction

between smoking, fruit and vegetable intake, and CRC, but hypothesised that some of the inconsistencies in the epidemiological literature may be explained by the failure of many studies to consider the interaction between smoking, fruit and vegetable intake and risk of CRC. Furthermore, they suggested that a combined analysis of data from two large US prospective cohort studies (The Nurses' Health Study and Health Professionals Follow-Up Study) may have failed to identify a modifying effect of smoking status on the protective effects of fruit and vegetables because they combined former smokers and current smokers together (two groups shown to have opposing effects in EPIC) into a single grouping of ever smokers (Michels *et al.* 2000; van Duijnhoven *et al.* 2009).

There are a number of other possible explanations for the inconsistencies in the literature. These include: errors in the dietary assessment techniques used, the modifying effects of genotype, failing to consider how processing may influence the biological actions of fruits and vegetables (these factors are discussed later in relation to cruciferous vegetables) and variation in the types of fruits and vegetables consumed by subjects in different studies (Koushik *et al.* 2007). Different botanical groups of fruits and vegetables and even different plants within a botanical grouping can vary substantially in their content of bioactive compounds and possibly in their ability to protect against cancer (Steinmetz & Potter 1996; van Duijnhoven *et al.* 2009). Although some studies suggest that fruits and vegetables act synergistically, it is also possible that considering fruits and vegetables as single groups may dilute the protective effect of a particular botanical group or individual fruit or vegetable (Lui, 2004; Koushik *et al.* 2007; van Duijnhoven *et al.* 2009). Among the different botanical groups, cruciferous vegetables (Table 1.2), which include the genus *Brassica*, have received much interest as possible anti-carcinogens (Verhoeven *et al.* 1996; Verhoeven *et al.* 1997).

1.4 Mechanisms through which cruciferous vegetables may protect against colorectal cancer

Cruciferous vegetables contain a number of bioactive components such as folate, vitamin C, tocopherols, carotenoids, and polyphenols (DeSouza & Eitenmiller 1986; Price *et al.* 1998; Kurilich *et al.* 1999). However, the anti-carcinogenic actions of cruciferous vegetables are most frequently attributed to their content of glucosinolates (GLS) (van Poppel *et al.* 1999; Lampe & Peterson 2002). GLS are a group of compounds that have a common basic structure of a β-D-thioglucose, and a sulfonated oxime moiety, but differ in respect to the structure of

Table 1.2 Cruciferous vegetables in the human diet

Genus	Species and variety	Common name
Brassica	B.oleracea var. botrytis	Cauliflower
	B.oleracea var. capitata	Cabbage, white cabbage
	B.oleracea var. costata	Portuguese cabbage
	B.oleracea var. gemmifera	Brussels sprouts
	B.oleracea var. gongyloides	Kohlrabi, turnip cabbage, stem turnip
	B.oleracea var. italica	Broccoli
	B.oleracea var. rubra	Red cabbage
	B.oleracea var. sabauda	Savoy cabbage
	B.oleracea var. sabellica	Curly kale
	B.oleracea var. viridis	Kale, collards
	B.oleracea var. alboglabra	Kai Lan, Chinese kale
	B.rapa var. Chinensis	Chinese cabbage, pak-choi, bok choi
	B.rapa var. oleifera	Turnip rape
	B.rapa var. pekinensis	Chinese cabbage, pe-tsai, Napa cabbage, celery, cabbage
	B.rapa var. rapa	Turnip
	B.rapa var. Parachinensis	Choi sum
	B,napus var. napobrassica	Swede, Swedish turnip, rutabaga
	B,napus var. Oleífera	Rape, canola, colza
	B.alba	White mustard
	B.juncea	Indian Mustard, brown mustard, spinach mustard
	B.juncea var. rugosa	Kai choi
	B.nigra	Black mustard
Raphanus	R.sativus	Radish
Armoracia	A.rusticana	Horseradish
Nasturtium	N.officinalis	Watercress
Lepidium	L.sativum	Cress, garden cress
Eruca	E.vesicaria	Arugula, rocket, Italian cress
Wasabia	W.japonica	Wasabi
Beta	B.vulgaris flavescens	Swiss chard
Crambe	C.abyssinica	Crambe

Source: IARC Handbooks of Cancer Prevention no 9.

their side chains, which may be aliphatic, aromatic or heterocyclic (Mithen *et al.* 2000; Holst & Williamson 2004). GLS have been regarded as biologically inert, but can be hydrolysed to a range of more bioactive products (Johnson, 2002). The hydrolysis of GLS is largely dependent on the activity of a plant based β-D-thioglucosidase enzyme called myrosinase (Bones & Rossiter 1996). GLS and myrosinase appear to be located in vacuoles in different cell types within the plant tissue. Myrosinase enzymes are present in specialised myrosin cells, whereas GLS are believed to be located nearby in other yet to be clearly defined cell types (Kissen *et al.* 2009). On disruption of the plant tissue, GLS and myrosinase come into contact with each other. Myrosinase then catalyses the hydrolysis of the thioglucose bond leading to the production of an unstable aglucone which undergoes spontaneous rearrangement to form a range of breakdown products such as isothiocyanates (ITC), nitriles and sulfur, oxazolidine-2-thiones and indolyl compounds (Holst & Williamson 2004). The product formed depends on the side chain structure of the parent GLS, pH, the presence of ferrous ions, and in the case of some GLS, the presence of a cofactor the epithiospecifier protein (ESP) (Fig 1.2; Bones & Rossiter 1996; Matuesheski *et al.* 2004).

The mechanisms through which cruciferous vegetables and their GLS breakdown products may protect against CRC include the modulation of XME, antioxidant effects, the induction of apoptosis, cell cycle arrest, and effects on the colonic microflora (Plumb *et al.* 1997; van Poppel *et al.* 1999; Smith *et al.* 2003; Humblot *et al.* 2005; Smith *et al.* 2005).

1.4.1 Modulation of XME

Chemical carcinogens can be classified as either direct acting or indirect acting, with the latter being the most common (Hodgson & Akunda 2001). The activation and detoxification of carcinogens is largely catalysed by the phase I and phase II enzyme families that comprise the XME system (Williams, 1967). Generally, phase I enzymes from the cytochrome P450 (CYP)

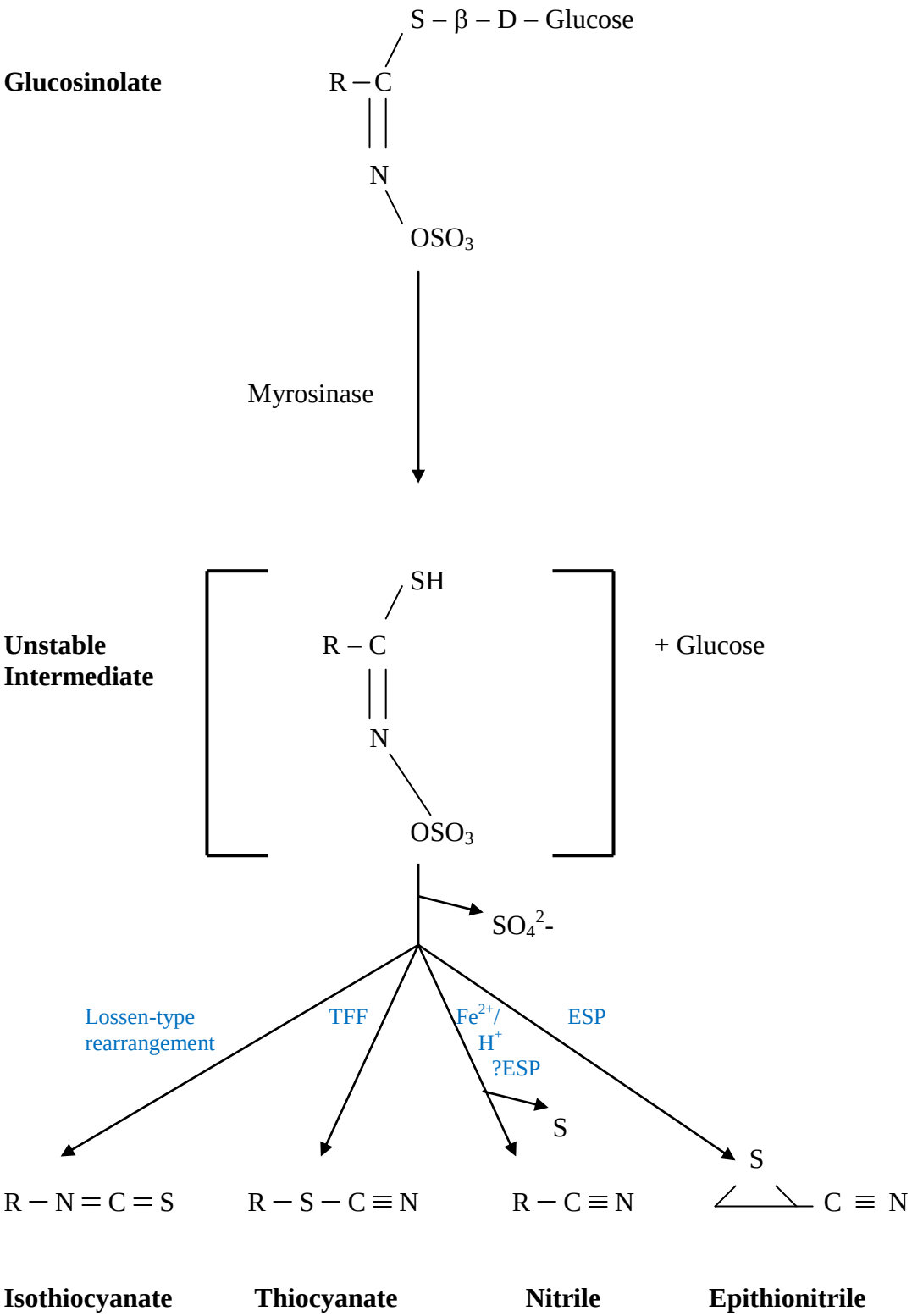


Fig. 1.2 General scheme of the hydrolysis of glucosinolates. Epithiospecifier protein (ESP), thiocyanate-forming factor (TFF; adapted from Matusheski *et al.* 2006).

family catalyse the activation of indirect acting carcinogens, whereas phase II enzymes such as the glutathione S-transferases (GST) catalyse the detoxification of both direct acting and indirect acting carcinogens (Talalay & Fahey 2001). Shifting the balance of phase I and phase II enzymes in favour of the latter might be an effective chemoprevention strategy (Talalay, 2000). However, this hypothesis has been challenged for a number of reasons including the fact that individual phase II enzymes often activate a specific class of chemicals (Paolini *et al.* 1999).

The induction of phase II enzymes has, however, been proposed as the major mechanism through which cruciferous vegetables protect against chemically induced tumours (Steinkellner *et al.* 2001; Jeffery & Stewart 2004). An extra-colonic increase in phase II enzyme activity could protect the colon from carcinogens by reducing the delivery of partially activated compounds from the systemic circulation (Pool-Zobel 1999). Kassie and associates have shown that in rodents exposed to the heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), the ability of juices prepared from cruciferous vegetables to protect against the induction of aberrant crypt foci (ACF) is correlated with their ability to increase the activity of the phase II enzyme UDP-glucuronosyl transferase (UGT) form 2 in the liver (Kassie *et al.* 2002; Kassie *et al.* 2003a). Cruciferous vegetables may also exert their protective effects through a local induction of phase II enzymes in the large intestine. In humans, the consumption of 300 g/d of Brussels sprouts for 1 week resulted in the elevation of rectal GST-α and –π isoenzymes (Nijhoff *et al.* 1995). Similarly, a diet containing 20% freeze-dried broccoli (cv. Majestic), potently induced quinone reductase (QR) activity in the colonic mucosa of rodents (Keck *et al.* 2003).

Although cruciferous vegetables induce phase II enzymes, they also tend to induce phase I enzymes, especially CYP1A1 and A2 (Vang *et al.* 1991; Wortelboer *et al.* 1992; Vang *et al.* 2001). The induction of CYP1A1 and A2 may sometimes be detrimental because they activate many carcinogens and may also increase the production of reactive oxygen species (ROS)

(Guengerich & Shimada 1998; Paolini *et al.* 2004). GLS breakdown products are probably the primary compounds responsible for the induction of XME after the consumption of cruciferous vegetables (Vang *et al.* 2001). Certain ITC, such as sulforaphane, appear to be potent monofunctional inducers of phase II enzymes (Fig 1.3) (Zhang *et al.* 1992). However, most cruciferous vegetables contain a range of GLS with variable modulatory effects on enzymes. For example, broccoli, which is the main dietary source of sulforaphane, also typically contains significant quantities of glucobrassicin and neo-glucobrassicin, which hydrolyse to form indole-3-carbinol (I3C) (Kushad *et al.* 1999; Mithen *et al.* 2000). On exposure to the acid environment of the stomach, I3C can undergo several condensation reactions to form a range of dimers, trimers, tetramers, and oligomers (McDanell *et al.*1988). In contrast to sulforaphane, I3C and its acid condensation products induce both phase I and phase II enzymes (Fig 1.3) (Shertzer & Sainsbury 1991; Nho & Jeffery 2004). The effect of cruciferous vegetable consumption, on phase I and phase II enzyme activities, is likely to be unpredictable, varying according to the GLS composition of the vegetable (which is governed by genotype, growing conditions, and post-harvest treatment) and possibly the content of other bioactive compounds present at the time of consumption (Vang *et al.* 2001; Jeffery & Stewart 2004; IARC 2004).

Evidence from animal models generally indicates that the joint induction of phase I and phase II enzymes by a variety of cruciferous vegetables results in a favourable metabolic profile for the elimination of certain chemical carcinogens (for a comprehensive review see IARC, 2004). However, it is unclear whether a similar chemoprotective effect would occur in free-living humans, who, in contrast to experimental animals, are chronically exposed to low doses of a wide variety of chemical carcinogens. The outcome of any shift in the balance of XME is likely to be unpredictable, depending on the range of chemical carcinogens to which an individual is exposed (Paolini *et al.* 1999).

1.4.2 Antioxidant effects

The colonic epithelium may be subject to oxidative damage mediated by free radicals generated near its surface by the faecal matrix (Babbs, 1990; Stone *et al.* 2002). Cruciferous vegetables may contribute to the antioxidant defence of the colonic mucosa through non-enzymatic and enzymatic mechanisms. Various GLS breakdown products can interact with a common antioxidant responsive element present in the upstream region of the genes encoding several phase II enzymes, with known antioxidant actions, such as GST and QR (Fig 1.3). Some cruciferous vegetables are rich sources of carotenoids and polyphenolic compounds (Kurilich *et al.* 1999). Both of these are poorly absorbed in the proximal gastrointestinal (GI) tract and are therefore likely to reach the colon in substantial quantities (Halliwell *et al.* 2000). Within the colonic lumen, carotenoids and polyphenolic compounds may protect the colonic epithelium from free radical attack. Polyphenolic compounds may chelate iron thereby helping to reduce the pro-oxidant effect of colonic iron and both carotenoids and polyphenols may directly scavenge free radicals (Halliwell *et al.* 2000). Little work appears to have been conducted on the ability of cruciferous vegetables to alter the antioxidant activity of the large bowel. However, preliminary work has indicated that pigs consuming broccoli have an increased faecal antioxidant level as measured by electron spin resonance (Ratcliffe *et al.* 2001).

1.4.3 Inhibition of the cell cycle and induction of apoptosis

Evidence derived primarily from studies performed *in vitro*, indicates that GLS breakdown products may be able to suppress cancer development in initiated cells by inhibiting cellular proliferation (Gamet-Payrastre *et al.* 2000; Frydoonfar *et al.* 2002; Hudson *et al.* 2003; Visanji *et al.* 2004; Frydoonfar *et al.* 2004; Smith *et al.* 2004; Neave *et al.* 2005; Pappa *et al.* 2006). The inhibition of cellular proliferation can result from cell cycle arrest, and/or an increase in apoptosis. In a variety of human colon cancer cell lines, ITC and indole compounds have

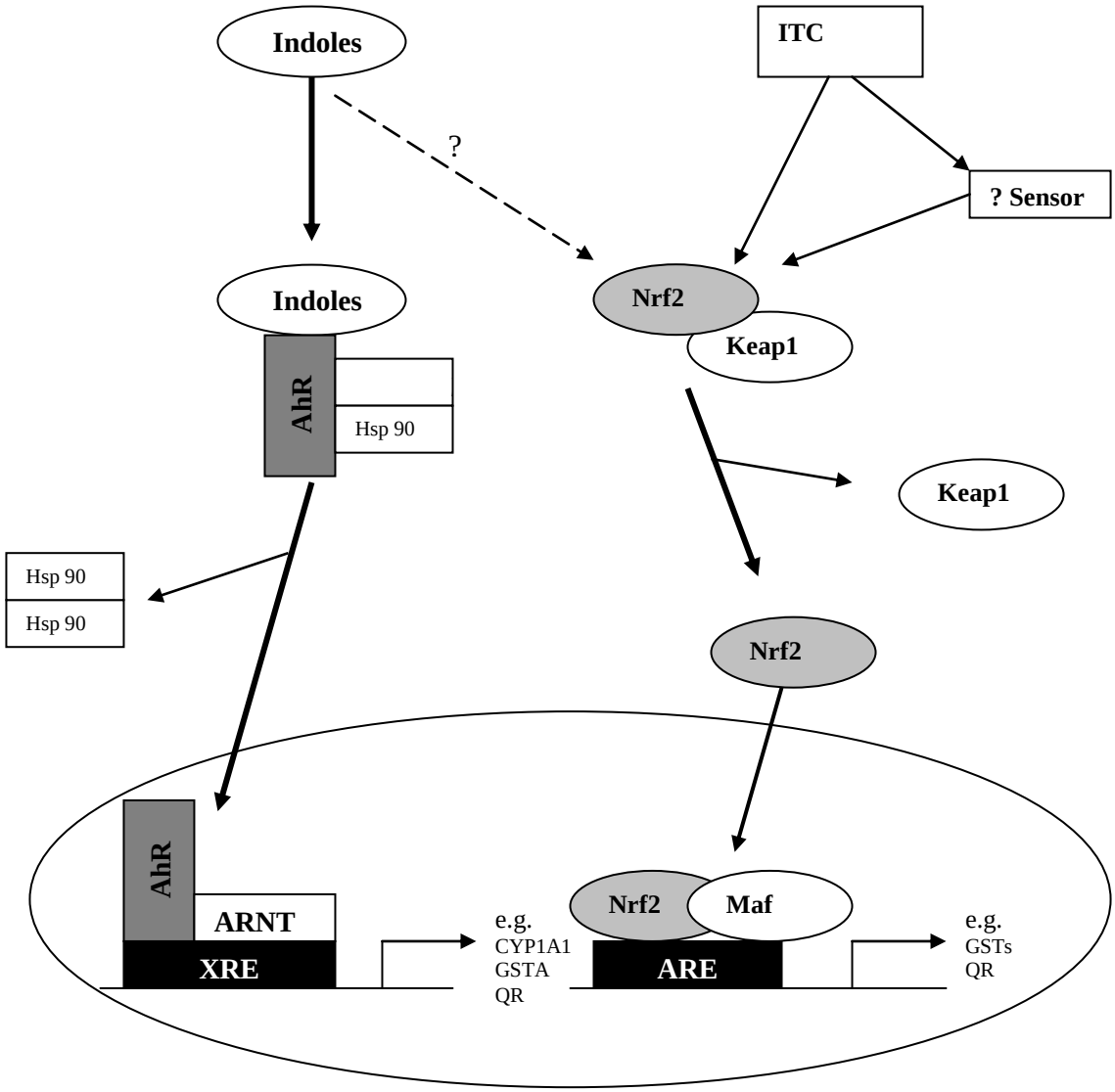


Fig. 1.3 Regulation of XME by glucosinolate breakdown products (adapted from Hayes & McMahon 2001; Lampe & Peterson 2002). Isothiocyanates (ITC) are monofuntional inducers that interact with an antioxidant responsive element (ARE) that is present in the upstream enhancer region of the genes encoding a number of phase II enzymes such as quinone reductase (QR) and several glutathione S transferase (GST) isoenzymes. Indoles have been classified as bifunctional inducers. Indoles enhance the expression of a number of genes with xenbiotic-responsive elements (XRE) in their upstream enhancer regions, such as cytochrome P450 (CYP) 1A1, CYP1A2, QR and GSTA. Indoles may also interact with the ARE possibly after metabolism by CYP450 or through some other as yet undefined mechanism (Hayes & McMahon 2001; Nho & Jeffery 2004). Hsp, heat-shock protein; AhR, aromatic hydrocarbon receptor; Nrf2, NF-E2-related factor-2; Keap 1, Kelch-like ECH-associated protein 1; Maf, musculoapo-neurotic fibrosarcoma; ARNT, aryl hydrocarbon receptor nuclear translator.

typically been shown to arrest the cell cycle and induce apoptosis (Bonnessen *et al.* 2001; Pappa *et al.* 2006). The effects of different ITC on cell cycle arrest appear to be similar with benzyl, phenethyl and allyl ITC (BEITC, PEITC, AITC) and sulforaphane all causing a G_2/M arrest (Gamet-Payrastre *et al.* 2000; Visjani *et al.* 2004; Smith *et al.* 2004). In contrast, limited evidence indicates that different indole compounds may block the cell cycle at different stages. For example, I3C appears to cause cells to arrest at G_0/G_1, whereas another indole, *N*-methoxyindole-3-carbinol (NI3C) causes cells to accumulate in G_2/M (Neave *et al.* 2005).

A recent study found that sulforaphane and PEITC were more potent inducers of apoptosis than I3C and its acid condensation product, 3,3'-diindolylmethane (DIM), in a p53 wild type colon cancer cell line (40-16) and its p53 knockout derivative (393.2, both derived from HCT116; Pappa *et al.* 2006). A low potency of certain indole compounds to induce apoptosis has also been reported in a number of other studies (Hudson *et al.*2003; Neave *et al.* 2005).

In contrast to the effects of other ITC, AITC appears to be unable to induce apoptosis *in vitro*. In a study conducted by Johnson's group, AITC failed to cause a significant increase in apoptosis in HT29 colon cancer cells, despite causing them to arrest at metaphase, and round up and detach from the flask surface (Smith *et al.* 2004). This appears surprising, as earlier work conducted *in vivo* had shown that sinigrin, the parent GLS of AITC, could increase the level of apoptosis in the colonic crypts of rats previously administered 1, 2-dimethylhydrazine (DMH; Smith *et al.* 1998). The same group also found that juice prepared from raw Brussels sprouts (a rich source of sinigrin) induced apoptosis in rats previously exposed to DMH, but only caused a minor non-significant induction of apoptosis *in vitro* (Smith *et al.* 2003, 2005). The authors speculated that, *in vivo*, interactions with neighbouring cells, especially interstitial lymphocytes might enhance the apoptotic effect.

The mechanisms through which GLS breakdown products cause cell cycle arrest and induce apoptosis have not been fully resolved, but a few studies have provided some insight. The progression of cells through the cell cycle depends on the activation and inactivation of a family of proteins called the cyclin dependent kinases (CDKs). The activation of CDKs is mediated by the binding of other proteins called cyclins. Each cyclin preferentially binds to a specific CDK. CDKs can be blocked by a group of proteins called cyclin dependent kinase inhibitors (CKIs; Phillips *et al.* 2001). Studies show that ITC and indoles alter the levels of various cell cycle regulatory proteins (Gamet-Payrastre *et al.* 2000; Neave *et al.* 2005; Gamet-Payrastre, 2006). For example, HCT-116 colon cancer cells treated with NI3C exhibit a general down-regulation in a range of cyclins and CDKs and an increase in the CKIs, p21 and p27 (Neave *et al.* 2005). HT29 cells treated with sulforaphane also show an increase in the levels of p21 and cyclin B1 and the activity of cdc2 kinase (see Gamet-Payrastre, 2006 for a review). How ITC and indoles regulate cell cycle regulatory proteins is not clear, but their initial effects on intra-cellular glutathione levels may be important. The delayed response of cells to ITC exposure is an up-regulation of antioxidant enzymes and an increased production of glutathione, however, immediately following exposure to ITC, there is a rapid, transient, depletion of intra-cellular glutathione that appears to increase intra-cellular stress. An increase in intra-cellular stress would be expected to influence the activity of various cell cycle regulatory proteins (Zhang *et al.* 2006). An alternative mechanism by which ITC may halt the cell cycle is by disrupting the polymerisation of tubulin, thereby halting mitosis (Smith *et al.* 2004).

In cultured cancer cells, ITC appear to be able to induce apoptosis through the activation of plasma membrane associated death receptors, and by mitochondrial mechanisms (Zhang *et al.* 2006). Little is known about how ITC trigger death receptors, because most studies have concentrated on exploring mitochondrial-mediated apoptosis (Zhang *et al.* 2006). In many types of cancer cells, ITC cause damage to the mitochondrial membrane leading to the release of cytochrome c into the cytoplasm. The release of cytochrome c is known to initiate

apoptosis. Released cytochrome c can bind to and activate Apaf1 (apoptotic protease activating factor), which in turn activates caspase 9. Caspase 9 is an initiator caspase that can act on a number of downstream effector caspases, including caspase 3. Caspase 3 can cleave key intra-cellular cytoplasmic and nuclear targets leading to apoptotic cell death (Cohen, 1997). Currently, it is unclear how ITC cause damage to the mitochondrial membrane. However, recent studies have shown that ITC can activate and inactivate various proteins belonging to the Bcl-2 family that are known to play a role in stabilising the mitochondrial membrane (reviewed by Zhang *et al.* 2006).

1.4.4 Effects on the luminal environment of the colon

Cruciferous vegetables are a rich source of dietary fibre (approximately 2-4 g per 100 g wet weight), with the fibre consisting primarily of pectic polysaccharides with lower quantities of hemi-cellulose, cellulose and lignin (Bourquin *et al.* 1993; FSA, 2002). The fermentation of fibre may protect against CRC by either increasing the production of SCFA or by altering the growth and metabolic activity of the colonic microflora. GLS present in cruciferous vegetables may also influence the growth of the colonic microflora as they appear able to suppress the growth of some bacteria such as *E.coli*, whilst acting as substrates for others such as *Bacteroides thetaiotaomicron*, and *Bifidobacterium spp*. (Park *et al.* 2000; Elfoul *et al.* 2001; Cheng *et al.* 2004).

A number of putative anti-carcinogenic actions have been identified for SCFA. These include inhibiting cellular proliferation, promoting cellular differentiation, inducing apoptosis, and lowering the pH of the colonic microflora (Augeron & Laboisse 1984; Cummings & MacFarlane 1991; Gamet *et al.* 1992; Hague & Paraskeva 1995). Fibre isolates of cruciferous vegetables are readily fermented when incubated with human faecal bacteria *in vitro* (McBurney & Thompson 1990; Weaver *et al.* 1992; Bourquin *et al.* 1993). Furthermore, on a weight for weight basis they produce more SCFA than most cereal fibres (McBurney &

Thompson 1990). However, host factors such as the physiology of the colon and transit time, probably influence the extent of fermentation that actually occurs *in vivo*. So far, no *in vivo* studies in humans appear to have been conducted, but one animal study has been published (Humblot *et al.* 2005). In male F344 rats harbouring a human microflora, Brussels sprouts (incorporated into the diet at a level of 10% w/w) failed to increase the total concentration of SCFA in the caecum. There was, however, a small alteration in the profile of SCFA in favour of acetate (108% of control value) (Humblot *et al.* 2005). If it is assumed that a change in production results in a change in concentration (which may not necessarily be the case, see Topping & Clifton 2001), it appears that the consumption of cruciferous vegetables probably does not have a major effect on SCFA production *in vivo*. However, the rat is thought to be a poor species for investigating the effect of diet on colonic fermentation because of major differences between their gastrointestinal tract and those of humans and because of their propensity for coprophagy (Topping & Clifton 2001). So, further studies in humans or at least a more suitable animal species such as the dog or pig are required to clarify the effect of cruciferous vegetable consumption on SCFA production.

Most substances that enter the large intestine are subject to some degree of metabolism by the colonic microflora. Sometimes this can result in the production of mutagens, carcinogens, or tumour promoters. The production of such compounds can be explained by the presence in the microflora of a range of XME (Goldin, 1986) (Table 1.3). Some bacteria such as *Bacteroides* and *E.coli* exhibit high levels of XME, whereas lactic acid producing species such as *lactobacilli* and *bifidobacteria* have relatively low levels (Rowland *et al.* 1985; Saito *et al.* 1992). Modifying the composition of the microflora in favour of species with relatively low levels of XME may provide some protection against CRC (Rowland, 1996).

Experimental studies in animals and humans have demonstrated that the composition of the microflora and activity of various bacterial XME can be altered by short-term dietary

Table 1.3 Examples of some bacterial XME and their possible role in carcinogen production (sourced from Goldin 1986 and Hughes & Rowland 2003).

Bacterial enzymes	Possible role in carcinogen production
β-glucuronidase	Hydrolyses biliary glucuronides releasing toxic parent compound or hepatic metabolite.
β-glucosidase	Hydrolyses various plant glycosides to release aglycones, some of which are carcinogens.
Nitroreductase	Reduces nitro compounds to amines, some of which may be carcinogens
Nitrate reductase	Converts nitrate present in drinking water and vegetables into nitrite. Nitrite can react with nitrogenous compounds in the lumen to produce N-nitroso compounds (NOC). Some NOC are DNA alkylating agents that are highly carcinogenic
Azo reductase	Hydrolyses azo bonds to generate substituted aromatic amines, some of which are established carcinogens
Cholylglycine hydrolase & 7α-dehydroxylase	Conversion of primary bile acids into secondary bile acids that may act as tumour promoters.

manipulation (Rowland & Tanaka 1993; Canzi *et al.* 1994; Rao *et al.* 1994; Silvi *et al.* 1999; Bouhnik *et al.* 2004; Costabile *et al.* 2008). Most studies have focused on investigating the effect of isolated non-digestible carbohydrates (CHO) such as inulin, guar gum and pectin (Rowland & Tanaka 1993; Canzi *et al.* 1994; Rao *et al.* 1994; Silvi *et al.* 1999). However, foods such as cruciferous vegetables that are naturally high in non-digestible CHO and other

bacterial substrates such as GLS might also be expected to have an effect. Limited evidence supports this theory. After 4 weeks of a 10% (w/w) Brussels sprouts diet, the faecal microbial diversity of human flora associated (HFA) rats was increased, whereas the heterogeneity of *Lactobacillus* populations was decreased. No changes however, were detected in the activity of β-glucuronidase in the caecal contents (Humblot *et al.* 2005). More recently, a diet supplemented with a variety of cruciferous vegetables (14 g/kg/bwt per d for 14 d) was shown to alter the bacterial community of the human colon (Li *et al.* 2009).

1.5 Cruciferous vegetables and experimental colon cancer

1.5.1 Cruciferous vegetables

In animal models of colon cancer, cruciferous vegetables have generally been shown to inhibit chemical carcinogenesis. Brussels sprouts in particular have been shown to be protective (Rijken *et al.* 1999; Smith *et al.* 2003; Kassie *et al.* 2003a; Uhl *et al.* 2004). Kassie and associates investigated whether juices prepared from two varieties of Brussels sprouts ('Maximus' and 'Cyrus') could provide protection during both cancer initiation and promotion (Kassie *et al.* 2003a; Uhl *et al.* 2004). In rats gavaged with IQ on 10 alternate days, the supplementation of their drinking water with Brussels sprout juices inhibited the development of ACF. In a further study using the same carcinogen-dosing schedule, the consumption of juice prepared from 'Maximus' Brussels sprouts inhibited the formation of ACF when consumed in the period after IQ exposure (Uhl *et al.* 2004). In contrast, juices prepared from two varieties of red cabbage ('Reliant' and 'Roxy') were much less effective, exerting only a small non-significant inhibiting effect during the initiation period and no effect during the promotion period. The authors hypothesised that the lack of a significant effect of red cabbage juice might be explained by its 2-3 fold lower content of GLS (Kassie *et al.* 2003a). However, in another study an extract of the colour from red cabbage (incorporated into the diet at a level of 5% w/w), devoid of GLS, was shown to inhibit adenoma and carcinoma formation in rats initiated with DMH and subsequently fed a diet containing the

heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhiP) at a concentration of 0.02%. The red cabbage colour extract was also found to protect against the formation of ACF in non-DMH treated rats fed a diet containing PhiP (Hagiwara *et al.* 2002).

In a recent study, diets supplemented with freeze-dried red cabbage, green cabbage or watercress all failed to inhibit the formation of ACF in rats administered DMH (Arikawa & Gallaher 2008). In contrast, diets containing fresh green cabbage, watercress or broccoli all reduced the number of ACF and mucin depleted foci (thought to be direct precursors of colonic tumours; Caderini *et al.* 1995) (Arikawa & Gallaher 2008). The authors suggested that the freeze-drying process may have led to the loss of GLS or inactivated myrosinase, thereby reducing exposure to chemoprotective GLS breakdown products. However, neither the content of GLS or activity of myrosinase was measured. Others have demonstrated that freeze-dried cruciferous vegetables retain myrosinase activity and their biological actions so it seems unlikely that either explanation adequately explains the differing effects of the fresh and freeze-dried cruciferous vegetable diets (Keck *et al.* 2003; Smith *et al.* 2003). Because the fresh and freeze-dried vegetables were sourced from different suppliers and not characterised for their content of GLS, it is possible that inherent differences in composition rather than freeze-drying may have been responsible for their differing effects.

Other cruciferous vegetables have received relatively little attention, but single studies have found that garden cress (*Lepidium sativum*) juice protects against IQ induced ACF and cruciferous seed meals and hulls protect against DMH induced colonic tumours (Barrett *et al.*1998; Kassie *et al.* 2002).

There are only two reports of a possible colon cancer promoting effect of cruciferous vegetables (Temple & El-Khatib 1987; Temple & Basu 1987). In these studies, female Swiss mice fed a diet supplemented with cabbage during initiation with DMH or throughout the

experimental period exhibited a modest increase in tumour formation, although in both cases the effect was not statistically significant.

1.5.2 Indoles and isothiocyanates

A variety of GLS hydrolysis products, including I3C, sulforaphane and PEITC have been investigated for their ability to inhibit chemically induced colon cancer (Pence *et al.* 1986; Pereira & Khoury 1991; Guo *et al.* 1995; Wargovich *et al.* 1996; Xu *et al.* 1996; Chung *et al.* 2000; Xu *et al.* 2001; Kim *et al.* 2003). There is consistent evidence that when heterocyclic amines are used to initiate colon cancer, I3C is an effective inhibiting agent (Guo *et al.* 1995; Xu *et al.* 1996). For example, I3C fed at a level of 0.1% in the diet, during the initiation phase, post-initiation or during the whole experimental period inhibited ACF in male F344 rats exposed to PhiP (Guo *et al.* 1995; Xu *et al.* 1996). Similarly, I3C fed at a level of 0.1% inhibited the formation of IQ induced ACF when fed to male F344 rats throughout the whole experimental period or during the post-initiation phase (Xu *et al.* 1996; Xu *et al.* 2001). However, there is some concern that when DMH is used to initiate colon cancer, I3C may promote tumour formation. In an early study, an increased incidence of tumours (combined tally of small intestinal and colonic tumours) was found in rats fed I3C in their diet at a level of 0.1% before, during, and after DMH administration (Pence *et al.* 1986). In contrast, in a more recent study, I3C failed to promote tumour formation when administered after DMH exposure (Xu *et al* 2001). A striking difference between the studies of Xu *et al.* (2001) and Pence *et al.* (1986) is the number of colon tumours found in the positive control group injected with DMH, 87 and 0% respectively. Possible explanations include the higher DMH dose (20 versus 10 mg/kg) and greater time lapse from the last dose to the rats being killed (45 v 16 weeks) in the study of Xu *et al.* (2001). No firm conclusion can be drawn regarding the promoting effect of I3C from the study of Xu *et al.* (2001) because the very high level of tumours in the positive control group would make it difficult to identify any tumour promoting effect of I3C. However, it is evident that I3C failed to exert a protective effect.

Interestingly, I3C fed during the post-initiation phase inhibited ACF formation in male mice administered AOM (a metabolite of DMH), perhaps indicating a species-specific effect (Kim *et al.* 2003).

Chung *et al.* (2000) compared the ability of sulforaphane, PEITC and their respective *N*-acetylcysteine (NAC) conjugates to prevent AOM induced ACF in male F344 rats. Sulforaphane and PEITC were both effective at reducing total ACF and crypt multiplicity when administered during the initiation and promotion periods. In contrast, their respective NAC conjugates were only effective when fed during the post-initiation period. Surprisingly, PEITC-NAC actually increased the formation of ACF when administered during the initiation period. In contrast to the study of Chung *et al.* (2000), two earlier studies found no protective effect of PEITC against AOM induced ACF (Pereira & Khoury 1991; Wargovich *et al.* 1996).

1.6 Epidemiological Studies

In the mid 1990s, a number of reviews of the epidemiological evidence concluded that the consumption of cruciferous vegetables is associated with a reduced risk of CRC (Steinmetz & Potter 1996; Verhoeven *et al.* 1996; Kohlmeier & Su 1997). However, at that time, few results from cohort studies had been published. At least ten cohort studies have now reported on the association between intakes of cruciferous or brassica vegetables and colonic, rectal or combined colorectal cancer risk (Summarised in Fig 1.4; Steinmetz *et al.* 1994; Hsing *et al.* 1998; Pietinen *et al.* 1999; Voorips *et al.* 2000; Michels *et al.* 2000; Flood *et al.* 2002; McCullough *et al.* 2003; Lin *et al.* 2005; Park *et al.* 2007; Nomura *et al.* 2008). Overall these provide little evidence for a protective effect of cruciferous vegetable intake against cancer of the large intestine. In fact, almost without exception the relative risks cluster around one and the confidence intervals encompass one (Fig 1.4). In only one study, in a Dutch population is there a significant negative association between brassica vegetable consumption and colon cancer, and even then, the effect is only observed in women (RR 0.51; 95% CI 0.50, 1.13,

P_{trend} = 0.05) (Voorrips *et al.* 2000). Furthermore, for rectal cancer, brassica vegetable consumption is associated with an increased risk in women (RR 1.66; 95% CI 0.94, 2.94, P_{trend} = 0.05). Of further concern, a study of male smokers in Finland, found a significant increase in colon cancer risk in the highest quintile of intake (RR 1.6; 95% CI 1.0, 2.4, P_{trend} = 0.05; Pietinen *et al.* 1999). However, the study population exhibited an atypical consumption pattern with almost no intake of green cruciferous vegetables and the highest overall consumption levels were very low, the median level in the highest category of intake being 38 g/d.

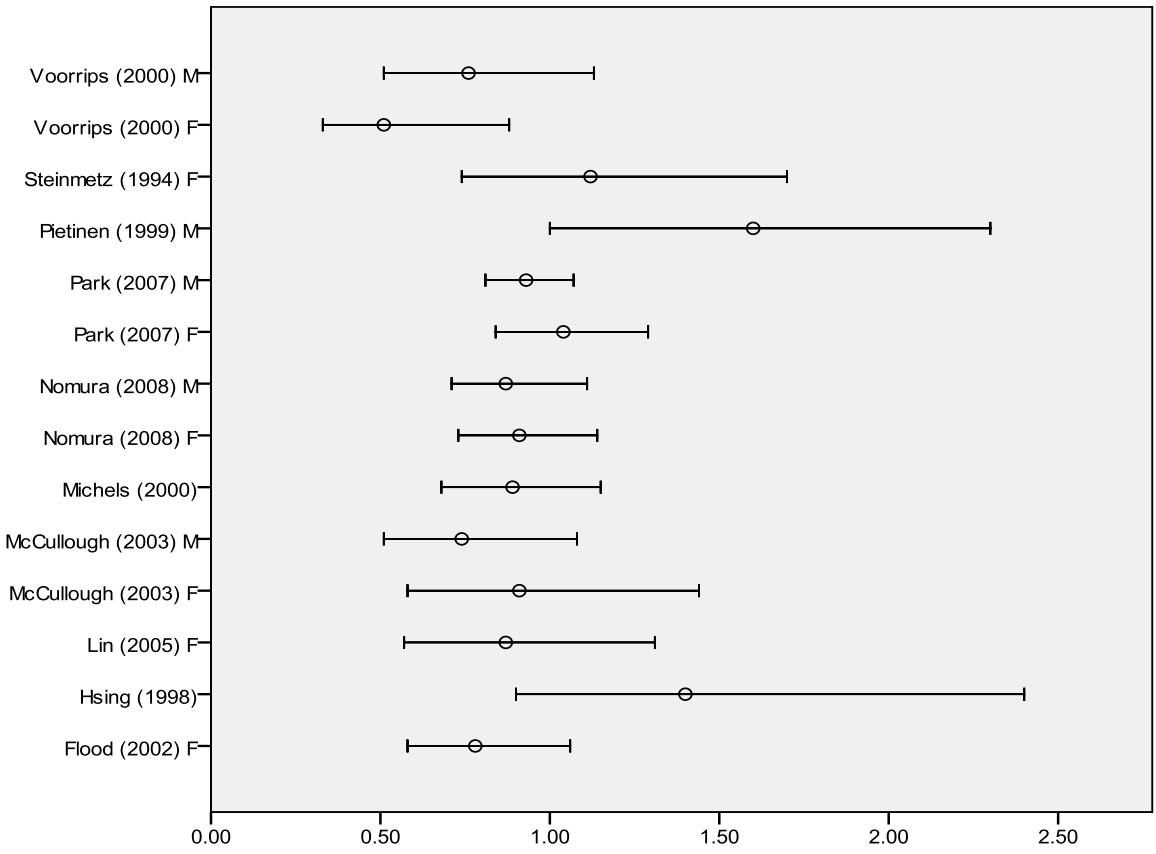


Fig. 1.4 Cohort studies of colon cancer and cruciferous vegetable consumption. Values are relative risks and 95% CI are represented by horizontal bars. F, females; M, males.

So what factors may explain why large-scale prospective epidemiological studies generally fail to support the hypothesis that cruciferous vegetables protect against colon cancer, whereas animal studies have generally observed chemoprotective effects and experimental studies have

identified a number of plausible mechanisms of action? One factor that may need to be considered is that relatively large doses of cruciferous vegetables or GLS exhibit chemoprotection, but normal habitual levels of intake of cruciferous vegetables have no effect. However, a number of limitations in the published cohort studies may have reduced their sensitivity to detect a modest protective effect of cruciferous vegetable consumption.

1.6.1 Measurement error

The cohort studies all used food frequency questionnaires (FFQs) to measure cruciferous vegetable intake. When validated against repeated weighed intakes, FFQs tend to perform badly in comparison to other methods of dietary assessment, overestimating vegetable consumption in particular (Bingham *et al.* 1994). The failure of FFQs to accurately assess vegetable intake could lead to an overestimation of vegetable intake and/or misclassification of subjects into the wrong category of consumption. Consistent over-estimation of intake among all subjects would not preclude studies from finding an association between cruciferous vegetable intake and colorectal cancer, but would create a false impression of the range of intake that does or does not have an effect on cancer risk. Despite the fact that cohort studies may have overestimated the intake of cruciferous vegetables, reported values are still low. For example, in the Cancer Prevention II study cohort, the highest quintile of cruciferous vegetable intake was half a portion or more a day in women and even lower in men (McCullough *et al.* 2003).

Misclassification of subjects is a potentially more serious problem than overestimation. Comparison of the ability of different methods of dietary assessment to classify individuals into the same quartile of consumption as 16 day weighed records indicated that FFQs correctly classify 30-50% of individuals for most nutrients (Bingham *et al.* 1994). Such an extent of misclassification would attenuate relative risk estimates and make modest protective effects very difficult to detect.

1.6.2 The modifying effect of genotype

The ability of cruciferous vegetables to inhibit CRC may be influenced by the GST genotype of the individual (Lin *et al.* 1998; Slattery *et al.* 2000; Seow *et al.* 2002). The failure of the published cohort studies to consider the modulatory effect of GST genotype may have weakened their sensitivity to detect a protective effect of cruciferous vegetable consumption. The GSTs are a family of isoenzymes involved in the detoxification of a range of chemical carcinogens. They are also responsible for the metabolism and excretion of ITC after cruciferous vegetable consumption (Kolm *et al.* 1995; Hayes & Pulford 1995). Approximately, 50% of individuals in most populations are homozygous for the null alleles of two GST isoenzymes (GSTM1 and T1) and therefore lack expression of some components of GST activity (Johnson & Lund 2007). At least 3 case-control studies and 3 nested case-control studies have explored the relationship between cruciferous vegetable and/or ITC exposure, genotype and risk of CRC or adenoma (Lin *et al.* 1998; Slattery *et al.* 2000; Seow *et al.* 2002; Turner *et al*. 2004; Epplein *et al.* 2009; Yang *et al.* 2010). The results have not been entirely consistent but tend to point towards a greater protective effect of cruciferous vegetables in individuals with GST M1 and T1 null genotypes (see Table 1.4).

A protective effect in individuals with null genotypes and therefore reduced GST activity may seem counterintuitive because the up-regulation of GST activity is a mechanism through which cruciferous vegetables appear to inhibit chemical carcinogenesis (Steinkellner *et al.* 2001). A possible explanation for this apparent discrepancy could be that individuals with GSTM1 and T1 null genotypes excrete ITCs more slowly leading to an accumulation of ITCs in target tissues (Seow *et al.* 2002; Johnson, 2002). ITCs may then induce other chemoprotective phase II enzymes or exert other anticarcinogenic effects such as blocking the cell cycle or inducing apoptosis in cells carrying DNA damage (Seow *et al.* 2002; Johnson, 2002).

Table 1.4 The modifying effect of GST genotype on the association between cruciferous vegetable intake and risk of adenoma or CRC

Study Type	**Population**	**Location**	**Measurement method**	**Outcome**	**Reference**
Case-control	459 cases (297 M & 162 F) 507 controls (338 M & 169 F) Age 50-74 y	Southern California	FFQ	*Adenoma* Total cruciferous vegetable intake GST M1present OR 0.95 (95% CI 0.72, 1.11 P_{trend}=0.71) GST M1 null OR 0.52 (95% CI 0.29, 0.93 P_{trend} =0.02) Broccoli intake GST M1present OR 0.74 (95% CI 0.4, 0.99 P_{trend}=0.43) GST M1 null OR 0.36 (95% CI 0.19, 0.68 P_{trend}=0.001)	Lin *et al.* (1998)
Case-control	1576 cases (890 M; 686 F) 1898 controls (1021 M; 877 F) Age 30-79 y	Northern California, an 8 county area in Utah & the metroplitan twin cities area of Minnesota	FFQ	*Colon cancer* GSTM1 null genotype strengthened the protective effect of a high intake of cruciferous vegetables in subjects <= 55 y of age. GSTM1 null OR 0.23 (95% CI 0.1, 0.54) GSTM1 present OR 0.44 (95% CI 0.19, 0.99) No effect observed in subjects >55 y.	Slattery *et al.* (2000)
Nested case-control	213 cases 1194 controls Age 45-70 y (Cantonese and Hokkien dialect speakers)	Singapore	FFQ	*Colorectal cancer* In those with a combined GSTM1 and T1 null genotype a high intake of ITC was protective OR 0.43 (95% CI 0.2, 0.96). No protection seen in individuals with GSTM1 and/or T1 genes present.	Seow *et al.* (2002)

Case-control	484 cases 742 controls Age 45-80 (Caucasian)	Four locations in the UK: Dundee, Perth, Leeds & York	FFQ	*Colorectal cancer* No modifying effect of GSTM1 genotype. GSTT1 null appeared protective OR 0.4 (95% CI 0.2, 0.8) Versus GSTT1 present OR 1.2 (95% CI 0.8, 2.0)	Turner *et al.* (2004)
Nested case-control	173 cases (106 M & 57 F) 313 controls (187 M & 126 F) Age 45-75	Hawaii & Los Angeles	Urinary ITC excretion	*Colorectal cancer* No modifying effect of GSTM1 or T1 genotype A trend towards a greater protective effect of ITC in those with GSTP1 AG or GG genotype versus AA genotype OR 0.47 (95% CI 0.19, 1.17) v 0.58 (95% CI 0.22, 1.48) P_{trend}= 0.09	Epplein *et al.* (2009)
Nested case-control	332 cases (F) 1251 controls (F) Age, 40-70 y	Shanghai	FFQ and urinary ITC excretion	*Colorectal cancer* No modifying effect of genotype when crucifer intake measured by FFQ. Modifying effect when exposure measured by urinary ITC concentration: GST M1 present OR 1.12 (95% CI 0.68, 1.83, P_{trend}=0.68) GST M1 null OR 0.66 (95% CI 0.44, 1.00, P_{trend}=0.04) GSTT1 present OR 0.92 (95% CI 0.59, 1.44, P_{trend}=0.71) GSTT1 null OR 0.67 (95% CI 0.44, 1.05 P_{trend}=0.07) GSTM1 and GSTT1 present OR 1.25 (95% CI 0.56, 2.78, P_{trend}=0.73) GSTM1 and GSTT1 null OR 0.51 (95% CI 0.27, 0.95 P_{trend}=0.03)	Yang *et al.* (2010)

A recent small experimental study appears to challenge the hypothesis that individuals with a GSTM1 null genotype excrete ITC more slowly than those with a GSTM1 positive genotype. Gasper *et al.* (2005) fed a standard broccoli and a high glucosinolate broccoli to nine individuals with a GSTM1 positive genotype and seven with a GSTM1 null genotype. They measured the urinary excretion of the ITC, sulforaphane, as free sulforaphane and as its various thiol conjugates. Surprisingly, rather than finding a slower rate of excretion in GSTM1 null genotypes they found a faster rate. The authors went on to argue that individuals with GSTM1 positive genotypes were more likely than those with GSTM1 null genotypes to gain protection from consuming broccoli. Although this seems to contradict most of the epidemiological literature they managed to quote a number of epidemiological studies examining the link between cruciferous vegetable consumption and cancer incidence at various body sites that appeared to support their argument. They suggested that GSTM1 deletion could have contrasting effects on the metabolism of alkenyl ITC such as 3-butenyl ITC and non-alkenyl ITC such as sulforaphane and that this could explain some of the conflicting results in the epidemiological literature. They argued that the studies showing a greater protection from cancer in individuals with a GSTM1 null genotype were conducted primarily in Asia where the main cruciferous vegetables consumed are Chinese cabbage and other forms of *B.rapa* that are high in alkenyl ITC. Whereas, those showing a greater protection in individuals with a GSTM1 positive genotype were conducted in Western populations, where consumption of *B.rapa* is low and the main cruciferous vegetable consumed is broccoli, a rich source of sulforaphane. Whilst this is an attractive hypothesis for explaining some of the inconsistencies in the epidemiology, there is currently too little data to draw any firm conclusions about possible interactions between the consumption of different types of cruciferous vegetables, different GSTM1 genotypes and CRC risk. In fact, the one colorectal adenoma study quoted by Gasper *et al.* (2005) as supportive of their hypothesis does not actually support it. The study by Lin *et al.* (1998) despite been conducted in a Western population (Southern California), still shows a greater protection against adenoma

formation in individuals with a GSTM1 null genotype (M1 null OR 0.36; 95% CI 0.19-0.68 versus M1 positive OR 0.74; 95% CI 0.40, 0.99) and is misquoted by Gasper *et al.* (2005).

1.6.3 The effect of processing

Before consumption, cruciferous vegetables are often stored or subjected to a range of treatments such as washing, chopping, blanching, freezing and cooking that may alter their content of bioactive compounds and consequently their biological actions (Goodrich *et al.* 1989; Rodrigues & Rosa 1999; Verkerk *et al.* 2001; Vallejo *et al.* 2002; Benner *et al.* 2003; Vallejo *et al.* 2003a; Vallejo *et al.* 2003b). Variations in storage and the degree of processing that cruciferous vegetables undergo prior to consumption may mean that individuals with apparently similar levels of intake are exposed to different amounts of bioactive compounds. Failing to consider this may mask any association between the intake of cruciferous vegetables and risk of CRC.

Numerous studies have investigated how processing and storage, alters the content of bioactive compounds present in cruciferous vegetables. Studies assessing the effect of refrigerated storage (4^{o}C) on GLS content have produced inconsistent results. Vallejo *et al.* (2003a) reported large losses of GLS after 6 d of storage, whereas Rodrigues & Rosa (1999) found only small losses (5-15%) and Rangkadilok *et al.* (2002) observed no effect of 7 d of cold storage (4^{o}C) on the glucoraphanin content of broccoli. In one study, cold storage also resulted in large losses of flavonoids, but had little effect on vitamin C levels (Vallejo *et al.* 2003a). In contrast to the variable effects of cold storage, storing broccoli at room temperature (15-20^{o}C) for as little as 3 d has consistently been shown to result in a substantial loss of GLS (Rodrigues & Rosa 1999; Rangkadilok *et al.* 2002; Vallejo *et al.* 2003a). No studies appear to have measured the level of GLS and GLS breakdown products after storage, so it is unclear whether losses result from the conversion of GLS to breakdown products or some other form of degradation (Jeffery & Stewart 2004).

Different cooking techniques have variable effects on the content of bioactive compounds present in cruciferous vegetables. Generally, steaming results in only minor losses of GLS, flavonoids and vitamin C, whereas boiling tends to result in moderate losses and cooking in a microwave with water causes large losses (Price *et al.* 1998; Conaway *et al.* 2000; Vallejo *et al.* 2002; Vallejo *et al.* 2003b). The different effects of steaming, boiling and microwaving probably reflect differences in cooking temperature, time, and degree of contact with the cooking water. Direct contact results in the leaching of compounds into the cooking water and higher temperatures may cause thermal degradation and increased evaporation of volatile compounds (Mithen *et al.* 2000; Vallejo *et al.* 2002; Vallejo *et al.* 2003b).

Cruciferous vegetables are often sold frozen. Before freezing, vegetables are blanched to inactivate spoilage enzymes. This may also result in the inactivation of myrosinase and the loss of bioactive compounds (Goodrich *et al.* 1989; Sarkamis *et al.* 2006). Commercial blanching techniques involve a period of steam or water blanching followed by cooling with air or water (Grandison, 2006). Goodrich *et al.* (1989) investigated the effect of steam and water blanching on the GLS content of Brussels sprouts and broccoli. Blanching followed by immersion in cold water resulted in GLS losses ranging between 7-22% for Brussels sprouts and 40-83% for broccoli. The authors hypothesised that variation in the physical structure of each vegetable was responsible for their different resistance to the effects of blanching. Losses of GLS during steam blanching appear to be much greater than those that occur when cruciferous vegetables are cooked by steaming (Goodrich *et al.* 1989; Conaway *et al.* 2000; Vallejo *et al.* 2002). This suggests that the termination of blanching by immersing vegetables in cold water results in a substantial amount of leaching. The subsequent freezing of blanched vegetables tends to preserve the level of most compounds (Galgano *et al.* 2007).

Processing may also beneficially or negatively alter the bioavailability of the bioactive compounds present in cruciferous vegetables. For example, the location and extent of GLS breakdown in the GI tract and the profile of breakdown products formed, is influenced by the

degree of thermal processing to which a vegetable is subjected before consumption (Johnson, 2002; Matusheski *et al.* 2004). When raw or lightly cooked cruciferous vegetables are consumed, if plant myrosinase and GLS come into contact with each other, then most GLS hydrolysis probably occurs in the proximal GI tract (Johnson, 2002). However, the profile of breakdown products formed after consumption of raw and lightly cooked cruciferous vegetables may vary. A heat labile cofactor, called ESP which is present in some cruciferous vegetables favours the formation of nitriles over ITC after the consumption of raw cruciferous vegetables; whereas, mild cooking may shift the ratio in favour of ITC by inactivating ESP whilst conserving some myrosinase activity (Mithen *et al.* 2003; Matusheski *et al.* 2004). If prolonged cooking inactivates myrosinase, intact GLS might be delivered to the colon and degraded by the colonic microflora (some of which have myrosinase activity), resulting in the production of ITC, nitriles and possibly other, as yet undetermined breakdown products (Combourieu *et al.* 2001; Johnson 2002).

Only a few studies appear to have investigated the effect of processing on the actions of cruciferous vegetables *in vivo*. In a rodent study, juice or tissue prepared from raw Brussels sprouts induced apoptosis and inhibited DMH induced ACF, whereas blanched Brussels sprouts (with inactive myrosinase) had no effect (Smith *et al.* 2003). In contrast, Kassie *et al* (2003a) found that juices prepared from raw Brussels sprouts or Brussels sprouts that had been cooked for 10 min at 100^{o}C were similarly as effective at inhibiting IQ induced ACF. Juice prepared from raw Brussels sprouts would be expected to contain GLS breakdown products, whereas juice prepared from cooked Brussels sprouts would be expected to contain intact GLS and no active myrosinase. The authors suggested that the colonic microflora or the acidic conditions of the stomach facilitated the hydrolysis of the GLS in the cooked Brussels sprouts juice. However, neither of these routes would be expected to be as effective at supplying anticarcinogenic GLS breakdown products as consuming juice containing hydrolysed GLS breakdown products, so it is surprising that both juices had similar effects. It

is possible that other bioactive compounds present in both juices may have contributed to the effect.

In work conducted prior to the start of the present project, the group investigated the effect of processing on the ability of whole raw broccoli to protect DNA in colonocytes from damage (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000). In an initial experiment, pigs fed whole raw broccoli (600 g/d x 12 d) exhibited lower levels of DNA damage (measured as strand breaks by the 'Comet assay') in their colonocytes, than pigs fed a cereal based control diet, or the control diet supplemented with raw broccoli that had been homogenised before consumption (Ratcliffe *et al.* 1999). The group hypothesised that after consumption of the whole raw broccoli, intact plant cell walls in large fragments of broccoli acted as a vehicle to deliver antioxidants and other non-defined anti-carcinogenic compounds to the colon. Once in the colon, it was suggested that the fermentative actions of the colonic microflora degraded the plant cell walls, thereby releasing anti-carcinogens directly into the colon, where they conferred protection against free radicals present in the faecal stream. It was argued that the disruption of the plant cell walls in the homogenised broccoli reduced the delivery of anti-carcinogens to the lumen of the colon by facilitating their release and absorption in the small intestine, and that systemic delivery to colonocytes was less protective than direct delivery to the lumen (Ratcliffe *et al.* 1999).

In a second study, the effect of whole raw broccoli was compared with the effect of microwaved broccoli (900 W x 9 min in 300 g water). In agreement with the earlier study, diets supplemented with whole raw broccoli (600 g/d x 12 d) reduced levels of damage to DNA in colonocytes, whereas, diets supplemented with microwaved broccoli had no effect. These results appeared to support the working hypothesis regarding the importance of intact cells walls, because microwaving would be expected to soften the broccoli and cause some disintegration of the plant cell structure. It was, however, acknowledged that other

explanations could exist to explain the contrasting effects of raw and microwaved broccoli (Ratcliffe *et al.* 2000).

The mechanisms underpinning the protective effect of the whole raw broccoli against damage to DNA in colonocytes was not fully explored. However, antioxidants or GLS could have contributed to the protective effect of the raw broccoli. Preliminary work using electron spin resonance indicated that the faeces of pigs consuming all of the broccoli diets had increased antioxidant ability, but the effect appeared to be more pronounced in pigs fed the whole raw broccoli (Ratcliffe *et al.* 2001). This was interpreted as support for the hypothesis that fragments of raw broccoli act as vehicles to transport greater quantities of antioxidants to the colonic lumen (Ratcliffe *et al.* 2001). It is however worth noting that many of the antioxidants present in broccoli, such as carotenoids and polyphenols are poorly absorbed in the small intestine and would be expected to reach the colon irrespective of whether they are trapped in a vegetable matrix or released by digestive processes (Halliwell *et al.* 2000).

The hypothesis that fragments of raw broccoli, act as a vehicle to transport protective compounds to the colon is attractive, but other factors may also explain the differing effects of raw and processed broccoli. Microwaving may have reduced the content of bioactive compounds present in the broccoli, and both microwaving and homogenisation may have altered the profile and amount of GLS breakdown products colonocytes were exposed to. The microwaving protocol used in the second study (900 W x 9 m in 300 g of water) probably caused substantial thermal degradation of GLS to unknown metabolites and the leaching of a range of water soluble compounds into the cooking water (Vallejo *et al.* 2002). The cooking water was poured onto the diet of the pigs fed the microwaved broccoli in an attempt to deliver any leached compounds. However, the high cooking temperature would be expected to cause the evaporation of at least some of these (Vallejo *et al.* 2002). Additionally, the harsh microwaving protocol would have almost certainly inactivated plant myrosinase (Rungapamestry *et al.* 2006). In the absence of active plant myrosinase, intact GLS may be

delivered to the colon, where the resident microflora appears capable of catalysing their hydrolysis (Shapiro *et al.* 1998; Getahun & Chung 1999; Conaway *et al.* 2000). The microbial hydrolysis of GLS however, appears to result in an approximate 3 fold lower yield of ITC, than hydrolysis catalysed by plant myrosinase (Conaway *et al.* 2000). The reason for this is uncertain, but the almost complete absence of GLS and ITC in faeces indicates that microbial hydrolysis/metabolism of GLS produces other compounds. These have yet to be fully characterised, although the *in vitro* incubation of sinigrin and glucotropaeolin with human faeces has been shown to result in the formation of allylamine and benzylamine, rather than ITC (Combourieu *et al.* 2001). It follows that pigs fed the microwaved broccoli may have been exposed to lower levels of ITC and possibly other protective compounds than those fed the raw broccoli.

Homogenisation disrupts the plant cell structure and allows myrosinase to come into contact with GLS; myrosinase then catalyses the hydrolysis of GLS, ultimately leading to the formation of a variety of breakdown products (Holst & Williamson 2004). When raw broccoli is homogenised, the main breakdown product formed from glucoraphanin is sulforaphane nitrile rather than sulforaphane (Mithen *et al.* 2003; Matuesheski *et al.* 2004). This is of interest, because while sulforaphane is a potent inducer of protective phase 2 enzymes, sulforaphane nitrile appears to have little, if any inducer activity (Matusheski & Jeffery 2001; Basten *et al.* 2002). It has been assumed that after the consumption of raw broccoli, sulforaphane nitrile is also the major breakdown product formed from glucoraphanin, however, one study indicates that this may not be the case (Keck *et al.* 2003). Keck and associates fed rats, diets supplemented with raw broccoli containing intact GLS, and raw broccoli containing hydrolysed GLS (essentially homogenised broccoli). They found a much greater urinary excretion of ITC and induction of phase 2 enzymes in rats fed the raw broccoli with intact GLS, than in rats fed the raw broccoli containing pre-hydrolysed GLS. These results indicate that the hydrolysis of GLS *in vivo* produces greater quantities of ITC than when GLS are hydrolysed in raw broccoli tissue *in situ*. This higher yield may partially

explain why the whole raw broccoli protected the colonocytes of pigs against DNA damage and the raw homogenised broccoli did not.

1.7 Hypothesis

A direct causal link between diet and CRC has yet to be fully established, but studies have identified a number of dietary factors that appear to modify risk (Johnson & Lund 2007; WCRF/AICR 2007). Among these, cruciferous vegetables have received much attention (Verhoeven *et al.* 1996; Verhoeven *et al.* 1997; IARC, 2004; Verkerk *et al.* 2009). In experimental studies, cruciferous vegetables and GLS and their breakdown products, have consistently been shown to exert chemoprotective effects (see pgs 17-31). The results from prospective cohort studies have however been unconvincing (see pgs 31-33). One possible explanation for this disparity is that the relatively large amounts of cruciferous vegetables used in experimental studies are chemoprotective, whereas, amounts consistent with normal human intakes are not. Conversely, the failure of cohort studies to detect an effect may reflect a lack of sensitivity of these studies. The protective effects of cruciferous vegetables against CRC may be influenced by the GST genotype of the individual (see pgs 34-38). The failure of most epidemiological studies to consider the modulatory effect of genotype may have weakened their findings. Most studies have assessed intake of cruciferous vegetables using FFQs. FFQs appear to be particularly poor at assessing vegetable intake, so a degree of misclassification of subjects into the wrong category of intake probably occurs (Bingham *et al.* 1994). This would attenuate any association between cruciferous vegetable intake and risk of CRC and make modest protective effects difficult to detect. Also, epidemiological studies rarely collect information on the amount of processing that vegetables have been subjected to prior to consumption. Processing may alter the content and bioavailabilty of the bioactive compounds present in cruciferous vegetables (see pgs 38-44). This may mean that individuals with apparently similar levels of crucifer intake may be exposed to different levels of bioactive compounds. Failing to account for this would add another source of error.

Work conducted by the group prior to the current project found that the ability of broccoli to protect against DNA damage in colonocytes was eliminated by both physical and thermal processing (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000). It is unclear how processing eliminated the protective effect of whole raw broccoli, but a number of possibilities exist. The bioavailability and concentration of the protective compounds may have been altered, as may the ability of plant compounds to induce hepatic and intestinal detoxification enzymes. Processing tends to increase the digestibility of vegetables and may therefore have also altered the location of their protective effect within the gastrointestinal tract (Ratcliffe *et al.* 1997).

The primary aim of this thesis was to further test the hypothesis that raw broccoli protects DNA in colonocytes from damage and that processing eliminates this effect. The secondary aims were to investigate:

- if the protective effect of raw broccoli is correlated with the induction of hepatic and/or colonic phase II enzymes, changes in the colonic microflora, or an increase in the production of SCFA and
- whether a non-cruciferous raw vegetable, rich in antioxidants also protects DNA in colonocytes from damage.

Chapter 2

Effect of raw broccoli and blanched-frozen broccoli on DNA damage in colonocytes

2.1 Introduction

Cruciferous vegetables and their bioactive constituents have been studied extensively for chemoprotective effects. In animal models of colon cancer they have consistently been shown to inhibit tumourigenesis (IARC, 2004). Furthermore, a number of plausible mechanisms of action have been identified (van Poppel *et al.* 1999; Smith *et al.* 2003; Smith *et al.* 2005). However, cohort studies investigating the association between cruciferous vegetable intake and CRC risk have found little evidence of protection (see Chapter 1, pgs 31-33). Possible explanations for this disparity were discussed in Chapter 1, and one of the factors identified as being of possible importance, was the failure of many epidemiological studies to consider the influence of processing.

Cruciferous vegetables are typically consumed after some type of processing. Processing generally results in the loss of bioactive compounds (Price *et al.* 1998; Vallejo *et al.* 2002). Processing may also beneficially or negatively alter the bioavailability of such compounds (Rock *et al.* 1998; Conaway *et al.* 2000). Therefore, variation in the degree of processing that cruciferous vegetables undergo before consumption may mean that individuals with similar levels of intake are exposed to different levels of bioactive compounds. Only a few studies have investigated the effect of processing on the actions of cruciferous vegetables *in vivo* (see Chapter 1, pgs 40-44). In work conducted by the group prior to this project it was found that homogenisation and microwave cooking (900 W x 9 min in 300 g water) eliminated the ability of raw broccoli to protect against DNA damage in colonocytes (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000). It was hypothesised that fragments of raw broccoli with intact cell walls

acted as a vehicle to transport protective compounds to the colon, where they were released by the fermentative activity of the colonic microflora (Ratcliffe *et al.* 1997; Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000).

The aim of this study was to further explore the effect of processing on the ability of raw broccoli to protect against DNA damage in colonocytes. Within the UK, a substantial proportion of vegetables is purchased frozen (Mintel, 2007). This experiment compared the effect of raw broccoli with broccoli that had been prepared to replicate the commercial preparation of frozen broccoli. It was hypothesised that raw broccoli would protect against damage to DNA in colonocytes, and that blanch-freezing would eliminate this protective effect.

The objectives of this experiment were to:

- develop a processing protocol that replicated the commercial preparation of frozen broccoli

- select appropriate groups of pigs for treatment with diets supplemented with raw and blanched-frozen broccoli

- measure damage to DNA in colonocytes using the 'comet' assay

- establish whether an intake of raw broccoli provides greater protection against DNA damage than broccoli that has been prepared to replicate commercially frozen broccoli

- measure and compare DNA damage in fresh colonocytes and in cryopreserved colonocytes

- measure plasma levels of antioxidant vitamins and compare any treatment differences with any treatment differences in colonocyte DNA damage.

2.2 Materials and Methods

2.2.1 ***Chemicals***

All chemicals were purchased from Sigma, Poole, UK except for the following: somulose from Arnolds, London, UK; zoletil from Virbac, Glasgow, UK; lymphoprep from Nycomed, Oslo, Norway; collagenase/dispase from Roche Diagnostics, Mannheim, Germany; low melting point (LMP) and high melting point (HMP) agarose from Gibco Life Technologies, Paisley, UK; NaCl and NaOH from Fisher Scientific, Loughborough, UK; modified RPMI 1640 from ICN Flow, Irvine, UK; DAPI (4'6-diamidine-2-phenylindole dihydrochloride) from Boehringer Mannheim, Lewes, UK; acetic acid, acetonitrile, ascorbic acid, benzene, EDTA, ethanol (Licrosolv), metaphosphoric acid, n-hexane from Merck Chemicals, Nottingham, UK; European and US vitamin standards from Promochem Ltd (Welwyn Garden city, Herts, UK).

2.2.2 ***Broccoli***

2.2.2.1 Broccoli for the pig experiment

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Marathon) for feeding in the pig trial was supplied by Sainsbury's, Garthdee, Aberdeen. New batches (delivered from Kettle Produce, Cupar, Fife) of broccoli were collected on Monday, Wednesday and Friday morning each week. No details were available on the time lapse between harvesting and delivery to Sainsbury's.

2.2.2.2 Broccoli for determining the effect of blanch-freezing on myrosinase activity

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Marathon) for determining the effect of blanch-freezing on myrosinase activity was supplied by Sakata UK Ltd, Boston, England. Broccoli was delivered within 2 d of being harvested from an experimental field trial in Lincolnshire, England.

2.2.2.3 Preparation of broccoli for the pig trial

To conform to the regulatory procedures of the Rowett Research Institute, minimal disease pig unit, broccoli was washed in a 1% Virkon solution and thoroughly rinsed with cold water prior to preparation.

2.2.2.4 Raw broccoli

Washed broccoli bunches were trimmed to size (~300 g) and stored in sealed plastic bags at 4^{o}C for a maximum of 4 d before use.

2.2.2.5 Blanched-frozen broccoli

Broccoli bunches (~300 g) were steam blanched at 95^{o}C for 4 min in a Convostar, Convotherm (model 0010, 10 Eglfing, Germany) steamer. After 4 min, the broccoli was removed from the steamer and rapidly immersed in ice water to halt the blanching process. After 5 min, the broccoli was removed from the ice water and gently shaken to remove excess water. The broccoli was then frozen for 50 min in a blast freezer, sealed in plastic bags and stored at –20^{o}C for a maximum of 4 d. Before feeding, each head of frozen broccoli was placed on a separate plastic tray and allowed to defrost at room temperature. During thawing a small amount of water collected on the plastic tray. This water was subsequently added to the feed of the relevant group of pigs.

2.2.2.6 Blanching test

A 60 sec peroxidase test was used to test the adequacy of the blanching process (protocol supplied by J.Dempsey of Esk Frozen Foods, Montrose, personal communication). Samples of blanched broccoli were removed from the ice water and cut in half through the length of the stem and florets. Each half was covered evenly with enzyme reagent (equal volumes of 0.5% w/v guiacol solution and 1.5% hydrogen peroxide (H_2O_2) solution). If active peroxidise is present it catalyses the oxidation of guiacol by H_2O_2 to produce products of unknown structure that have a reddish brown colour (Miller, 1998). The blanching process was accepted as adequate if no brown colouration occurred in the broccoli florets within 60 sec.

2.2.3 ***Myrosinase activity***

2.2.3.1 Preparation of broccoli for determining the effect of blanch-freezing on myrosinase activity

To replicate the handling of the broccoli in the pig trial, three raw broccoli heads (approx 300 g) were stored at 4^{o}C and three were blanched (as described earlier, except that a different steamer was used; Hobart model CSD1012E, Ohio, USA), blast frozen and stored at -20^{o}C. In both cases, the broccoli was stored for a maximum of 4 d prior to preparing extracts for measuring myrosinase activity.

2.2.3.2 Preparation of broccoli extracts for the determination of myrosinase activity

All steps were conducted at 0-4^{o}C or on ice. Samples of approximately 2 g in weight were cut from the stalk and the outer and inner florets of 3 raw and 3 blanched-frozen broccoli heads. Each sample was transferred to ice-cold 33 mM potassium phosphate buffer (5 ml/g plant tissue) and then homogenised with 2 x 5 sec bursts of a rotor stator homogeniser (Ystral D-79282, Ballrechten-Dottingen, Germany). Homogenates were centrifuged at 2990 **g** x 10 min (Hermle Labortechnik Z36HK, Germany). Supernatants were carefully removed and

centrifuged again at 2990 **g** for a further 10 min. Myrosinase activity was determined immediately in the resulting supernatants.

2.2.3.3 Determination of myrosinase activity

Myrosinase activity was determined by the spectrophotometric method of Shapiro *et al.* (2001) as modified by Rungapamestry *et al.* (2006). The assay measures the rate at which plant myrosinase catalyses the decomposition of the GLS, sinigrin. The reaction was initiated by adding 100 µl of broccoli extract to 1 ml of reaction mixture (150 µM sinigrin, 500 µM ascorbic acid, 1mM EDTA in a 33 mM phosphate buffer, pH 7.0) in a quartz cuvette (Hellma 114-QG). After a 1 min lag time, the rate of reaction was measured as a linear decrease in absorbance at 227 nm for 3 min (Cecil CE9500 Super Aquarius, Cambridge, England). A standard curve was constructed using isolated myrosinase (0.012 to 0.3 units, R^2=0.998-0.999) and the myrosinase activity in each vegetable extract was calculated by linear regression and expressed as units/g of plant tissue (see Appendix, Fig A.1, pg 152). Myrosinase standards and samples were measured in duplicate.

2.2.4 ***Animals and Experimental Design***

Fifteen male Landrace X Large White pigs (Rowett Research Institute, Aberdeen, Scotland) were divided into five age (79 (SD 3) d) and weight (34.7 (SD 3.9) kg) matched cohorts. Each cohort consisted of siblings to minimise the effect of genetic variation on subsequent treatment outcome. Each animal was individually housed within a temperature-controlled room (20^{o}C) with a 12 h light-dark cycle with the light phase beginning at 07.00 h. Within each cohort, siblings were randomly assigned to one of three diet groups: (1) control diet (Rowett Grower feed, see Table 2.1); (2) the control diet plus 600 g/d of whole raw broccoli; (3) the control diet plus 600 g/d of blanched-frozen broccoli.

After a 3 d adaptation period to experimental conditions and diets, animals were maintained on the experimental diets for a further 12 d. The control diet and broccoli were provided in two daily feeds of equal size at 08.00 h and 16.00 h, with each pig receiving the control diet at a level of 5% of their bodyweight. Within each cohort, feed intake was monitored and corrected where necessary to ensure a similar intake between animals. No correction was made to the control diet to account for the macronutrient content of the supplemental vegetables. Throughout the trial, pigs were allowed access to water *ad libitum*. The starting date of each cohort was staggered to allow time at the end of the experimental period to process and analyse samples that could not be frozen.

Table 2.1 Composition of Rowett Standard Grower Feed

Component	kg/1000 kg
Barley	228
Wheat	430
Hipro Soya	225
Super Soya	50
Soya Oil	10
Salt	5
Grower Vitamin and Mineral Mix*	30
Molasses	20
De-oderase®	2

* The vitamin and mineral mix contained (per kg) calcium 250 g, copper 8.925 g, phosphorus 7 g, sodium 5 g, selenium 13 mg, retinol 113, 636 µg, α-tocopherol acetate 3750 mg, vitamin D, 1875 µg. De-oderase® is a preparation that contains glycocomponents derived from the *Yucca shidigera* plant. It is thought to reduce odour and ammonia emissions from livestock (Amon *et al.* 1995).

At the end of the experimental period, the pigs were weighed, sedated (intramuscular injection of Zoletil 100®; 1 vial reconstituted in 5 ml of di.H_2O), anaesthetised (with an intravenous injection of Somulose®; 1 ml per 10 kg BW), and then killed by exsanguination. Within each

cohort, pigs were killed in a randomised sequence, at hourly intervals, commencing approximately 1 h after the morning feed.

2.2.5 ***Collection of blood samples and plasma isolation***

Immediately after slaughter, venous blood was collected into 10 ml lithium-heparin coated vacutainers (SIS, Nottingham, UK). Vacutainers were gently rotated to mix the blood with lithium-heparin to prevent clotting and then stored on ice prior to plasma isolation. Plasma was isolated from the samples by centrifugation (2400 **g** x 15 min, 4^{o}C). For the analysis of ascorbic acid content, a portion of plasma (600 µl) was diluted 50:50 v/v with 10% metaphosphoric acid, snap frozen in liquid N_2 and stored at -80^{o}C. The remaining plasma was divided into 600 µl samples, snap frozen in liquid N_2 and stored at -80^{o}C for the analysis of retinol, carotenoids and vitamin E.

2.2.6 ***Collection of colon and isolation of colonocytes***

The entire colon from the ileocaecal junction to the rectum was excised. A section of the mid colon, approximately 200 mm in length was carefully flushed with modified Ca^{2+}/Mg^{2+}-free Hanks Balanced Salt Solution (HBSS, 37^{o}C) and then transferred to fresh HBSS solution (37^{o}C) for transportation to the laboratory for colonocyte isolation. Colonocytes were removed from the section of mid colon by a modification of the method of Brendler-Schwaab *et al.* (1994).

One end of the colon was clamped shut and the colon was filled with approximately 50 ml of freshly prepared ice-cold HBSS solution containing 30 mM dithiothreitol (DTT). The open end was tied shut and the colon segment was left on a Petri dish for 10 min during which time excess connective tissue was removed. The colon was then flushed with ice cold HBSS, tied at the base, filled with 40 ml of digestion buffer (HBSS containing 1 mg/ml collagenase/dipase, pH 7.3), tied at the top, and suspended in a beaker containing HBSS. The beaker was placed in a shaking water-bath (37^{o}C, 60 rpm x 30 min) to gently agitate the

colon. The colon was cut longitudinally and the colonocyte containing fluid from inside was collected. Large clumps of tissue were removed from the colonocyte containing fluid and the remaining cell suspension was centrifuged for 6 min (200 **g**, 20^{o}C). The supernatant was decanted and the pellet was resuspended in RPMI 1640-glutamine (1% w/v). Cell concentration and viability was determined by trypan blue exclusion (cell viability typically 70 – 80%). The cell suspension was separated for fresh comet analysis and for cold storage.

For fresh comet analysis, five aliquots (200 µl) were centrifuged for 3 min (200 **g**, 4 ^{0}C), the supernatant was decanted and cells were resuspended in 85 µl of 1% LMP agarose. For cold storage, aliquots were centrifuged for 3 min (200 **g**, room temperature), the supernatant was decanted and cells were resuspended in freezer mix (90% FCS, 5% DMSO, 5% RPMI) at a cell concentration of 3 x 10^{6} per ml. Cells were then stored overnight at –80^{o}C in thick walled polystyrene trays to lower the gradient of temperature drop, before being transferred to liquid N_2 for long-term storage.

2.2.7 ***Comet analysis***

In its simplest form the comet assay measures DNA strand breaks in isolated cells. After isolation, cells are embedded in agarose on a microscope slide and lysed with detergent and salt. This produces nucleoids that consist of supercoiled loops of DNA attached to the nuclear matrix. When subjected to electrophoresis at a high pH, these nucleoids form structures that resemble comets. These can be viewed by fluorescence microscopy. The relative intensity of the tail reflects the number of DNA strand breaks present (Fig 2.1; Collins, 2004).

In the current experiment, the comet assay was used to measure DNA strand breakage in fresh (on day of isolation) and frozen colonocytes (after storage at –196^{o}C in liquid N_2). Colonocytes suspended in 85 µl of 1% LMP agarose were pipetted onto a frosted glass microscope slide pre-coated with 1% HMP agarose (3 slides per animal, 2 gels per slide). The

agarose was allowed to set by incubating at 4°C for 5 min. Slides were then incubated in lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA and 1% (v/v) Triton X-100, adjusted to pH 10 with 10 M NaOH) for at least 1 hour at 4°C to remove cellular constituents such as membranes and various proteins.

Slides were then placed in an electrophoresis tank (containing 0.3 M NaOH & 1 mM EDTA, approximately pH 13) in continuous rows and incubated for 40 min to allow time for DNA unwinding. Subsequently, slides were subjected to electrophoresis in the same solution at a fixed voltage of 25 V and 300 mA for 30 min. The slides were then washed 3 times for 5 min with neutralising buffer (0.4 M Tris, adjusted to pH 7.5 with HCl, 4°C), before staining with 20 µl of DAPI (1 µg/ml).

2.2.7.1 Quantification of Comet Assay

DAPI stained nucleoids were examined with a fluorescence microscope. One hundred nucleoids on each slide were examined visually and scored according to tail density and length using an integer scale between 0 (no damage) and 4 (maximal damage) (Fig 2.1). Therefore the total score for 100 comets could range from 0-400.

2.2.8 ***Determination of plasma vitamin C***

Samples of plasma stabilised with MPA (see "Collection of blood samples and plasma isolation") were allowed to thaw on ice for 1 h and then centrifuged (1000 **g** x 10 min, 4°C). Supernatants were removed and transferred into amber vials. Each plasma sample was then analysed for vitamin C by reversed phase HPLC using an ion-pairing reagent with UV detection (Ross, 1994). Under the pH conditions of the assay (pH 5.5), ascorbic acid (p*K*a 4.19) exists primarily as the ascorbate ion. The ascorbate ion pairs with an ion-pairing reagent forming neutral compounds that can be separated by reversed phase HPLC.

The equipment used consisted of a Gilson model 305 pump (Anachem, Luton, UK) and a 20 µl loop volume automated sampler (Gilson 231; chamber maintained at 4°C) connected to a Gilson 116 UV detector set to 262 nm. Vitamin C was separated on a Nucloesil ODS 5-µm column (25 x 0.46 cm, Jones, Henygoed, Mid Glamorgan, UK) with 2 x 0.4cm guard column packed with pellicular C18 reversed phase packing 30-40 µM. The mobile phase consisted of 25 mM myristyltrimethylammonium bromide, 0.05 M NaOH, 0.06 M acetic acid, 7.5% (v/v) acetonitrile, pH 5.5. Prior to use, EDTA (200 mg/l; to chelate any transition metals present in the sample) and homocysteine (100 mg/l; to stabilise the ascorbic acid in its reduced form) were added. The mobile phase was pumped through the column at a flow rate of 0.55 ml/min at an ambient temperature. The run time was 12 min. Data was collected and analysed using Gilson 715 software.

Solutions of ascorbic acid diluted in 10% (w/v) MPA were used to construct a standard curve (0-100 µM). The peak area of each sample was determined using Gilson 715 software and was quantified against a curve of the areas produced by the standards to produce a vitamin C concentration. For quality control, a pooled plasma sample was analysed alongside every 25 samples and European and USP reference standards of ascorbic acid were analysed with each run.

2.2.9 ***Determination of retinol, carotenoids and tocopherols***

Plasma levels of retinol, α-carotene, β-carotene, zeaxanthin/lutein, α-tocopherol and γ-tocopherol were measured simultaneously using reversed phase HPLC. Carotenoids were detected by a spectrophotometric detector in the visible region and retinol and tocopherols were detected with a fluorometer (Hess *et al.* 1991).

Frozen samples of plasma were defrosted at 4°C. Samples of plasma (200 µl) were pipetted into 2 ml centrifuge tubes to which distilled water (200 µl) and ethanol (400 µl) were then added. The tubes were mixed for 10 sec in a Whirlimix **(**Water, Watford, Herts, UK), hexane

(700 µl) and echinone (100 µl; internal standard) were then added. The tubes were shaken for 10 min (Vortex Genie**,** Scientific Industries Inc, USA) and centrifuged for a further 5 min (2000 **g**). A 600 µl sample of the hexane layer was removed and dried for 9 min on a Speed Vac Concentrator (Savant Instruments, Farmingdale, USA). The resulting dried sample was dissolved in DEA (200 µl), shaken for 5 min, and then loaded onto the HPLC column.

The HPLC equipment consisted of a Walters 717 autosampler (injection volume 150 µl), a Walters model HPLC 515 pump connected to a 2487 UV/Vis detector and 2475 fluorescence detector and a Beckman Ultrasphere ODS i.d. column (250 mm x 4.6 mm, particle size 5 µm, Beckman, High Wycombe, Bucks, UK). The mobile phase (acetonitrile-tetrahydrofuran-methanol containing BHT-ammonium acetate (10 g/l; 67.4:22:6.8:3.8 by volume)) was pumped through the column at a rate of 1.05 ml/min. Run time was 30 min and during the run, wavelengths were changed as follows: visible detection program 0-11.9 min, 450 nm; 12-17.4 min, 472 nm; 17.5-30 min, 450 nm; fluorescence detection program (excitation and emission settings) 0-5.1 min, 330 and 470 nm; 5.2-14.6 min, 298 and 328 nm; 14.7-30.0 min, 249 and 480 nm. Appropriate concentrations of mixed standards containing carotenoids, retinol and tocopherol were included in each run. A pooled plasma sample was included after every 5 samples for quality control. Data was collected and analysed using Walters Millennium software. Quantification was by the external standard method. The peak area of each compound was quantified against the relevant reference standard.

2.2.10 ***Statistics***

2.2.10.1 Effect of blanch-freezing on myrosinase activity

The myrosinase data was $\log_{10}$ transformed after Levene's test indicated the data had unequal variances (P<0.001). Log transformation partially stabilised the variance (P=0.049). Standardised residuals calculated from the log-transformed data were tested for normality using the Shapiro-Wilk W test (P=0.064). A two-way ANOVA using the log-transformed data

was used to investigate the effect of blanch-freezing on myrosinase activity, and whether myrosinase activity varied in different sections of broccoli. Results are expressed as the geometric mean and 95% CIs of the antilog transformed data.

2.2.10.2 Pig trial

Standardised residuals for each dependent variable were tested for normality using the Shapiro-Wilk *W* test. Data that deviated significantly from normality were $\log_{10}$ transformed prior to testing for treatment effects. The significance of differences between treatment means on each outcome variable was tested by two way ANOVA for a randomised block design. When a significant difference was found, this was followed with a Dunnett's test (Ruxton & Beauchanp, 2008). Non-transformed data are expressed as means (SD); transformed data are expressed as geometric means (95% CI).

Student's paired t-test was used to assess the difference in DNA damage between fresh and cryopreserved colonocytes after Shapiro-Wilk *W* test revealed the differences between the paired colonocytes were normally distributed. The correlation between DNA damage in fresh and cryopreserved colonocytes was determined using Pearson's product moment correlation co-efficient.

All statistical analyses were conducted on SPSS Release 17.0 (2008) and a *P* value of <0.05 was used as the critical level of significance.

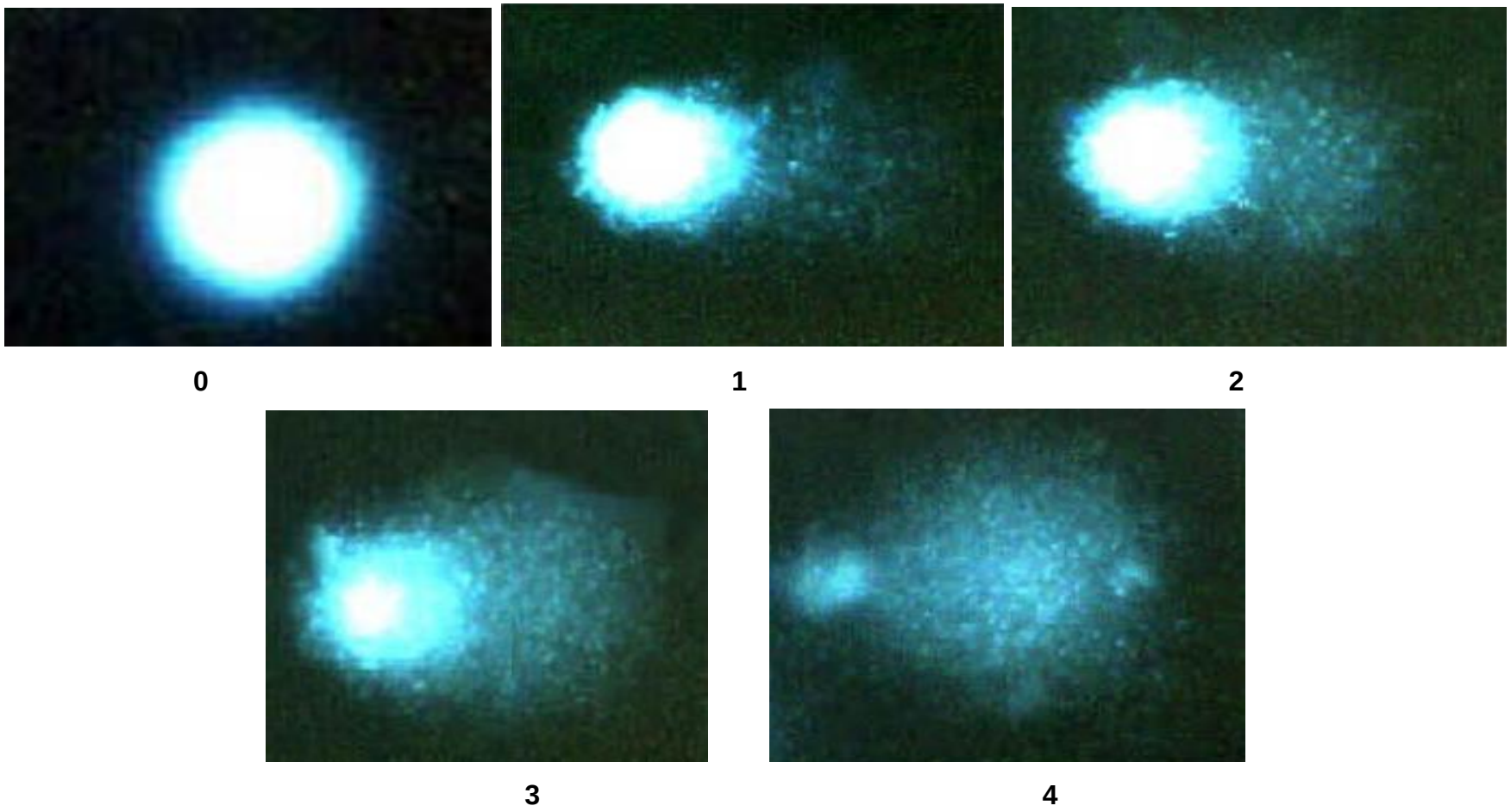


Fig 2.1 DNA damage in Caco-2 cells determined by single cell gel electrophoresis. Images represent differences in DNA damage scores 0-4.
Source: Kindly supplied by Professor Paul Kong, School of Pharmacy and Life Sciences, The Robert Gordon University, Aberdeen.

2.3 Results

2.3.1 Effect of blanch-freezing on myrosinase activity

Blanched-frozen broccoli had significantly less myrosinase activity than raw broccoli ($P<0.001$). Myrosinase activity did not differ significantly between inner florets, outer florets and stalk ($P=0.147$) and there was no significant interaction between the effects of blanch-freezing and the part of the plant that each section was sampled from ($P=0.765$) (Fig 2.2).

2.3.2 Pig trial

The mean intake of basal diet (1273 (SD 167.1) g/d) did not differ among treatment groups and the raw broccoli and blanched-frozen broccoli groups consumed similar amounts of broccoli. All groups of animals gained weight, but the raw broccoli ($P=0.028$) and blanched-frozen broccoli ($P=0.043$) groups both gained significantly more weight (approximately 1 kg) than the control group (Table 2.2).

2.3.2.1 Plasma concentrations of vitamins and carotenoids

Broccoli supplementation failed to have any significant effect on plasma concentrations of retinol (pooled geometric mean 0.309 (95% CI 0.2323, 0.4121) μg/ml), lutein/zeaxanthin (pooled mean 0.0029 (SD 0.00046) μg/ml), ascorbic acid (pooled geometric mean 33.1 (95% CI 23.28, 47.21) μM), α-tocopherol (pooled mean 1.46 (SD 0.450) μg/ml) or γ-tocopherol (pooled mean 0.0231 (SD 0.0076) μg/ml) (Table 2.3).

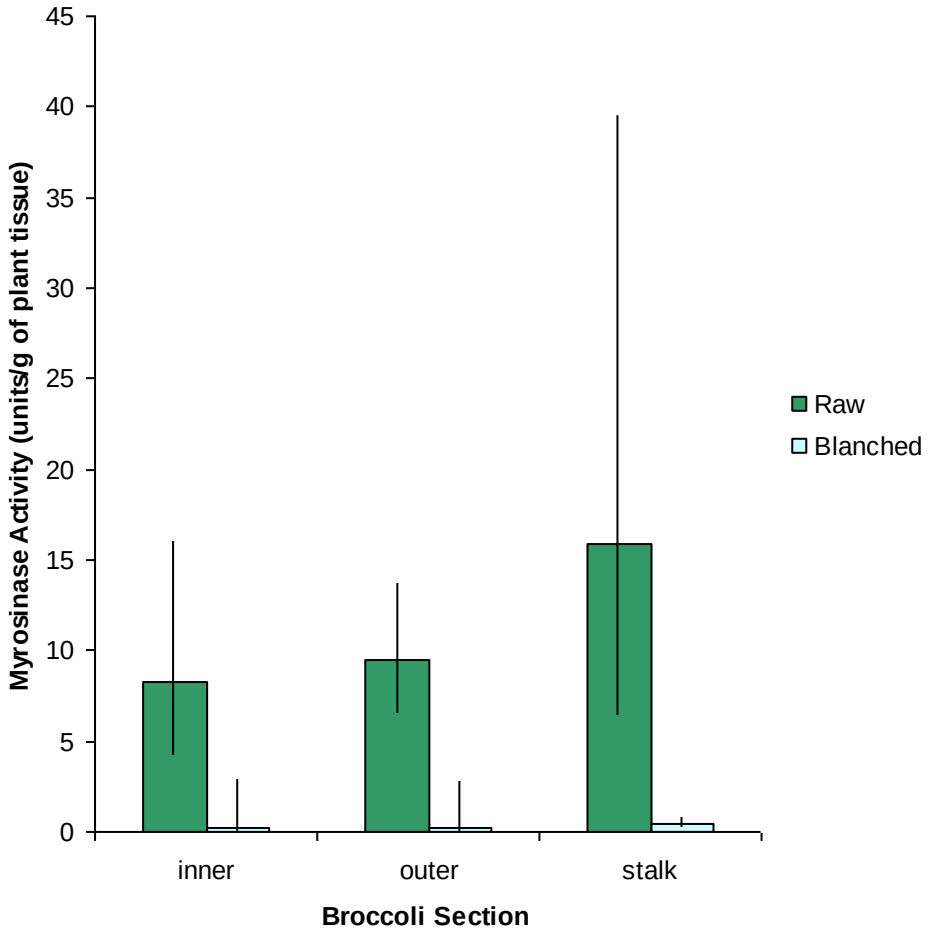


Fig 2.2 Effect of blanch-freezing on myrosinase activity in broccoli. Myrosinase activity is expressed as units/g of plant tissue in sections sampled from the stalk and the inner and outer regions of heads of whole raw and blanched-frozen broccoli. Bars represent 95% CI of the geometric means of three replicates. Two way ANOVA with interaction was used to assess the significance of differences. Blanched-frozen broccoli had significantly less myrosinase activity than raw broccoli ($P<0.001$). Myrosinase activity did not differ significantly in samples from different parts of the broccoli plant ($P=0.147$). There was no interaction between the effects of blanch-freezing and part of the plant that the sample was taken from ($P=0.765$).

Table 2.2 Mean values (SD) of weight gain and daily intake of basal diet and broccoli over the 12 d test period.

Treatment	Basal diet g/d	Broccoli g/d	Initial body weight kg	Final body weight kg	Body weight gain kg
Raw broccoli	1273 (167.1)	577.4 (39.81)	33.90 (1.828)	41.58 (3.518)	7.68* (1.788)
Blanched-frozen broccoli	1273 (167.1)	585.4 (29.46)	34.32 (6.317)	41.92 (6.978)	7.60* (1.233)
Control	1273 (167.1)	___	35.86 (2.904)	42.64 (3.905)	6.78 (1.997)

Basal diet, Rowett standard grower feed (see Table 2.1 for composition). Two way ANOVA (for a randomised block design) and Dunnett's test were used to assess statistical differences in body weight gain. An asterisk within a column indicates a significant difference between a treatment group and the control group (P<0.05); n=5 in each diet group in all cases.

Table 2.3 Mean plasma concentration of vitamins and carotenoids in groups of pigs fed cereal diets unsupplemented (control) or supplemented with 600 g of raw or blanched-frozen broccoli (cv. Marathon) for 12 d.

Treatment	Retinol µg/ml	Zeaxanthin/lutein µg/ml	Ascorbic acid µM	α-tocopherol µg/ml	γ-tocopherol µg/ml
Raw broccoli CI/SD	0.348 (0.211, 0.572)	0.0031 (0.0006)	28.6 (15.6, 52.8)	1.19 (0.209)	0.021 (0.0047)
Blanched-frozen broccoli CI/SD	0.280 (0.170, 0.461)	0.0029 (0.0006)	43.2 (23.4, 79.4)	1.52 (0.326)	0.024 (0.0062)
Control CI/SD	0.304 (0.185, 0.501)	0.0027 (0.0002)	29.4 (16.0, 54.2)	1.68 (0.636)	0.025 (0.0115)

Two way ANOVA (for a randomised block design) was used to assess statistical differences, n=5 in each diet group for all variables, except zeaxanthin/lutein, where n=4. Non-transformed data are expressed as mean (SD), data that were $\log_{10}$ transformed before analysis are expressed as geometric mean (95% CI).

2.3.2.2 Colonocyte DNA damage

Colonocyte DNA damage was measured in freshly prepared colonocytes (fresh) on *post mortem* days and in colonocytes isolated on *post mortem* days and subsequently stored at -196^{o}C prior to analysis (cryopreserved). In both fresh and cryopreserved colonocytes, the consumption of raw broccoli significantly increased DNA strand breakage in comparison to the control group (mean increase 14% for fresh colonocytes (Fig. 2.3A; P=0.020) and 27% for

cryopreserved colonocytes (Fig. 2.3B; P=0.025)). The number of DNA strand breaks in the blanched-frozen broccoli group was not significantly different to the control group (Fig 2.3A, P=0.999 for fresh colonocytes, Fig 2.3B, P=0.243 for cryopreserved colonocytes).

Analysis of individual comet classes in cryopreserved colonocytes revealed a significantly greater percentage of maximally damaged class 4 comets (42 v 29%, P=0.03) and a significantly lower percentage of colonocytes exhibiting no damage (class 0; 15 v 26%, P=0.019) in pigs fed raw broccoli than in pigs fed the control diet. Comet classes in pigs fed blanched-frozen broccoli did not differ significantly from the control group (Table 2.4B). Analysis of individual comet classes in fresh colonocytes revealed no significant treatment effects (Table 2.4A).

A comparison between fresh and cryopreserved colonocytes revealed that cryopreserved colonocytes had significantly fewer DNA strand breaks than fresh colonocytes (mean 237 (SD 40.6) vs. 302 (SD 57.5), P=0.001). There was a weak non-significant positive correlation between DNA damage measured in fresh and cryopreserved colonocytes (r=0.21, P=0.464, Fig 2.4).

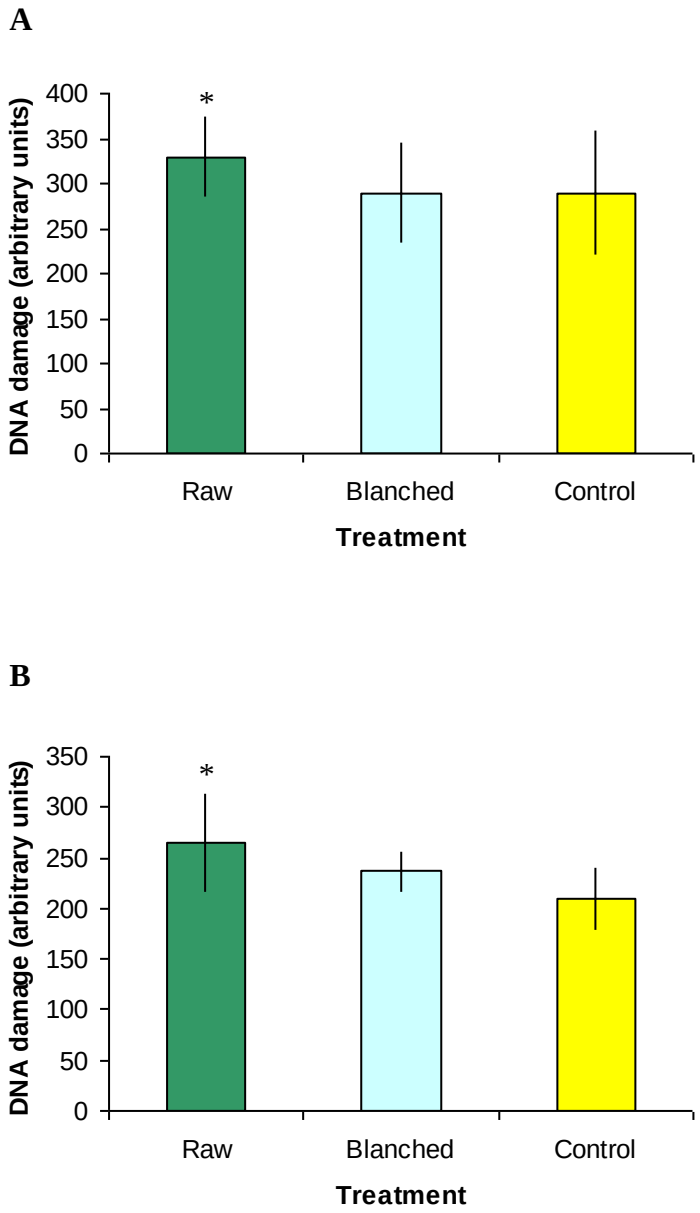


Fig. 2.3 The effect of raw and blanched-frozen broccoli on the level of DNA damage in (A) fresh and (B) cryopreserved colonocytes. The animals (five per treatment group) were fed cereal diets unsupplemented (control) or supplemented with 600 g/d of raw or blanched-frozen broccoli (cv. Marathon) for 12 d. Results are expressed as mean values (in arbitrary units, scored 0-400) for each treatment group and standard deviations represented by vertical bars. Two way ANOVA (for a randomised block design) and Dunnett's test were used to assess statistical difference. An asterisk indicates that the treatment group differs significantly from the control group (P<0.05).

Table 2.4 Comet classes in (A) freshly prepared and (B) cryopreserved colonocytes isolated from pigs fed one of the following diets: cereal diet unsupplemented (control), or cereal diet supplemented with 600 g/d of raw and blanched-frozen broccoli for 12 d.

A

Treatment	**Comet Classes**				
	0	**1**	**2**	**3**	**4**
Raw broccoli	2.7 (3.11)	7.7 (6.50)	7.1 (3.18)	23.1 (8.44)	59.4 (20.46)
Blanched-frozen broccoli	7.5 (7.79)	14.8 (9.77)	8.7 (2.19)	19.7 (5.27)	49.3 (14.73)
Control	11.5 (14.91)	10.3 (6.09)	6.3 (2.70)	21.7 (10.07)	50.2 (17.14)

B

Treatment	**Comet Classes**				
	0	**1**	**2**	**3**	**4**
Raw broccoli	15.0 (12.30) *	11.5 (2.01)	9.3 (2.07)	22.1 (6.06)	42.2 (9.25) *
Blanched-frozen broccoli	17.7 (5.26)	15.2 (2.79)	12.4 (1.32)	21.6 (3.46)	33.2 (6.47)
Control	26.0 (6.78)	15.4 (4.58)	11.40 (3.24)	18.4 (3.18)	28.9 (8.54)

In tables A and B, results are expressed as the number of colonocytes in each class of damage and are the mean (SD) of 3 slides (each containing two gels) for each animal and 5 animals in each treatment group. Two way ANOVA (for a randomised block design) and Dunnett's test were used to assess significance of differences. Within each column an asterisk indicates that the treatment group differs significantly from the control group ($P<0.05$).

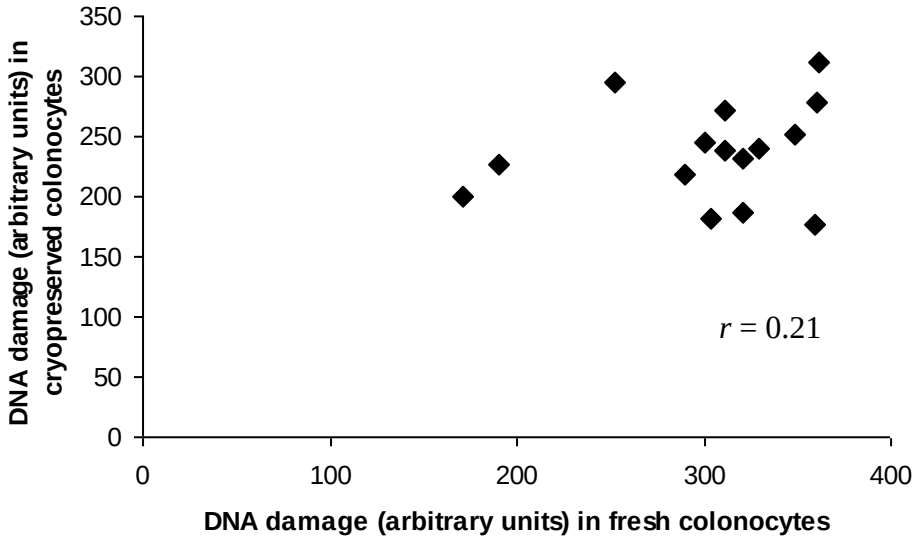


Fig 2.4 The correlation between DNA damage measured in freshly prepared colonocytes and cryopreserved colonocytes. Cryopreserved colonocytes were stored at -196^{o}C prior to analysis. Colonocytes were isolated from pigs fed an unsupplemented cereal diet (control) or 600 g/d of raw or blanched-frozen broccoli for 12 d.

2.4 Discussion

In two experiments conducted by the group before this project, colonocytes isolated from pigs fed a diet supplemented with raw broccoli had less DNA damage (measured as strand breaks) than those isolated from pigs fed a diet supplemented with processed broccoli, or a diet containing no broccoli (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000). In contrast, in the present experiment, DNA damage was increased in colonocytes isolated from pigs fed raw broccoli. This was surprising because the same experimental design was used as in the earlier experiments. A possible explanation is that the broccoli fed in the present experiment differed in some important aspect from that fed in the two earlier experiments. No information exists regarding the cultivar of broccoli fed in the previous experiments. However, as the broccoli was supplied from a different source, and the experiments were conducted at a different time of year, 3-4 years earlier, it is not unreasonable to assume that it may have been a different cultivar and/or grown under different conditions.

Different cultivars of broccoli, or even the same cultivar, grown under different conditions, can vary markedly in their content of bioactive compounds. For example, an analysis of 50 different cultivars of broccoli, found a greater than 27 fold difference in glucoraphanin content, 9 fold difference in indole GLS and α-tocopherol content, 4 fold difference in β-carotene content and a 2 fold difference in vitamin C content (Kurlich *et al.* 1999; Kushad *et al.* 1999). Also, in another study, an approximate 2-fold range in total polyphenol content was reported (Eberhardt *et al.* 2005). In addition to differences between cultivars, 2 to 3 fold differences in the content of a range of GLS have been found in the same cultivar of broccoli grown at different sulphur and nitrogen fertiliser levels (Vang *et al.* 2001). Such variations in the content of bioactive compounds could mean that two different cultivars or batches of broccoli may exert very different biological effects. Indeed, differences in composition have been shown to correlate with the ability of broccoli to induce XME *in vivo* and protect against H_2O_2 induced DNA strand breaks in cultured human hepatoma HepG2 cells (Kurlich *et al.* 1999; Kushad *et al.* 1999; Vang *et al.* 2001; Eberhardt *et al.* 2005).

In contrast to the effect of raw broccoli, the blanch-frozen broccoli had no significant effect on DNA damage. This suggests that the blanch-freezing protocol either reduced the level of the genotoxic component or its delivery to colonocytes. Freezing appears to preserve the level of many of the bioactive compounds present in broccoli, but blanching followed by immersion in cold water typically causes moderate losses, possibly as a result of leaching into the cooling water (Goodrich *et al.* 1989; Howard *et al.* 1999; Galgano *et al.* 2007). For example, Galgano *et al.* (2007) reported a 32% loss of vitamin C and a 13% loss of sulforaphane in broccoli subjected to a steam blanching protocol similar to that used in the current study (96^oC x 3min, followed by immersion in cold water for 1.5 min). Whereas, Goodrich *et al.* (1989) reported that a slightly harsher steam blanching protocol (99^oC x 5.5 min) followed by immersion in cold water for 3.5 min resulted in an approximate 50% loss of GLS. So, it is possible that the blanching protocol reduced the level of the unidentified genotoxic compound present in the broccoli.

The hydrolysis of GLS to their breakdown products is largely dependent on the presence of active plant myrosinase. When cooked broccoli with inactive myrosinase is consumed, the urinary excretion of ITC (as their mercapturic acid metabolites) is approximately three fold lower than when similar levels of GLS are supplied in raw broccoli with active plant myrosinase (Conaway *et al.* 2000). The urinary excretion of ITC was not measured in the current study, but the blanch-freezing protocol almost completely inactivated the plant myrosinase present in the broccoli. The combination of, inactive myrosinase and the possible loss of GLS during the blanching protocol implies that pigs consuming the blanched-frozen broccoli were exposed to lower levels of ITC than those fed the raw broccoli. ITC are generally regarded as anti-carcinogens, however, genotoxicity and other toxic effects have occasionally been reported (Murata *et al.* 2000; Paolini *et al.* 2004; Visanji *et al.* 2004). Under certain circumstances, the –N=C=S group of ITC appears to be able to undergo spontaneous hydrolysis leading to the production of superoxide and H_2O_2. It is possible that the presence of these prooxidants in colonocytes could lead to oxidative DNA damage and explain the effect of raw broccoli in the current study.

Other GLS breakdown products may also have contributed to the genotoxic effect of raw broccoli. Broccoli typically contains substantial quantities of the indolyl GLS, glucobrassicin and neoglucobrassicin, which on contact with myrosinase are hydrolysed to indole 3-carbinol (I3C) (Kushad *et al.* 1999; Mithen *et al.* 2000). Indolo(3, 2-b) carbozole, one of the acid condensation products formed from I3C in the stomach has been shown to induce CYP1A1 in Hepa1c1c7 cell lines, resulting in the generation of reactive oxygen species and subsequently oxidative DNA damage (McDanell *et al.*1988; Park *et al.* 1996). A local induction of CY1A1 enzymes in colonocytes could explain the effect of raw broccoli in the current experiment. The hydrolysis of GLS in raw broccoli may generate substantial quantities of nitriles (Matusheski *et al.* 2004). Cruciferous nitriles have been shown to exert toxic effects in the kidney, liver and pancreas of rats (Wallig *et al.* 1988; VanSteenhouse *et al.* 1989). So, it is possible that they contributed to the genotoxic effects of the raw broccoli. Other compounds

present in broccoli such as the flavonoids, quercetin and kaempferol and the sulphur compound, *S*-methyl cysteine sulfoxide (SMCSO) have also been shown to increase DNA strand breakage or cause oxidative damage *in vitro* (Smith, 1980; Duthie *et al.* 1997; Niering *et al.* 2005; Wätjen *et al.* 2005). The concentrations of these individual compounds shown to exert a detrimental effect in various experiments were greater than the amount that 600 g/d of broccoli could conceivably supply. However, it is possible that the different compounds present in raw broccoli acted in synergy to promote a genotoxic effect. Conversely, other unidentified compounds present in broccoli or bacterial metabolites formed in the colon may have been responsible.

In addition to causing the loss of various bioactive compounds and reducing the formation of ITC, the blanching-freezing protocol may have altered the location of release of the genotoxic component in the gastrointestinal tract. Freezing leads to the formation of ice crystals within plant cells, which can cause cell walls to rupture, an effect that is increased when vegetables are blanched before freezing (Crivelli *et al.* 1971; Brown, 1976; Fernandez *et al.* 2006). Ruptured cell walls would be expected to facilitate the release of a range of cytoplasmic compounds from the broccoli in the upper GI tract. Whereas, the release of compounds from fragments of raw broccoli with intact cell walls may have been more difficult. Previously, it was hypothesised that fragments of raw broccoli with intact cell walls resist digestion in the stomach and small intestine and act as a vehicle to transport various anti-carcinogenic compounds to the lumen of the colon, where the fermentative activity of the colonic bacteria hydrolyse the plant cell walls and release various compounds trapped within the plant matrix (Ratcliffe *et al.* 1997). Presumably, fragments of raw broccoli may have also acted as a vehicle to transport the unidentified genotoxic compound (or compounds that are converted to genotoxins by the colonic microflora) to the lumen of the colon.

Although the current study appears to be the first to find an increase in DNA damage in colonocytes after the consumption of raw broccoli, there is at least one report in the literature

of cruciferous vegetables damaging DNA *in vivo*. In a rodent study, Sorensen *et al.* (2001) reported that a cooked Brussels sprouts extract increased oxidative damage (measured as 8-oxo-dG) in the liver. Interestingly, scaled for body weight the daily intake of Brussels sprouts was similar to the daily intake of broccoli in the present experiment (25.6 v 17.3 g/kg/body weight). Together these results offer some cause for concern and indicate that high intakes of cruciferous vegetables may sometimes have unpredictable detrimental effects.

A possible criticism of the present study is the very high level of DNA strand breaks in the colonocytes isolated from the pigs fed the control diet (Fig 2.3A & 2.3B). Colonocytes from control pigs that were analysed on the day of isolation contained an average of 50% class 4 comets, whereas those cryopreserved and analysed at a later date contained an average of 28% class 4 comets. In the absence of exposure to a genotoxin, cells should contain few if any class 4 comets. This suggests that a substantial amount of damage occurred during the isolation process, especially in the freshly prepared colonocytes (Personal communication, A Collins). The higher level of class 4 comets in the freshly prepared colonocytes may be explained by the fact that after the cryopreserved colonocytes were frozen (presumably halting DNA damage), the fresh colonocytes spent further time on ice awaiting analysis, whilst samples for other analyses were being collected and processed. A certain amount of damage is probably unavoidable when isolating colonocytes and the overall comet score of the control group of pigs is comparable with previously published values for rat colonocytes (289 fresh and 208 cryopreserved versus 210 in the study of Duthie *et al.* (2002), value derived from a graph). The expected effect of a large amount of damage occurring during the isolation process is that any treatment effect could be masked. This appears to have occurred to a certain extent because a smaller genotoxic effect of raw broccoli treatment was observed in freshly prepared colonocytes than in cryopreserved colonocytes (14% increase versus 27% increase). Furthermore, there were no significant differences in individual comet classes in freshly prepared colonocytes, whereas in cryopreserved colonocytes there were significant differences in the number of class 0 and class 4 comets in raw broccoli group versus the control group

(Tables 2.4A & B). So, it is possible that the genotoxic effect of raw broccoli may actually be greater than that observed in the present experiment. It is also possible that a more modest genotoxic effect of the blanched-frozen broccoli may have been masked.

A further criticism of the present study is that the diet of the control pigs was not adjusted to account for the increased energy intake supplied by the supplemental raw and blanched-frozen broccoli (estimated to be approximately 2700 kcal over the 15 d trial period). This led to a slightly greater weight gain in the groups of pigs fed the diets supplemented with broccoli than in the control group. However, it seems unlikely that the minor differences in energy intake and weight gain explains the effect of the raw broccoli, because the blanched-frozen broccoli group gained almost the same amount of weight as the raw broccoli group, but exhibited no significant increase in DNA damage.

Literature values indicate that 600 g of broccoli (cv. Marathon) would be expected to contain an average of 8.2 mg of total carotenoids (5.5 mg β-carotene, 2.6 mg lutein, 0.1 mg other carotenoids), 8.7 mg α-tocopherol, 5.4 mg γ-tocopherol, and 730 mg of ascorbic acid (Vallejo *et al.* 2003; Eberhardt *et al.* 2005). Despite this, neither broccoli-supplemented diet increased the concentration of retinol, zeaxanthin/lutein, α-tocopherol, γ-tocopherol or ascorbic acid in the plasma of the pigs. The failure of either broccoli diet to increase the concentration of retinol and α-tocopherol in the plasma is probably because the basal diet (Rowett Grower Feed, see Table 2.1) was enriched with substantial quantities of retinol and α-tocopherol. In humans, the consumption of 200-300 g/d of broccoli causes an increase in the concentration of lutein in the plasma (Micozzi *et al.* 1992; Granado *et al.* 2006). The lack of any effect in the present study may reflect species differences between humans and pigs. The pig can synthesise ascorbic acid (Pond & Houpt, 1978). Studies investigating the effect of supplemental ascorbic acid in pigs are inconsistent with some finding an elevation in plasma ascorbate and others showing no effect (Yen & Pond, 1981; Mahan & Saif, 1983; Pointillart *et al.* 1997; Audet *et al.* 2004). The reason for this is unclear, but limited evidence indicates that

supplying high amounts of ascorbic acid to pigs, suppresses l-gulono-gamma-lactone oxidase (GLO) the hepatic enzyme responsible for synthesising ascorbic acid (Mahan *et al.* 2004). This could partially explain the failure of broccoli to significantly elevate plasma ascorbate in the present study. Alternatively, the lack of effect may reflect oxidation of ascorbic acid due to poor sample handling and preparation (Chung *et al.* 2001). The plasma ascorbic acid results of the pigs varied widely (range 7.9 to 58.7 μM) and included some suspiciously low values, such as 7.9 and 11.3 μM, perhaps indicating some loss of ascorbic acid occurred during the processing of samples.

In conclusion, the feeding of 600 g/d of raw broccoli for 12 d caused a significant increase in the number of DNA strand breaks in isolated colonocytes. No effect was seen in the colonocytes of pigs fed blanched-frozen broccoli, indicating that processing caused the genotoxic compound(s) to be lost, or reduced its formation *in vivo* or delivery to the colonocytes. It is unclear why the result of this experiment differed from work carried out by the group prior to this project, but the feeding of a different batch of broccoli may have been responsible. This study indicates that the biological effects of consuming broccoli may depend on the cultivar consumed and the degree of thermal processing it has been subjected to before consumption. The effect of different cultivars of raw broccoli on DNA damage in colonocytes warrants further investigation.

Chapter 3

Effect of raw broccoli and raw carrot on DNA damage in colonocytes

3.1 Introduction

In experimental studies, cruciferous vegetables and a number of their constituents typically exert chemoprotective effects (see Chapter 1). Occasionally, however, negative effects, such as an induction of chromosomal damage, tumour promotion, and the generation of reactive oxygen species have been observed (Pence *et al.* 1986; Kassie *et al.* 1996; Paolini *et al.* 2004). The experiment reported in Chapter 2 found a significant increase in DNA damage in colonocytes isolated from pigs fed 600 g/d of raw broccoli. This was unexpected, because work conducted by the group prior to the current project, found that raw broccoli conferred protection against DNA damage (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000). The factors responsible for this disparity are unclear, but it was suggested that the composition of the broccoli may have differed in some important aspect (see Chapter 2).

Several epidemiological studies have reported an inverse association between the consumption of vegetables rich in carotenoids and the incidence of CRC (Steinmetz & Potter 1996). It is unclear, whether this association is mediated by carotenoids or other bioactive compounds common to such vegetables. However, a number of anti-carcinogenic actions of carotenoids have been identified. These include increasing cell-to-cell communication, modulating the immune response and acting as antioxidants (Olsen, 1999). Much research has focused on the antioxidant actions of carotenoids, but the absorption of carotenoids is generally poor and the concentrations typically reached in the plasma and most body tissues may be insufficient to exert an antioxidant effect (Halliwell *et al.* 2000). Unabsorbed carotenoids may, however, reach sufficient concentrations in the lumen of the large intestine

to act as antioxidants (Halliwell *et al.* 2000). The faecal matrix can generate free radicals (Babbs *et al.* 1990; Stone *et al.* 2002). Unabsorbed carotenoids that reach the colon may scavenge reactive oxygen and nitrogen species generated by the faecal matrix, thereby protecting the colonic epithelium from oxidative DNA damage.

In most western countries, carrots are the main dietary source of carotenoids (O'Neill *et al.* 2001). The main carotenoids in carrot are β-carotene, α-carotene and lutein/zeaxanthin (Mangels *et al.* 1993). The absorption of carotenoids from raw carrots appears to be low. Studies using different methods to assess absorption have reported values between 10 and 50% (Erdman *et al.* 1993; Livny *et al.* 2003). The low bioavailability of carotenoids from raw carrot has been largely attributed to the food matrix resisting disruption during transit through the upper GI tract (Olsen, 1999). The consumption of raw carrots would be expected to deliver large quantities of carotenoids to the lumen of the large intestine. In the large intestine the fermentative activities of the microflora may degrade plant cell walls and release any carotenoids and other bioactive compounds that have remained trapped within the carrot matrix during their transport through the upper GI tract. Released carotenoids may then be available to act as antioxidants in the lumen of the colon.

The aims of this experiment were: (1) to investigate whether a different cultivar of broccoli (cv. Monoco) to that fed in the experiment reported in Chapter 2 would also exert a genotoxic effect on colonocytes; (2) to investigate whether a raw vegetable high in poorly absorbable antioxidants (carotenoids), and devoid or low in many of the putative genotoxic compounds present in raw broccoli would protect colonocytes from DNA damage. It was hypothesised that raw Monaco broccoli would damage DNA in colonocytes and that raw carrots would protect colonocytes from DNA damage.

The objectives of this experiment were to:

- conduct a dietary trial feeding supplemental raw broccoli and raw carrots to appropriate groups of pigs

- to determine whether either raw vegetable supplemented diet altered the level of DNA damage in the colonocytes of the experimental pigs

- to determine whether either raw vegetable supplemented diet increased the plasma concentration of retinol, ascorbic acid, α-tocopherol and γ-tocopherol.

3.2 Materials and Methods

3.2.1 ***Chemicals***

The chemicals used in this experiment were the same as those used in the experiment reported in Chapter 2 (see pg 48 for details).

3.2.2 ***Broccoli and carrots***

Broccoli (*Brassica oleracea* L. *var.* Italica cv. Monaco) and carrots (*Daucus carota* L. cv. Nairobi) were supplied by Sainsbury's, Garthdee, Aberdeen. New deliveries of broccoli and carrots were collected on Monday, Wednesday and Friday morning. No details were available on the time lapse between harvesting and delivery to Sainsbury's. To conform to the regulatory procedures of the Rowett Research Institute's, minimal disease pig unit, both vegetables were briefly washed in a 1% Virkon solution and thoroughly rinsed with cold water prior to preparation.

3.2.2.1 Preparation of broccoli and carrots

Raw broccoli

Washed broccoli bunches were trimmed to size (~300 g) and stored in loose fitting plastic bags at 4^{o}C for a maximum of 3 d before use.

Raw carrots

Washed whole carrots were stored in loose fitting plastic bags (~300 g per bag) at 4^{o}C for a maximum of 3 d before use.

3.2.3 ***Animals and experimental design***

Eighteen male Landrace X Large White pigs (Rowett Research Institute, Aberdeen, Scotland) were divided into six age (83 (SD 5) d) and weight (35.4 (SD 3.89) kg) matched cohorts. Each cohort consisted of siblings to minimise the effect of genetic variation on subsequent treatment outcome. Each animal was individually housed within a temperature-controlled room (20^{o}C) with a 12 h light-dark cycle with the light phase beginning at 07.00 h. Within each cohort, siblings were randomly assigned to one of three diet groups: (1) control diet (Rowett Grower feed, see Chapter 2, Table 2.1, pg. 52); (2) the control diet plus 600 g/d of whole raw broccoli; (3) the control diet plus 600 g/d of raw carrots.

After a 3 d adaptation period to experimental conditions and diets, animals were maintained on the experimental diets for a further 12 d. The control diet, broccoli and carrots were provided in two daily feeds of equal size at 08.00 h and 16.00 h, with each pig receiving the control diet at a level of 5% of their bodyweight. Raw broccoli was provided in 300 g individual bunches at each feed. Raw carrots were provided as whole carrots. Where necessary, one of the carrots was chopped to an appropriate size to bring the total meal intake to 300 g. Within each cohort, feed intake was monitored and corrected where necessary to

ensure a similar intake between animals. No correction was made to the control diet to account for the macro-nutrient content of the supplemental vegetables. Throughout the trial, pigs were allowed access to water *ad libitum*. The starting date of each cohort was staggered to allow time at the end of the experimental period to process and analyse samples that could not be frozen.

At the end of the experimental period, the pigs were weighed, sedated (intramuscular injection of Zoletil 100®; 1 vial reconstituted in 5 ml of di.H_2O), anaesthetised (intravenous injection of Somulose®; 1 ml per 10 kg BW), and then killed by exsanguination. Within each cohort, pigs were killed in a randomised sequence, at hourly intervals, commencing approximately 1 h after the morning feed.

3.2.4 ***Collection and isolation of samples***

Colon tissue and plasma were collected and prepared as outlined in Chapter 2 pgs 53-54

3.2.5 ***Experimental analyses***

All analyses were carried out as outlined in Chapter 2 pgs 54-57.

3.2.6 ***Statistical analyses***

The significance of differences between treatment means was tested by two way ANOVA for a randomised block design and where appropriate post hoc Dunnett's test. Shapiro-Wilk *W* tests revealed that the standardised residuals of all the outcome variables were normally distributed. Data are presented as means (SD).

The Shapiro-Wilk *W* test was used to confirm that the difference between paired fresh and cryopreserved colonocytes had a normal distribution. Student's paired t-test was then used to assess the significance of differences in DNA damage between fresh and cryopreserved colonocytes. Pearson's product moment correlation coefficient was used to determine the

correlation between fresh and cryopreserved colonocytes. All statistical analyses were conducted on SPSS Release 17.0 (2008) and a *P* value of <0.05 was set as the critical level of significance.

3.3 Results

One cohort of three pigs was excluded from all analyses because two pigs within the cohort developed diarrhoea just before the end of the intervention period. The mean intake of basal diet did not differ among treatment groups and the broccoli and carrot groups consumed similar amounts of their allocated vegetables (Table 3.1). The broccoli group gained 4.6% more weight than the control group (*P*=0.014) and the carrot group gained 3.1% more weight (*P*=0.055), but the difference was only significant for the broccoli group.

3.3.1 Plasma concentrations of vitamins

Neither vegetable supplement significantly increased plasma concentrations of retinol (pooled mean 0.34 μg/ml, SD 0.068), ascorbic acid (pooled mean 59.9 μM, SD 13.45), α-tocopherol (pooled mean 1.43 μg/ml, SD 0.295) or γ-tocopherol (pooled mean 0.026 μg/ml, SD 0.0126) relative to the control group (Table 3.2).

3.3.2 Colonocyte DNA damage

DNA damage was measured in colonocytes prepared on *post mortem* days (fresh) and in colonocytes isolated on *post mortem* days and subsequently stored at -196^{o}C prior to analysis (cryopreserved). Analysis of fresh colonocytes revealed that raw broccoli consumption caused a significant 54% increase in DNA strand breakage in comparison to the control group (Fig. 3.1A, *P*<0.001). A similar 51% increase was seen in cryopreserved colonocytes (Fig. 3.1B,

P<0.001). The consumption of raw carrot did not significantly alter the number of DNA strand breaks in fresh or cryopreserved colonocytes (Fig 3.1A & Fig 3.1B).

Table 3.1 Mean values (SD) of weight gain and daily intake of basal diet, broccoli and carrots over the 12 d experimental period.

Treatment	Basal diet	Broccoli	Carrot	Body weight gain
	g/d	g/d	g/d	kg
Raw broccoli	1085 (120.8)	600 (0)	___	5.6 (2.59)*
Carrot	1085 (120.8)	___	578 (49.6)	5.2 (2.27)
Control	1085 (120.8)	___	___	4.2 (1.80)

Basal diet, Rowett standard grower feed (see Chapter 2, Table 2.1 for composition). Two way ANOVA (for a randomised block design) and *post hoc* Dunnett's test were used to assess statistical difference in weight gain. An asterisk within a column indicates that the vegetable supplemented group differs significantly from the control group (*P*<0.05), *n*=5 for each diet group in all cases.

Table 3.2 Mean (SD) plasma concentration of vitamins in groups of pigs fed cereal diets unsupplemented (control) or supplemented with 600 g/d of raw broccoli (cv Monaco) or raw carrots (cv. Nairobi) for 12 d.

Treatment	Retinol	Ascorbic acid	α-tocopherol	γ-tocopherol
	μg/ml	μM	μg/ml	μg/ml
Raw broccoli	0.36 (0.104)	70.1 (10.64)	1.40 (0.477)	0.029 (0.0079)
Carrot	0.34 (0.051)	53.0 (15.20)	1.38 (0.183)	0.031 (0.0036)
Control	0.32 (0.043)	56.6 (9.29)	1.51 (0.176)	0.019 (0.0196)

Two way ANOVA (for a randomised block design) was used to assess the significance of differences between treatment group means, *n*=5 for each diet group in all cases.

The distribution pattern of DNA damage expressed as the number of cells in the five comet classes (from undamaged class 0 to maximally damaged class 4) varied significantly among diet groups (Tables 3.3A & B). Freshly prepared colonocytes isolated from pigs fed raw broccoli contained 79% fewer undamaged class 0 comets (P=0.01) and 129 % more class 3 (P=0.026) and 50% more maximally damaged class 4 comets (P=0.007) than those isolated from pigs fed the control diet (Table 3.3A). Colonocytes isolated from pigs fed raw carrot also contained significantly less undamaged class 0 comets, than the control group (P=0.049). A similar distribution pattern of DNA damage was observed in cryopreserved colonocytes, except that there were no significant differences among treatment groups in the number of class 3 comets and the number of class 0 comets in the raw carrot group was not significantly different to the control group (Table 3.3B).

When colonocytes from all treatment groups were pooled, a paired t test revealed no difference in the amount of DNA damage between freshly prepared and cryopreserved colonocytes (mean 229 (SD 52.4) vs. 226 (SD 49.2), P=0.558). There was a moderate significant positive correlation between DNA damage measured in fresh and cryopreserved colonocytes (r=0.6, P=0.018, Fig 3.2).

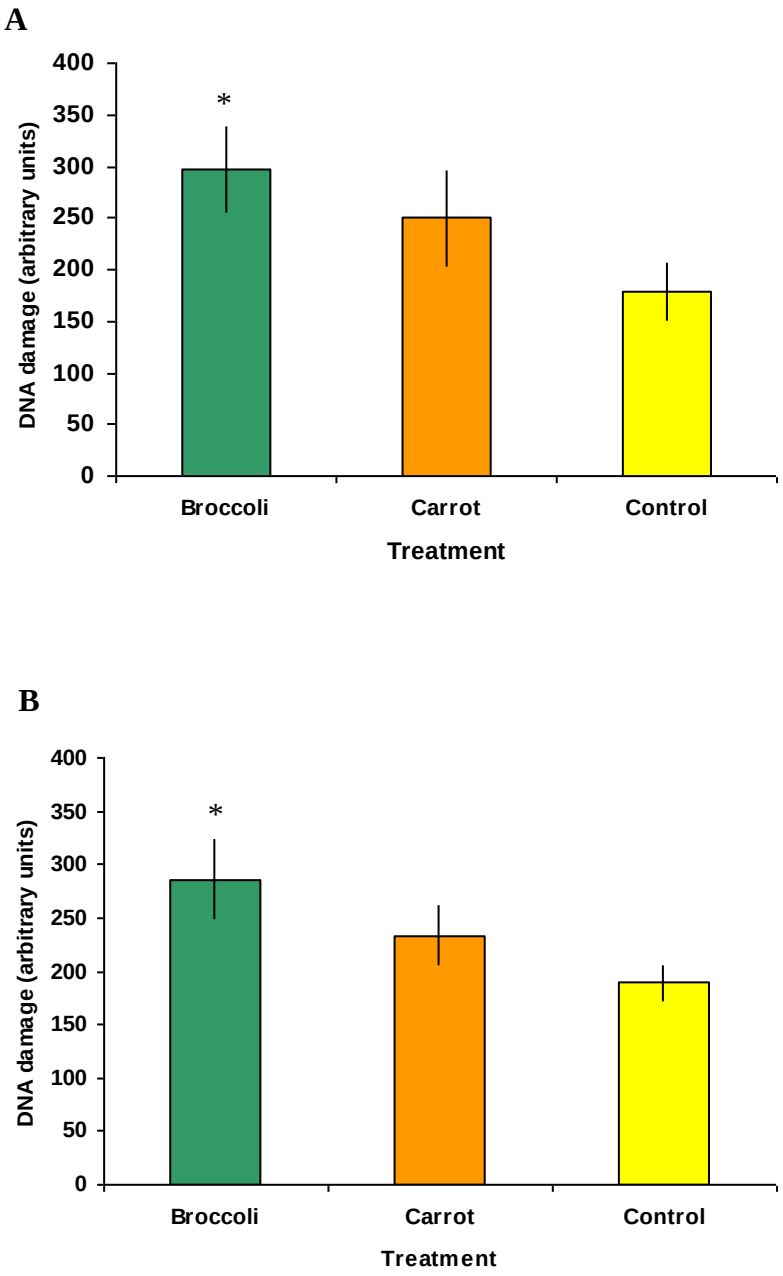


Fig. 3.1 The effect of raw broccoli and raw carrots on the level of DNA damage in (A) fresh and (B) cryopreserved colonocytes. The animals (five per treatment group) were fed cereal diets unsupplemented (control) or supplemented with 600 g of raw broccoli (cv. Monaco) or raw carrots (cv. Nairobi) for 12 d. Results are expressed as mean values (in arbitrary units, scored 0-400) for each treatment group and standard deviations represented by vertical bars. Two way ANOVA (for a randomised block design) and *post hoc* Dunnett's test were used to assess statistical difference. *Treatment groups with an asterisk differ significantly from the control group ($P<0.05$).

Table 3.3 Comet classes in (A) freshly prepared and (B) cryopreserved colonocytes isolated from pigs fed one of the following diets: cereal diet unsupplemented (control), or cereal diet supplemented with 600 g/d of raw broccoli or raw carrot for 12 d.

A

Treatment	**Comet Classes**				
	0	**1**	**2**	**3**	**4**
Raw broccoli	7 (3.74)*	13.2 (5.02)	13.7 (3.57)	23.7 (8.50)*	42.4 (8.73)*
Raw carrot	20.5 (11.02)*	18.2 (3.36)	12.3 (3.52)	15.1 (6.75)	33.9 (10.50)
Control	32.7 (7.90)	18.3 (2.42)	10.7 (1.96)	10.2 (3.73)	28.1 (6.14)

B

Treatment	**Comet Classes**				
	0	**1**	**2**	**3**	**4**
Raw broccoli	10.2 (7.10)*	12.1 (3.80)	10.2 (2.47)	16.5 (4.07)	51.0 (9.31)*
Raw carrot	21.5 (6.08)*	15 (1.56)	10.0 (1.14)	15.4 (2.47)	38.2 (8.39)
Control	33.2 (5.52)	15.1 (1.50)	9.6 (2.20)	13.6 (3.21)	28.5 (3.59)

In tables A and B, results are expressed as the number of colonocytes in each class of damage and are the mean (SD) of 3 slides (each containing 2 gels) for each animal and 5 animals in each treatment group. Two way ANOVA (for a randomised block design) and *post hoc* Dunnett's test were used to assess statistical difference. Within a column an asterisk indicates a significant difference between a treatment group and the control group ($P<0.05$).

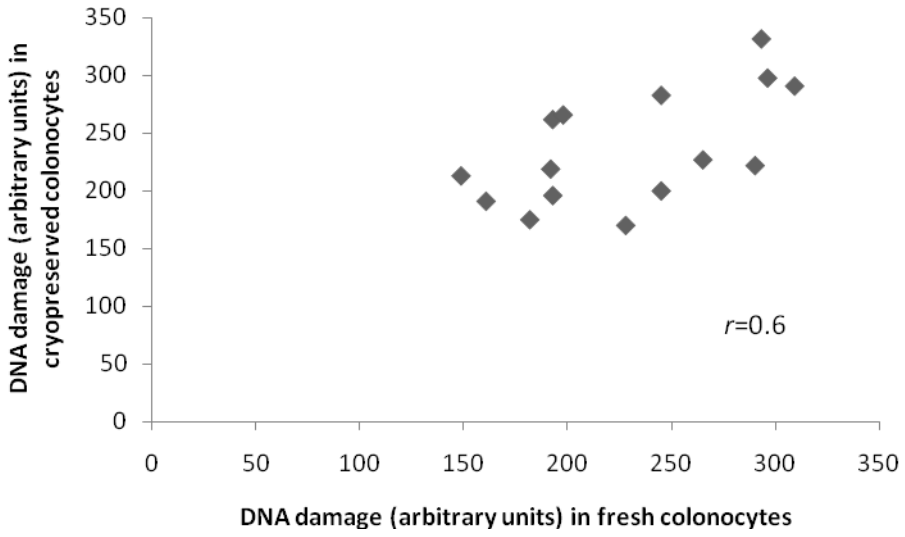


Fig 3.2 The correlation between DNA damage measured in freshly prepared colonocytes and cryopreserved colonocytes. Cryopreserved colonocytes were stored at -196^{o}C prior to analysis. Colonocytes were isolated from pigs fed an unsupplemented cereal diet (control) or 600 g/d of raw broccoli or carrots for 12 d.

3.4 Discussion

In the experiment reported in Chapter 2, the consumption of raw broccoli caused an increase in DNA damage in colonocytes, although earlier work had shown that raw broccoli protected colonocytes against DNA damage (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000). The reason for the disparity is unclear, but it was suggested that different cultivars of broccoli might have different biological effects. To investigate this possibility, the present experiment fed a different cultivar of broccoli. In agreement with the experiment reported in Chapter 2, raw broccoli consumption caused an increase in DNA damage. Furthermore, the magnitude of the effect was larger than that reported in Chapter 2 (cv. Monaco 54% increase over control pigs vs. cv. Marathon 14% increase over control pigs, calculated from data for fresh colonocytes, i.e. colonocytes analysed on the day of isolation). This may indicate that Monaco broccoli contains higher levels of the genotoxic compound(s) than Marathon broccoli. However, in Chapter 2 it was argued that the genotoxic effect of Marathon broccoli might have been

partially masked by the high amount of damage that occurred during the isolation of the colonocytes. Colonocytes isolated from control pigs in the present experiment exhibited much less DNA damage than control pigs in the previous experiment (fresh colonocytes 183 versus 289; cryopreserved colonocytes 189 versus 209). Whilst acknowledging that different groups of pigs may have different basal levels of DNA damage, the large differences suggest that less damage occurred during the isolation process. This may in part explain the greater apparent genotoxicity of Monaco broccoli in the present experiment.

Raw carrot consumption failed to protect colonocytes against DNA damage. It was proposed that the fermentative activity of the microflora would release carotenoids from the vegetable matrix, allowing the released carotenoids to protect colonocytes against DNA damaging free radicals by acting as extra-cellular antioxidants in the lumen of the colon. The lack of protection against DNA damage may indicate that the carotenoids did not become available to quench free radicals generated by the faecal matrix, or that the dose supplied by 600 g/d of raw carrots was ineffective. In a human study, carrot juice (330 ml/d) failed to influence the level of malonaldehyde in the faeces (a measure of lipid peroxidation) despite causing a large increase in the concentration of carotenoids in the faeces (Briviba *et al.* 2004). The authors suggested that carotenoids in the faeces were not solubilised and were therefore unable to quench reactive species at the sites of lipid peroxidation. It is possible that the failure of raw carrot consumption to protect against DNA damage in the present experiment was due to poor solubility of carotenoids in faeces. It is also possible that raw carrot fragments reaching the colon were resistant to breakdown by the microflora, resulting in carotenoids and other bioactive compounds remaining trapped within the vegetable matrix.

Recently, raw carrot consumption was shown to reduce the ability of AOM to induce preneoplastic lesions in rat colon. However, rather than carotenoids, falcarinol (a polyacetylene) appeared to be the compound responsible for the protective effect (Kobaek-Larson *et al.* 2005). It is uncertain how falcarinol protects against chemically induced colon

cancer, but it is probably not through an antioxidant effect (Kobaek-Larson *et al.* 2005; Young *et al.* 2007). So, antioxidant mechanisms may not be responsible for driving the inverse association between carrot consumption and CRC that has been seen in some epidemiological studies (Steinmetz & Potter 1993; Franceshi *et al.* 1997).

Most experimental studies have found that consumption of cruciferous vegetables is associated with protection against colon cancer (see Chapter 1 for a review), so it is worth reflecting whether an increase in DNA damage detected by the comet assay could be interpreted as a chemoprotective effect. The standard comet assay used in the present experiment and the experiment in Chapter 2 is a non-specific marker of DNA damage that detects strand breaks that are present at cell isolation and alkali labile sites that are converted to strand breaks under the alkaline conditions of the assay (Collins *et al.* 1997; Moller, 2006). Both strand breaks and alkali labile sites can be present because of exposure to genotoxins or as transient intermediates during nucleotide and base excision repair processes (Collins *et al.* 1997). Therefore, theoretically an increase in DNA strand breaks could actually represent an increase in DNA repair (Collins *et al.* 1997). However, the level of repair occurring *in vivo* at any one time would be very low if detectable at all and is very unlikely to account for the 50% increased level of DNA damage found in the pigs consuming raw broccoli in this experiment or even the smaller increase in DNA damage found in the experiment reported in Chapter 2 (Personal communication, A Collins). In the present experiment and the experiment reported in Chapter 2, colonocytes isolated from pigs fed the control diet contained substantial amounts of DNA damage. This may indicate that some DNA damage occurred during the cell isolation process. It is possible that the large amount of damage in colonocytes from pigs fed raw broccoli resulted from the colonocytes being more sensitive to damage during the isolation process. The implication of this is unclear. However, it is worth noting that putative dietary anti-carcinogens generally increase the resistance of isolated cells to damage by compounds such as H_2O_2 (Duthie *et al.* 1996; Riso *et al.* 1999; Boyle *et al.* 2000) although it is uncertain

whether such cells would be protected from mechanical damage and other types of stress encountered during isolation.

Some evidence indicates that GLS breakdown products can induce apoptosis (Gamet-Payrastre *et al.* 2000; Bonnesen *et al.* 2001). It is possible that the increase in DNA damage in this experiment reflects cells undergoing apoptosis but it is very unlikely. During apoptosis, fragmentation of DNA typically results in the formation of nucleosome oligomers. During the comet assay such small pieces of DNA would be expected to disappear during the cell lysis or electrophoresis steps (Collins, 2004). So, if the increased strand breaks cannot be explained away as reflecting an increase in repair or an induction of apoptosis then this experiment and the one reported in Chapter 2 suggests that large quantities of raw broccoli damage the DNA in colonocytes.

What is the implication of raw broccoli inducing DNA damage? It has been argued that the strand breaks detected by the comet assay are normally repaired within a few hours, before cells replicate and any mutation becomes fixed (Faust *et al.* 2004). However, it is logical to assume that the greater the amount of DNA damage, the greater the likelihood that some damage may go unrepaired. Also, it is worth noting that known carcinogens, including diet derived heterocyclic amines such as IQ typically produce positive results in the comet assay *in vivo*, whereas anti-carcinogens generally do not (Anderson *et al.*1998; Kassie *et al.* 2002; Park *et al.* 2003).

In the present experiment, fresh and cryopreserved colonocytes exhibited similar levels of total DNA damage. This contrasts with the experiment reported in Chapter 2 where freshly analysed colonocytes had significantly more DNA damage than cryopreserved colonocytes. This may reflect improved colonocyte isolation techniques in the present experiment. The finding of no significant difference in DNA damage between fresh and cryopreserved

colonocytes is consistent with the study of Duthie *et al.* (2002) who reported no effect of cryopreservation on DNA damage in lymphocytes.

Despite being relatively rich sources of carotenoids and tocopherols (FSA, 2002), neither vegetable supplement increased the plasma concentration of retinol or α and γ-tocopherol. This can probably be explained by the high level of fortification of the pig's basal diet with retinol and α-tocopherol (see Chapter 2 for further discussion) and possibly the low bioavailability of β-carotene from raw vegetables. Pigs consuming diets supplemented with raw broccoli exhibited a modest non-significant increase in plasma ascorbic acid. The lack of significance may partly reflect a lack of statistical power to detect a modest increase due to the small numbers of animals in each treatment group. An exploratory analysis of all 6 cohorts of pigs including the cohort removed from all analyses because they developed diarrhoea revealed a significantly higher plasma ascorbic acid concentration in the raw broccoli group than in the control group (data not shown).

In conclusion, the feeding of 600 g/d of raw broccoli (cv. Monaco) led to a significant increase in DNA damage in colonocytes, whereas a similar quantity of raw carrots (cv. Nairobi) had no significant effect. The finding of an increase in DNA damage was consistent with the experiment reported in Chapter 2 and raises doubts as to whether the consumption of large quantities of raw broccoli is advisable. The standard comet assay used in this experiment provides no information as to the mechanism by which raw broccoli exerted its genotoxic effect. Further investigation into the effect of raw broccoli consumption on colonocyte DNA damage is warranted to clarify potential mechanisms of action.

Chapter 4

Effect of broccoli and carrot supplemented diets on hepatic and colonic phase II enzymes

4.1 Introduction

The induction of phase II enzymes may be an important mechanism through which cruciferous vegetables protect against chemically induced colonic cancer (Talalay & Fahey 2001; also see review in Chapter 1). In rodents, diets high in freeze-dried raw broccoli (20-25% w/w) have been shown to induce phase II enzymes in a variety of tissues (Aspry & Bjeldanes 1983; Keck *et al.* 2003; Hwang & Jeffery 2004). This has been attributed to broccoli being a rich source of the aliphatic GLS, glucoraphanin, which on hydrolysis can form sulforaphane, a potent inducer of phase II enzymes (Zhang *et al.* 1992; Keck *et al.* 2003). However, the glucoraphanin content of different cultivars of broccoli is known to vary considerably (Kushad *et al.* 1999). Therefore, different cultivars of broccoli may differ in their ability to induce phase II enzymes.

The specific product(s) formed from the breakdown of GLS depends on a number of factors, including the presence of active plant myrosinase and a cofactor protein, ESP (Bones & Rossiter 1996; Matuesheki *et al.*2004). In the UK, a substantial quantity of vegetables is sold frozen (Mintel, 2007). The commercial preparation of frozen broccoli involves a blanching step that probably inactivates ESP and myrosinase (Matusheski *et al.* 2004; Grandison, 2006; Sarikamis *et al.* 2006; also see Chapter 2). In the absence of active plant myrosinase, the GLS in frozen broccoli may be hydrolysed in the colon by the resident microflora. The identity of the compounds formed *in vivo* from the microbial hydrolysis of GLS has not been completely resolved but appears to include nitriles, amines, small amounts of ITC and probably other, yet to be identified metabolites (Shapiro *et al.* 1998; Getahun & Chung 1999; Combourieu *et al.*

2001; Cheng *et al.* 2004). Except for ITC, it is uncertain whether these compounds induce phase II enzymes.

In a recent rodent study, carrot consumption reduced the ability of AOM to induce preneoplastic lesions in the colon (Kobaek-Larson *et al.* 2005). The mechanisms through which carrots exert chemoprotection remain relatively unexplored, but may include the induction of phase II enzymes. No studies appear to have investigated whether carrot consumption can induce phase II enzymes in the colon, but there is some evidence of a modest capacity to induce these enzymes in the liver (Bradfield *et al.* 1985; Anilakumar *et al.* 2000).

Most studies in rodents that have investigated the ability of vegetables to induce phase II enzymes have incorporated vegetables into experimental diets at levels far in excess of what might be realistically consumed by humans (see for example Keck *et al.* 2003). Also, vegetables were fed as freeze-dried powders, whereas humans eat whole vegetables. This experiment aimed to explore whether proportionally lower intakes of whole broccoli and carrots induce phase II enzymes in the liver and colon of pigs.

It was hypothesised that:

- raw broccoli would induce phase II enzymes, but the potency of different cultivars of broccoli would differ
- blanch-freezing would alter the ability of broccoli to induce phase II enzymes
- raw carrot would induce phase II enzymes but it would be less potent than raw broccoli.

The objectives of this experiment were to:

- prepare cytosolic samples from frozen porcine hepatic and colonic tissue and analyse them for glutathione S-transferase (GST) and quinone reductase (QR) activity
- compare the effects of two different cultivars of raw broccoli (cv. Marathon and cv. Monaco) on GST and QR activity, and to correlate any effect with differences in their GLS content
- compare the effect of raw broccoli and broccoli that had been prepared to replicate commercially frozen broccoli on GST and QR activity.
- investigate whether raw carrots induce GST and QR activity.

4.2 Materials and Methods

4.2.1 ***Chemicals***

All chemicals were purchased from Sigma Aldrich Company Ltd, (St Louis, USA), Fisher Scientific Supplies (Loughborough, UK) or BDH Laboratory supplies (Poole, UK), except for glucoraphanin which was obtained from The Royal Veterinary and Agricultural University (KVL), Frederiksberg, Denmark.

4.2.2 ***Animals and Diets***

This experiment used tissue collected from the Landrace x Large White pigs described in the materials and methods section of Chapters 2 and 3. Very briefly, pigs were fed either a cereal diet with no vegetables (control) or: experiment (1) a cereal diet supplemented with 600 g/d of raw broccoli (cv. Marathon) or blanched-frozen broccoli (cv. Marathon); experiment (2) a cereal diet supplemented with 600 g/d of raw broccoli (cv. Monaco) or raw carrot (cv. Nairobi) for 12 d and then killed on day 13.

4.2.3 ***Collection of liver samples***

The entire liver was removed from each pig and excess connective tissue was removed. The liver was then chopped into smaller pieces, placed in labelled plastic freezer bags, rapidly frozen in liquid N_2 and stored at -80^{o}C until use. Sections of the lower left lobe were used for preparation of cytosolic fractions for GST and QR analysis.

4.2.4 ***Collection of colon tissue***

The entire colon from the ileocaecal junction to the rectum was removed. A section of colon from the mid point moving proximal was excised and its contents were gently removed. The section of colon was then further divided into smaller pieces weighing approximately 10 g. Each piece of colon was flushed with distilled water to remove any remaining faeces, placed in a labelled freezer bag, rapidly frozen in liquid N_2 and stored at -80^{o}C until use.

4.2.5 ***Analytical Methods***

4.2.5.1 Preparation of hepatic cytosols

Hepatic cytosolic fractions were prepared as described by Nijhoff & Peters (1992), with minor modifications. Sections of liver (approximately 6 g) in plastic bags were defrosted under cold running water. All subsequent steps were conducted at 0-4^{o}C or on ice. Defrosted liver segments were rinsed with ice cold homogenising buffer (0.25 M sucrose, 20 mM Tris, 1 mM dithiothreitol (DTT), adjusted to pH 7.4 with 12 M HCl) blotted dry, minced with scissors and rapidly transferred to homogenising buffer (4 ml/g of tissue). Liver segments were homogenised with one 45-60 sec burst of a rotor stator homogeniser (8000 rpm; Status X620, CAT, Germany). Homogenates were centrifuged at 10,000 **g** x 20 min (ALC PK121R, Cologno Monzese, Italy). The resulting supernatants were removed and centrifuged at 100,000 **g** x 60 min (Beckman Coulter LE-70 ultracentrifuge, Fullerton, USA). The fat layer

was aspirated and the supernatants (cytosolic fractions) were divided into 0.5 ml aliquots, snap frozen in liquid nitrogen and stored at -80^{o}C until use.

Liver samples from experiment 2 were prepared in the same way except they were homogenised with a different homogeniser (Janke & Kunkel, Ultra-turrax T25, Germany).

4.2.5.2 Preparation of colonic cytosols for glutathione S-transferase analysis

Colonic cytosolic fractions were prepared as described by Nijhoff & Peters (1992), with minor modifications. For each pig a section of tissue from the same location within the mid colon was defrosted under cold running water. All subsequent steps were conducted at 4^{o}C or on ice. Colons were cut longitudinally and their lumens rinsed with homogenising buffer (0.25 M sucrose, 20 mM Tris, 1 mM DTT, adjusted to pH 7.4 with 12 M HCl). Mucosae were removed by gentle scraping of the exposed luminal wall with a glass microscope slide. Scrapings of mucosal cells were homogenised in ice-cold homogenising buffer (4 ml/g of tissue) by 10-12 strokes of a motor driven (Status R100C, CAT, Germany) Potter-Elvehjem Teflon/glass homogeniser (1000 rpm). Homogenates were centrifuged at 10, 000 **g** x 20 min (ALC PK121R, Cologno Monzese, Italy). The resulting supernatants were diluted 1:4 with PBS to provide a volume sufficient for ultra-centrifugation in the available rotor (Beckman Coulter, Type 70Ti) and centrifuged at 100, 000 **g** x 60 min (Beckman Coulter LE-70 ultracentrifuge, Fullerton, USA). The supernatants (cytosolic fractions) were then divided into 0.5 ml aliquots rapidly snap frozen in liquid N_2 (BOC) and stored at -80^{o}C until use.

4.2.5.3 Preparation of colonic cytosols for quinone reductase analysis

Colonic cytosolic fractions for quinone reductase determination were prepared in the same way as those for GST analysis except for the following modifications. Colons were allowed to thaw in KCl-potassium phosphate buffer (0.15 M KCl, 0.25 M KH_2PO_4/K_2HPO_4, pH 7.25) at 4^{o}C, lumens were rinsed with 1.15% KCl and scrapings of mucosal cells were homogenised in

KCl-potassium phosphate buffer (4 ml/g of tissue). Post mitochondrial supernatants were diluted 1:3 with PBS before ultra-centrifugation.

4.2.5.4 Protein determination of hepatic and colonic cytosols

Protein was determined by the colorimetric method of Lowry *et al.* (1951) as modified by Ohnishi & Barr (1978). The assay is a two-step procedure that uses two reagents, a diluted Biuret solution and the Folin-Ciocalteu reagent (Table 4.1, reagent B and C). In the first step, $Cu2^{+}$ ions in the Biuret solution, bind to peptide bonds producing a light blue copper-protein complex. In the second step, the copper-protein complex reduces the phosphotungstic and phosphomolybdic acids contained in the Folin-Ciocalteau phenol reagent resulting in the development of a strong blue colour. Tyrosine and tryptophan residues in the protein also contribute to the reduction (Holme & Peck 1998).

Table 4.1 Composition of protein reagents

Reagent A	Reagent B	Reagent C
In a final volume of 1 litre: 1.5 g $CuSO_4 \cdot 5H_20$ 6 g $KNaC_4H_4O_6 \cdot 4H_20$ 30 g NaOH	Reagent A diluted 1 vol to 7 vol with 2.3% Na_2CO_3	Undiluted Folin- Ciocalteau solution

Protein standards were prepared over the range 50-400 μg/ml from bovine serum albumin (BSA) diluted in PBS. Then 1 ml of cytosol, (diluted in PBS, 200 x dilution for liver, 40 x dilution for colon), standard or blank (PBS) was added to each test tube. Following this, 4 ml of reagent B was added to each test tube, which was immediately mixed on a vortex mixer for 5 sec. After 10 min, 0.125 ml of undiluted Folin-Ciocalteau solution was added to each tube and it was immediately mixed on a vortex mixer for 5 sec. After 30 min, the contents were mixed again and partially transferred to a semi-micro cuvette (BDH, Poole Dorset, UK). Absorbance was measured and recorded at 750 nm and 25^{o}C (Unicam 8700 series UV/VIS

spectrophotometer, Cambridge, UK). Protein standards were measured in duplicate, and cytosol samples were measured in triplicate.

The determination of protein content in colonic samples prepared for GST analysis needed a minor modification to account for the contribution of the homogenising buffer (0.25 M sucrose, 20 mM Tris, 1 mM DTT, pH 7.4) to final colour development. This was achieved by preparing a blank containing the homogenising buffer (diluted in PBS to the same concentration as the colonic samples, a 40 x dilution) and analysing it in triplicate alongside colonic samples. The mean absorbance value of the homogenising buffer blank was then subtracted from the mean sample values. The effect of the homogenising buffer was below the limit of detection in the more dilute liver samples (200 x dilution in PBS).

The Lowry reaction is inherently non-linear (Ohnishi & Barr 1978; Peterson, 1979). The protein standards were plotted on a log-log scale to produce a linear standard curve (r^2>0.995; see Appendix, Fig A.2, pg 153). Samples were log transformed and protein concentration was determined by linear regression (see Appendix A.2.1, pg 153). Calculated values were then anti-log transformed and the resulting value was multiplied by the appropriate dilution factor to give the protein concentration of the cytosolic fraction. The mean intra-assay coefficient of variation (cv) and inter-assay cv were <4% and <6%.

4.2.5.5 Validation of protein assay

To check the validity of calculating protein content using a log transformed standard curve, a series of samples was analysed in triplicate at three different concentrations and values were compared; the calculated values differed by less than 2%.

4.2.5.6 Glutathione S-transferase determination

GST was determined spectrophotometrically at 25^{o}C with 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate according to the method of Habig *et al.* (1974) with minor modifications. The assay measures the change in absorbance of CDNB when it conjugates with glutathione, to form *S*-2, 4-dinitrophenylglutathione. The reaction was initiated by the addition of 0.1 ml of cytosol to a UV grade Kartell brand semi-micro cuvette (Fisher Scientific Supplies, Loughborough, UK) containing 0.1 ml glutathione (final concentration 5 mM) and 0.8 ml reaction mixture (final concentration 0.1 M KH_2PO_4/K_2HPO_4 pH 6.5, and 1 mM CDNB, 2% ethanol). The glutathione and reaction mixtures were incubated separately at 25^{o}C prior to use. Cytosolic fractions were diluted in PBS to ensure assay linearity (a 100 x dilution for liver and 5 x dilution for colon). The linear increase in absorbance at 340 nm was recorded for 3 min (Unicam 8700 series UV/VIS spectrophotometer, Cambridge, UK) and GST activity was calculated using the molar extinction coefficient of 9.6 mM/cm and expressed as nmol of product formed/min/mg cytosolic protein. GST activity was corrected by the subtraction of an appropriate blank (see Appendix A2.2, pg 154). The mean within day assay cv was < 3% and between day cv was < 6%.

4.2.5.7 Quinone reductase determination

Quinone reductase activity was determined by the spectrophotometric method of Ernster (1967), as modified by Benson *et al.* (1980), using 2, 6-dichlorophenolindophenol (DPIP) as the electron acceptor and NADPH as the electron donor. The assay measures the reduction of DPIP in the presence and absence of dicumoral, an inhibitor of quinone reductase. On reduction, DPIP decolourises, and the rate of the reaction can be measured as a linear decrease in absorption. The portion of the reaction that is inhibited by dicumoral is a reasonably selective measure of quinone reductase activity (Lind *et al.* 1990).

The reaction was initiated by the addition of 10 µl of 12 mM DPIP to a Fisher brand semi-macro cuvette (Fisher Scientific Supplies, Loughborough, UK) containing 0.3 ml of cytosol (100 x dilution for liver, 20 x dilution for colon) and 2.65 ml of the reaction mixture (final concentrations: 25 mM Tris, 0.7 mg BSA, 0.01% Tween 20, 5 µM FAD, 0.2 mM NADPH). The rate of reaction was measured as a linear decrease in absorbance at 600 nm over 90 sec (Unicam 8700 series UV/VIS Spectrophotometer, Cambridge, UK). Activity was measured in triplicate in the presence and absence of dicumarol (10 µM). Quinone reductase activity was calculated from the dicumarol sensitive portion of the assay, using a molar extinction coefficient of 21 mM/cm and expressed as nmol of product formed/min/mg cytosolic protein (see Appendix A.2.3, pg 154). The mean within day assay cv was < 3% and mean between day assay cv was < 7%.

4.2.5.8 Analysis of the GLS content of broccoli

Whole broccoli heads collected during experiment 2 (see Chapter 3 for details) were freeze-dried prior to GLS analysis. From each broccoli head, a representative 50 g was sampled and placed in liquid N_2 (BOC). Samples were then freeze-dried for 5-7 d. Freeze-dried samples were ground to a fine powder by 10-12 short bursts of a mini-grinder (Revel model CCM101CH, UK). The resulting powder was then stored in airtight containers, in a box with desiccant at –20^{o}C prior to extraction.

4.2.5.9 Extraction of GLS

GLS were extracted from the freeze-dried broccoli and analysed using a method adapted from Minchinton *et al.* (1982) and the European Community (1990) as described by Rungapamestry *et al.* (2006 & 2008). Freeze-dried broccoli (1 g) or glucoraphanin standard in a freeze-dried watercress matrix (1 g) were added to a heat resistant test tube containing 15 ml of boiling methanol and 200 µl of an internal standard of benzyl GLS (12.5 mM). The blank consisted of methanol and internal standard. The tube was capped, mixed and incubated in a

water bath for 15 min at 80°C. The tube was then cooled for 10 min at room temperature and centrifuged at 2500 **g** x 5 min at 22°C. The supernatant was removed and stored at room temperature. The remaining pellet was resuspended in 10 ml of 70% methanol and incubated for 15 min at 80°C, allowed to cool at room temperature (10 min) and then centrifuged at 2500 **g** x 5 min at 22°C. The resulting supernatant was pooled with the first supernatant. The pooled supernatants were transferred to a round bottom flask and the methanol was removed by rotary evaporation (5-10 min at 60°C). The resulting aqueous residue was transferred to a volumetric flask and made up to a volume of 10 ml by the addition of distilled water. Samples were then transferred to scintillation vials for on-column desulfation

4.2.5.10 Desulfation

Prepared columns of DEAE-Sephadex A-25 were prewashed with 0.5 M pyridine acetate buffer. The GLS extracts (4 ml) were then added through the columns. The columns were washed with 0.02 M pyridine acetate buffer and then a 0.2% aryl sulfatase solution was added. The columns were allowed to stand overnight (12 h) at room temperature and desulfoglucosinolates were eluted with 2 ml of distilled H_2O and collected into HPLC vials.

4.2.5.11 Analysis of desulfoglucosinolates by reverse-phase HPLC

Each extract of desulfoglucosinolates was separated according to their hydrophobic nature by reverse-phase HPLC. The equipment used consisted of an Agilent 1110 Series HPLC system (Waldbronn, Germany) with a LiCrosphere RP-18 encapped column, 250 mm x 4 mm and particle size 5 μm (Merck, Darmstadt) and a diode array detector set at 228 nm. The mobile phase (water and acetonitrile) was pumped through the column at a flow rate of 1 ml/min starting with 100% water rising to 20% acetonitrile at 46 min for 8 min and descending to 100% water at 58 min for a further 7 min (Rungapamestry *et al.* 2006 & 2008).

Individual GLS were quantified using ChemStation Rev. A.10.02 and identified by comparing their retention time with experimentally determined published response factors (see Appendix A.2.4, pg 155 for a sample calculation; EU, 1997). A standard curve of glucoraphanin prepared in a freeze-dried watercress matrix (0-20 μmol/g of dry matter, r^2=0.9905) was constructed to check the linearity of the assay over the analytical range. The cv for extraction and analysis of total GLS from broccoli was 5.2% (*n*=6) (Rungapamestry *et al.* 2008).

4.2.6 ***Statistical Analysis***

Data are presented as means (SD). The significance of differences between treatment means was tested by two way ANOVA for a randomised experimental block design. Standardised residuals for each outcome variable were normally distributed (Shapiro-Wilk *W* test).

All statistical analyses were conducted on SPSS Release 17.0 (2008) and a *P* value of <0.05 was used as the critical level of significance.

4.3 Results

4.3.1 ***Experiment 1***

The raw and blanched-frozen broccoli (cv. Marathon) diets failed to significantly increase hepatic GST (pooled mean 2167 (SD 438.4) nmol/min/mg of protein) and QR (pooled mean 160 (SD 72.2) nmol/min/mg of protein) or colonic GST (pooled mean 131 (SD 21.4) nmol/min/mg of protein) and QR (pooled mean 124 (SD 28.8) nmol/min/mg of protein) activities (Tables 4.2A & B).

4.3.2 ***Experiment 2***

Raw Monaco broccoli and raw carrot (cv. Nairobi) supplemented diets also had no significant effect on hepatic GST (pooled mean 1808 (SD 628.4) nmol/min/mg of protein) and QR (pooled mean 202 (SD 74.3) nmol/min/mg of protein) or colonic GST (pooled mean 136 (SD 22.9) nmol/min/mg of protein) and QR (pooled mean 207 (SD 58.5) nmol/min/mg of protein) activities (Tables 4.2A & B). Analysis of the GLS content of the raw broccoli (cv. Monaco) revealed that indolyl GLS predominated. Glucobrassicin was present in the greatest concentrations accounting for approximately 57% of the total content of GLS. Other GLS present in substantial amounts were the indolyl GLS, neoglucobrassicin, and 4-methoxyglucobrassicin, and the aliphatic GLS, glucoraphanin accounting for 15%, 10% and 10% respectively of the total GLS content (Table 4.3).

Table 4.2 Glutathione S-transferase and quinone reductase activity in cytosolic preparations from the liver (A) and colonic mucosa (B) of groups of pigs fed cereal diets unsupplemented (control) or supplemented with broccoli or carrots for 12 d.

A

Experiment 1	Glutathione S-transferase nmol/min/mg protein	Quinone Reductase nmol/min/mg/protein
Raw Broccoli (cv. Marathon)	2109 (549.2)	160 (89.2)
Blanched-Frozen Broccoli	2178 (546.9)	171 (56.5)
Control	2213 (254.7)	149 (94.3)
Experiment 2		
Raw Broccoli (cv. Monaco)	1915 (541.7)	194 (69.6)
Raw Carrots (cv. Nairobi)	1731 (524.6)	222 (71.4)
Control	1777 (889.6)	191 (93.1)

B

Experiment 1	Glutathione S-transferase nmol/min/mg protein	Quinone reductase nmol/min/mg protein
Raw Broccoli (cv. Marathon)	120 (15.7)	134 (25.9)
Blanched-Frozen Broccoli	138 (23.2)	131 (27.1)
Control	135 (24.5)	107 (30.8)
Experiment 2		
Raw Broccoli (cv. Monaco)	145 (34.8)	189 (96.1)
Raw Carrots (cv. Nairobi)	138 (14.0)	236 (74.5)
Control	126 (14.0)	194 (25.3)

In tables A and B, results are expressed as means (SD). Hepatic and colonic GST activity was determined in triplicate. Hepatic and colonic quinone reductase activity was determined in triplicate in the presence and absence of dicumarol. Two way ANOVA (for a randomised block design) was used to assess significance of differences; n=5 in each diet group in all cases.

Table 4.3 Glucosinolate content of broccoli (cv. Monaco)

Glucosinolate	μmol/g DM
Glucoraphanin	0.99 (1.82)
Glucobrassicin	4.32 (0.52)
Neo-glucobrassicin	1.12 (0.31)
4- methoxy-glucobrassicin	0.76 (0.21)
Other glucosinolates	0.42
Total Glucosinolates	7.61 (2.55)

Values are means (SD), *n*=5; DM=dry matter.

4.4 Discussion

The induction of phase II enzymes has been identified as an important mechanism by which broccoli may confer protection against cancer (Talalay & Fahey 2001). Furthermore, in rodent studies, the degree of induction has been shown to be greater in the colon than liver, leading to the suggestion that broccoli may be especially protective towards colon cancer (Keck *et al.* 2003). However, in this experiment, 600 g/d of raw or blanched-frozen broccoli had no effect on the activity of GST or QR in the liver or colon of pigs. The most likely explanation for this disparity is the proportionally lower level of broccoli fed in this study in comparison to the published rodent studies. Rodent studies reporting an induction of phase II enzymes, fed diets containing 20-25% (w/w) freeze-dried raw broccoli (Aspry & Bjeldanes 1983; Ramsdell & Eaton 1988; Keck *et al.* 2003; Hwang & Jeffery 2004). If the broccoli in the present experiment was freeze-dried before being incorporated into the diet of the pigs, it would equate to an approximately 5% (w/w) broccoli diet. So, the lack of effect may simply indicate that higher intakes of broccoli are required to invoke a significant induction of phase II enzymes. Whilst the level of broccoli fed in the present study was proportionally lower than the rodent studies it was still high in comparison to normal human intakes. An average serving

size of broccoli is 85 g, and estimates of total daily intakes of cruciferous vegetables in human populations range from less than 20 g/d in some Southern European populations to 100 g/d in Chinese populations (MAFF 1994; Chiu *et al.* 2003; IARC, 2004). The pigs used in the present experiment weighed approximately 50 to 60% of an average human adult. Scaled up to a 70 kg human male, the level of intake in the present study would equate to over 1 kg of broccoli per day, far in excess of the habitual intake of human populations.

There is some support in the literature for the argument that only very high intakes of broccoli significantly induce GST enzymes. In a study that found an induction of hepatic GST activity in rodents fed a 25% (w/w) broccoli diet, a 10% (w/w) broccoli diet failed to have any significant effect (Aspry & Bjeldanes 1983). Furthermore, in a human study, the consumption of 300 g/d of steamed broccoli for 5 d had no effect on the content of GST-α in plasma or GST activity in plasma or saliva (Steinkellner *et al.* 2000). Also, in a more recent human study, the consumption of a standard broccoli soup or a high GLS broccoli soup (both prepared to maximise sulforaphane production) failed to upregulate the expression of GST genes in the gastric mucosa, 6 hours post consumption. The high GLS soup did however, upregulate a small number of other XME genes (Gasper *et al.* 2007).

Other factors that may have contributed to the present experiment failing to detect an induction of phase II enzymes include the form in which the broccoli was fed, the cultivar fed, tissue storage before analysis, the method used to kill the pigs, the composition of the basal diet, and species differences in the inducibility of phase II enzymes. Raw and blanched-frozen may not be the optimal state in which to consume broccoli to induce phase II enzymes. The induction of phase II enzymes by broccoli has been largely attributed to the effects of sulforaphane, the breakdown product of the aliphatic GLS, glucoraphanin (Zhang *et al.* 1992). Recent evidence indicates that when raw broccoli is homogenised in water, the main breakdown product formed is sulforaphane nitrile rather than sulforaphane (Mithen *et al.* 2003; Matusheski *et al.* 2004). While sulforaphane is a potent inducer of phase II enzymes, it

appears that sulforaphane nitrile is not (Matusheski & Jeffery 2001; Basten *et al.* 2002). The preferential formation of sulforaphane nitrile rather than sulforaphane may have contributed to the lack of effect of the raw broccoli on phase II enzyme induction. However, there is some evidence to indicate that the release of sulforaphane from raw broccoli may be higher *in vivo* than *in vitro*. Keck *et al.* (2003) measured the excretion of sulforaphane mercapturic acid (SF-MA) in the urine of rats fed raw broccoli containing pre-hydrolysed glucoraphanin and raw broccoli containing intact glucoraphanin, and found a five times greater excretion of SF-MA in the rats fed raw broccoli containing intact glucoraphanin. This appears to indicate that the formation of sulforaphane from raw broccoli is greater *in vivo* than when raw broccoli is homogenised *in vitro*. Furthermore, they found that on a mole for mole basis, intact raw broccoli was a more potent inducer of phase II enzymes than purified sulforaphane (Keck *et al.* 2003). So far, no single *in vivo* study appears to have measured the release of sulforaphane from raw broccoli and broccoli prepared in a manner that would, based on data from homogenising broccoli, be expected to maximise sulforaphane production. However, one study that fed human volunteers meals containing broccoli, which had been lightly cooked to inactivate ESP, but retain myrosinase activity (mode of preparation that maximises sulforaphane production *in vitro*) reported a similar proportion of glucoraphanin excretion as SF-MA as another study that fed raw broccoli (20 vs. 24.9%, Conaway *et al.* 2000; Rungapamestry *et al.* 2007). So it is currently unclear whether broccoli prepared to optimise the conversion of glucoraphanin to sulforaphane *in vitro*, actually leads to any greater conversion of glucoraphanin to sulforaphane *in vivo*. It is also worth noting that even if it does, humans do not typically prepare broccoli in such a manner.

As discussed in Chapter 2, the blanching step used in the preparation of the frozen broccoli almost completely inactivated myrosinase. After the consumption of broccoli containing inactivated myrosinase, it is commonly assumed that most GLS reach the colon intact (Johnson, 2002). However, there is some evidence that limited degradation of GLS may occur in the stomach and small intestine, and that small quantities of GLS may be absorbed intact

from the proximal GI tract (Maskell & Smithard 1994; Michaelson *et al.* 1994). In the colon, the endogenous microflora may hydrolyse GLS, resulting in the generation of some ITC (Shapiro *et al.* 1998; Getahun & Chung 1999). This would be expected to result in a localised exposure of the colonic epithelium to ITC, and might be expected to result in the induction of phase II enzymes in the colon. However, a variety of *in vitro* studies have found that amines or nitriles are the major compounds formed from the microbial hydrolysis of GLS, with ITC only constituting a small proportion of the products (Combourieu *et al.* 2001; Krul *et al.* 2002). Although these studies *in vitro* may not necessarily model what occurs *in vivo,* they suggest that the colonic epithelium of the pigs fed the blanched-frozen broccoli may have been exposed to only small quantities of ITC.

The two cultivars of broccoli fed in the present experiment were supplied by the local supermarket and therefore were representative of the type of broccoli consumed by the general public, at least in North East Scotland. In contrast, studies showing an induction of phase II enzymes in the colon of rats used a cultivar of broccoli (cv. Majestic) that was selected because of its high content of glucoraphanin (7.03 μmol/g DM v. 0.99 μmol/g for the Monaco broccoli in the current work; Keck *et al.* 2003; Hwang & Jeffery 2004). Furthermore, the broccoli was grown on an experimental plot and freeze-dried immediately after harvest. In contrast, a number of days may pass between harvest and delivery of commercial broccoli to the supermarket, during which time substantial losses of GLS may occur (Vallejo *et al.* 2003). An objective of this study was to analyse the two cultivars of broccoli for their content of GLS and to relate any differences to their ability to induce phase II enzymes. Previously, differences in GLS content have been shown to correlate with the ability of different cultivars to induce phase 1 enzymes in rodents (Vang *et al.* 2001). Unfortunately, a freezer breakdown meant that the Marathon cultivar was lost before a successful analysis could be completed. Analysis of the Monaco cultivar revealed a low total content of GLS in comparison to a published mean value based on the analysis of 50 cultivars of broccoli (7.61 v 12.8 μmol/g/DM Kushad *et al.* 1999). It is possible that this may have been the result of the

broccoli being stored for an extended period prior to analysis, or may simply indicate that Monaco broccoli available in supermarkets in North East Scotland is low in GLS. Also, rather than glucoraphanin being the predominant GLS, the indole GLS, glucobrassicin was present in the greatest concentration. Reports in the literature also indicate that glucobrassicin is typically the major GLS present in Marathon broccoli (Vallejo *et al.* 2002 & 2003). On hydrolysis, glucobrassicin forms I3C, DIM or ascorbigen (McDanell *et al.* 1988). While these compounds have some ability to induce phase II enzymes, they appear to be more potent inducers of phase I enzymes (Shertzer & Sainsbury 1991; Bonnesen *et al.* 1999). Therefore, the cultivars of broccoli fed in the present experiment may not have had the optimal GLS profile to induce phase II enzymes.

Freezing and long-term storage of body tissues may result in the loss of activity of some XME (Reinke & Stohs 1976). In the present experiment, liver and colon tissue was frozen and stored at -80^{o}C for 4 to 18 months before cytosolic fractions were prepared for the analysis of phase II enzyme activities. Samples of tissue were frozen because it was not logistically possible to prepare cytosolic fractions on the days that the pigs were killed. The time lapse between the tissues being frozen and their subsequent analysis for phase II enzyme activities reflects the time needed to develop/establish the various enzyme analysis techniques. If freezing and storage leads to a loss of QR and GST activity in the liver and colon this may have contributed to the lack of affect of the broccoli diets in the present experiment. There appears to be no studies that have investigated the effect of freezing and storage on QR activity, so a negative effect cannot be ruled out. However, GST activity in rat liver and kidney does not appear to be significantly affected when tissue is frozen in liquid N_2 and stored for 1 month at -70^{o}C (Jung *et al.* 1993). Also, GST activity is stable in cytosolic fractions stored at -20^{o}C and -70^{o}C for up to 2 years (Peters *et al.* 1997). No studies appear to have investigated the effect of freezing on the stability of phase II enzymes in colonic tissue. However, a number of researchers have detected dietary induction of GST or QR activity in cytosolic fractions prepared from frozen colonic tissue (Nijhoff *et al.*1993; van Lieshout *et al.*

1996; Keck *et al.* 2003). So, it appears unlikely that freezing and storing liver and colon tissue before the preparation of cytosolic fractions results in a substantial loss of GST or QR activity.

Another possible confounding factor in the present experiment was the method of killing. The pigs were sedated and anaesthetised with Zoletil® and Somulose® before being killed by exsanguination. Ideally drugs should not be administered to animals when the induction of XME is being investigated, because they may induce or inhibit the enzymes that are being measured (Gibson & Skett 1986). Zoletil® and Somulose® were used in previous work conducted before this project to investigate the effect of broccoli on DNA damage (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000). Because other aspects of this project (see Chapters 2 & 3) were a continuation of that work, the killing procedure had to be kept the same. In the present experiment, Zoletil® was administered intramuscularly approximately thirty minutes before death and Somulose® was then administered intravenously immediately prior to death, by exsanguination. No studies appear to have investigated whether either drug induces or inhibits GST or QR, but if they do, the short time lapse between the first drug administration and the removal and freezing of tissue samples (approx 30-40 min) would have precluded any substantial effect. In studies investigating the administration of large doses of prototypic inducers, maximal induction of XME activity typically takes 16-24 h, with no increase seen in the first few hours (Gozukara *et al.* 1984; personal communication with P. Guengerich). However, to confirm whether the drugs affected GST and QR activity, a second control group of pigs killed without the use of any drugs should have been included in this experiment. In theory literature values for GST and QR activity in pigs could provide a rough indication as to whether the drug administrations caused a large degree of induction or inhibition of GST and QR. However, no literature values for QR activity in pigs exist, and reported values for GST vary over 40,000 fold and thus provide little useful information (Desille *et al.* 1999; Diaz & Squires 2003; Szotakova *et al.* 2004). Of note, other investigators have used the anaesthetic, ketamine/xylazine prior to killing by cervical dislocation and still observed large inductions of QR activity in the colon and liver of rats fed broccoli (Hwang & Jeffery 2004).

It has been argued that a non-enzyme inducing control diet should be fed when investigating the effects of inducing compounds on the XME system (McDanell & McLean 1984). In one study, a standard chow diet was shown to have similar potency as a 25% (w/w) broccoli diet to increase hepatic GST activity (towards CDNB, the surrogate substrate used in the present study) above the level found in rats fed a purified diet (Ramsdell & Eaton 1988). The basal diet fed in the present experiment was a cereal based diet consisting of 43% (w/w) wheat, 25% (w/w) soya, 23% (w/w) barley and small amounts of other ingredients, including supplemental vitamins and minerals (see Chapter 2, pg 52, Table 2.1 for full composition). In rodents, wheat and soya have been shown to have opposing effects on hepatic GST activity (Mirsalis *et al.* 1993; Helsby *et al.* 2000). Mirsalis *et al.* (1993) found that adding soya flakes (8 and 25% w/w) to the diet of rats induced hepatic GST activity; whereas Helsby *et al.* (2003) found that the addition of wheat bran (20% w/w) inhibited hepatic GST activity. The consumption of a basal diet containing substantial quantities of ingredients with contrasting abilities to modulate the activity of XME may have obscured a possible enzyme inducing effect of the vegetable treatments. However, humans consume diets that contain numerous dietary components that may also have contrasting abilities to modulate XME, so it could be argued that if broccoli can only induce phase II enzymes when added to a purified or semi-purified diet, then the induction of phase II enzymes is unlikely to be an important mechanism, whereby, broccoli confers protection against CRC in humans consuming mixed diets.

Mechanistic studies have shown that GLS breakdown products induce phase II enzymes via the antioxidant responsive element (ARE), and possibly in the case of indolyl compounds, via the xenobiotic responsive element (see Chapter 1, pgs 21-22). Studies comparing the regulatory region of GSTα1 have found some differences in both the number and sequence of AREs among different species (Friling *et al.* 1990; Rushmore *et al.* 1993). Such differences could cause species differences in the response of phase II enzymes to the feeding of

cruciferous vegetables. The regulatory regions of porcine QR and GST enzymes do not appear to have been sequenced. However, it is possible that the phase II enzymes of pigs may have lower inducibility than those of rodents. If so, this could have contributed to the lack of effect of the broccoli diets in the present experiment.

Given the lack of effect of the broccoli diets, the failure of raw carrots to induce phase II enzymes is not particularly surprising. In a study comparing the relative ability of extracts of different vegetables to induce QR activity in murine Hepa 1c1c7 cells, carrot was a much poorer inducer than broccoli (Prochaska *et al.* 1992). Also, in rodents, diets containing proportionally 4 times greater amounts of raw carrot than the present study have been shown to have only a very modest and variable ability to induce GST activity in the liver (Bradfield *et al.* 1985; Anilakumar *et al.* 2000).

In summary, 600 g/d of raw carrots and raw or blanched-frozen broccoli failed to induce GST and QR activity in the colon and liver of pigs. A number of confounding factors may have reduced the ability of the present experiment to detect an induction of phase II enzymes. However, the most likely explanations for finding no effect is that the diets supplied too low a level of enzyme inducing compounds, or that the compounds supplied, failed to reach the target tissues in sufficient quantities to exert an effect. As the level of vegetables consumed would equate to an average human intake of approximately 1 kg/d, it seems unlikely that the addition of raw carrot, raw broccoli or blanched-frozen broccoli to the human diet is likely to exert chemoprotection through an induction of GST or QR activity in the liver or colon. Although caution should be taken in extrapolating these findings to humans, it is worth noting that the premise that broccoli protects against cancer through the induction of phase II enzymes is built almost entirely on evidence from *in vitro* studies, and from rodent studies that fed unrealistically high intakes of specific cultivars of broccoli, known to be rich in glucoraphanin.

Chapter 5

Effect of vegetable supplementation on fermentation and the microbial populations in the colon of pigs

5.1 Introduction

The colonic microflora is a complex ecosystem consisting of at least several hundred bacterial species (Goldin *et al.* 1994). These bacterial species exhibit a wide range of xenobiotic metabolising enzyme (XME) activities, many of which appear able to convert dietary derived pro-carcinogens into carcinogens (Rowland, 1996). Certain bacterial species such as *Bacteroides* and *E. coli* have high xenobiotic metabolising activity, whereas others, such as *Lactobacilli* and *Bifidobacteria*, have relatively low activity (Rowland *et al.* 1985; Saito *et al.* 1992). Modifying the composition of the microflora in favour of those species with low xenobiotic metabolising activity may offer some protection against CRC (Rowland, 1996).

The fermentation of non-digestible carbohydrates by the microflora produces short-chain fatty acids (SCFA) (Cummings, 1984). SCFA may modify CRC risk by influencing the proliferation, differentiation and apoptosis of colonic epithelial cells (Augeron & Laboisse 1984; Gamet *et al.* 1992; Hague & Paraskeva 1995). Also, an increased production of SCFA can lower luminal pH (Cummings & MacFarlane 1991). A reduction in luminal pH may protect against colon cancer by decreasing the activity of a number of potentially pro-carcinogenic bacterial XME enzymes, such as 7α-hydroxylase and nitroreductase (Ballongue *et al.* 1997).

The main exogenous substrates for microbial growth are believed to be dietary carbohydrates that resist digestion in the upper gastrointestinal tract (e.g. non-starch polysaccharides, resistant starch and oligosaccharides) and become available for fermentation in the colon

(Cummings *et al.* 1989). These non-digestible carbohydrates may modify the composition of the colonic microflora (Rowland & Tanaka 1993; Canzi *et al.* 1994; Rao *et al.* 1994; Silvi *et al.* 1999). For example, a diet containing the dietary fibre, guar gum (10% w/w), has been shown to increase counts of bifidobacteria in the caecal contents of rats (Canzi *et al.* 1994). Also, in some studies, an increased consumption of various isolated non-digestible carbohydrates has been shown to alter the profile, or increase the concentration of SCFA, or both (McIntyre *et al.* 1991; Younes *et al.* 1997; Bird *et al.* 2007).

An alternative approach to studying the effect of isolated non-digestible carbohydrates is to investigate the effect of consuming foods that are naturally rich in such compounds. Vegetables typically contain substantial quantities of dietary fibre (Anderson & Bridges 1988). Only a few studies appear to have investigated the effect of vegetable consumption on the colonic microflora or SCFA production. Diets supplemented with raw and processed broccoli exerted minor effects on the colonic microflora of pigs, but tended to increase the ratio of lactobacilli to coliforms (Ratcliffe *et al.* 1999; Kemble, 2000; Ratcliffe *et al.* 2000). In another study, freeze-dried Brussels sprouts (incorporated into the diet at a level of 10% w/w) altered the faecal microbial diversity and profile of SCFA in the caecum of human flora associated rats (Humblot *et al.* 2005).

In addition to being a rich source of non-digestible carbohydrates, vegetables typically contain a range of phytoalexins. Many of these, such as the oxygenated terpimoids present in carrots, and certain ITC present in cruciferous vegetables exhibit potent anti-microbial activity *in vitro* (Batt *et al.* 1983; Park *et al.* 2000). Whether such compounds exhibit anti-microbial activity *in vivo* remains largely unexplored, but it is a possibility.

The aims of this experiment were to investigate whether adding broccoli or carrots to the diets of pigs could modify the composition of the colonic microflora, alter SCFA production, or both. It was hypothesised that the vegetable supplements would increase the amount of

fermentable carbohydrate and anti-microbial compounds entering the colon, leading to a change in the composition of the colonic microflora, and an increased production of SCFA.

The objectives of this experiment were to:

- collect digesta samples from the colon of pigs fed different experimental diets
- enumerate the major bacterial groups present in the digesta of pigs fed different experimental diets
- investigate whether any of the experimental diets altered the composition of the colonic microflora
- investigate the effect of the experimental diets on colonic fermentation by measuring the concentration of SCFA present in the colonic contents.

5.2 Materials and Methods

5.2.1 ***Chemicals***

All media and supplements were purchased from Oxoid, Basingstoke, Hampshire, UK, except for: vancomycin, which was purchased from Sigma-Aldrich Chemicals, Poole, Dorset, UK; metaphosphoric acid which was purchased from BDH laboratories, Poole, Dorset, UK; acetic acid, propionic acid, *n*-butyric acid and sulphuric acid which were purchased from Fisher Scientific, Loughborough, UK.

5.2.2 ***Animals and Diets***

The pigs used in this study were those described in experiments 1 and 2. For comprehensive details of the animals, diet, and experimental design see the Materials and Methods sections of Chapters 2 and 3. In both experiments the control groups consumed a cereal diet (600 g/d). In experiment 1, the treatment groups consumed the control diet supplemented with raw (600 g/d) or blanched-frozen (600 g/d) broccoli (cv. Marathon). In experiment 2, the diets of the treatment groups were supplemented with raw broccoli (cv. Monaco; 600 g/d) or raw carrot (cv. Nairobi; 600 g/d). In both experiments the pigs were fed the experimental diets for 12 d and then killed on the morning of day 13.

5.2.3 ***Sample collection***

At post-mortem, two samples of digesta (approx 5 g) from the mid-colon were collected from each pig, one for microbial analysis and one for short-chain fatty acid (SCFA) analysis. After collection, each sample was rapidly transferred to sealable plastic containers and placed inside an anaerobic jar (Oxoid, Basingstoke, UK). A gas generating kit (Oxoid, Basingstoke) was used to create anaerobic conditions inside the jar. Samples were then transported to the lab for immediate processing. Microbial analysis was carried out immediately. Samples for SCFA analysis were diluted 1:1 (w/v) with reverse osmosis purified water and mixed by vortex to homogeneity (determined visually). Samples were then strained through a double layer of muslin. An internal standard (0.2 ml of 1% w/v 2-ethyl-n-butyric acid) and 0.4 ml of 21.5% (w/v) metaphosphoric acid solution (21.5 g MPA, 2.2 ml concentrated sulphuric acid made up to 100 ml with distilled water) were added to 2 ml of each sample. Samples were then mixed by vortex and stored at –80^{o}C until analysis.

5.2.4 ***Bacteriological Counts***

For each animal the following agar were used: MacConkey no. 3, De Man, Rogosa, Sharpe (MRS), Wilkins-Chalgren Anaerobe (WCA) agar with 10% defibrinated horse blood,

Columbia agar with 5% defribrinated sheep blood, and kanamycin-vancomycin agar with 10% defibrinated horse blood. These were prepared as outlined below:

5.2.4.1 MacConkey no.3

MacConkey no.3 (51.5 g) was added to 1 litre of distilled water, autoclaved for 15 minutes at 121^{o}C, allowed to cool to 45^{o}C, and then poured into sterile Petri dishes.

5.2.4.2 DeMan Rogosa and Sharpe (MRS)

MRS (62 g) was added to 1 litre of distilled water, autoclaved for 15 minutes at 121^{o}C, allowed to cool to 45^{o}C, and then poured into sterile Petri dishes.

5.2.4.3 Wilkins-Chalgrin Anaerobe (WCA) agar with 10% defibrinated horse blood

WCA (43 g) was added to 900 ml of distilled water, autoclaved at 121^{o}C for 15 minutes. The agar was left to cool to 48^{o}C in a water bath, then 100 ml of sterile defribinated horse blood was added aseptically, and the resulting solution was mixed by inversion and poured into sterile Petri dishes.

5.2.4.4 Columbia agar with 5% defibrinated sheeps blood

Columbia agar (39 g) was added to 950 ml of water, autoclaved at 121^{o}C for 15 minutes. The agar was allowed to cool to 48^{o}C in a water bath, then 50 ml of sterile defribrinated sheep blood was added, and the resulting solution was mixed by inversion and poured into sterile Petri dishes.

5.2.4.5 Kanamycin-Vancomycin agar with 10% defibrinated horse blood

The kanamycin-vancomycin agar was prepared as outlined by Draser & Roberts (1991). First, 23.5 g of brain-heart infusion agar was added to 450 ml of distilled water and autoclaved at 121^{o}C for 15 minutes. The agar was left to cool to 48^{o}C in a water bath, then 5 ml of

Kanamycin (10 mg/ml), 3.75 ml of vancomycin (1 mg/ml), and 50 ml of defribrinated horse blood was added aseptically, the agar was mixed by inversion and the resulting solution was poured into sterile Petri dishes.

5.2.4.6 *Incubation times and enumeration technique*

Each faecal sample was serially diluted in maximum recovery diluent down to 10^{-8}. Three different dilutions (10^{-6}-10^{-8}) were dropped in duplicate with a 50-dropper Pasteur pipette onto each agar plate, and plates were incubated as described. The media incubated aerobically were: Columbia agar with 5% defibrinated blood (total aerobes, 39^{o}C, for 48 h), MacConkey no. 3 agar: (total coliforms, 39^{o}C for 18-24 h; presumptive *E.coli*, 44^{o}C, for 24 h) and MRS agar: (aerotolerant lactobacilli, 39^{o}C for 48 h). The media incubated anaerobically were: kanomycin-vancomycin agar (Bacteroides, 39^{o}C for 96 h), WCA agar with 10% defribrinated horse blood (total anaerobes, 39^{o}C for 48 h) and MRS agar: (lactobacilli, 39^{o}C, 48 h).

After incubation, the number of colonies of each colony type growing on each media type was counted. The dilution chosen for enumerating the colonies was the lowest dilution showing growth that was countable.

5.2.5 ***SCFA analysis***

Samples of digesta collected as outlined in "*Sample collection"* were allowed to defrost at room temperature. Defrosted samples were centrifuged at 10,000 **g** x 10 min. The supernatant was removed and filtered through a 0.2 micron syringe filter fitted to a HPLC sample vial. Each digesta sample was then analysed by HPLC. The equipment consisted of a Gilson model 203 HPLC pump (Gilson Incorporated, Middleton, USA), Aminex HPX-87H ion exclusion column (7.7 mm x 30 cm; Biorad, Richmond, California, USA), a cation H^+ guard column (Biorad, Richmond, California, USA), and a Shodex refractive index SE-61 detector (Showa Denko, Kawasaki, Japan) set to a wavelength of 210 nm. The standard solution consisted of

acetic acid (5mg/ml), propionic acid (2.5 mg/ml) and n-butyric acid (2.5 mg/ml). The internal standard was 2-ethyl-n-butyric acid. The mobile phase was 5 mM sulphuric acid, which was filtered, degassed, and pumped at a flow rate of 0.5 ml/min through the column heated to 48^{o}C.

The peaks were quantified by height using a Kontron 450 data system (Kontron, Munich, Germany) with reference to the internal standard and standard chromatograms.

5.2.6 ***Statistical Analysis***

Bacterial counts are expressed as $\log_{10}$ transformed counts of colony forming units per gram of colon contents. SCFA concentrations are expressed as mmol/kg of wet weight of digesta. The significance of differences between treatment means was tested by two way ANOVA (for randomised block experiments), when a significant effect was found this was followed by post hoc Dunnett's test. Standardised residuals for each outcome variable were normally distributed (Shapiro Wilk *W* test). In experiment 1, the mean number of *E.coli* in the two broccoli groups, was compared against the control group using a linear contrast. All statistical analyses were conducted on SPSS Release 17.0 (2008) and a *P* value of <0.05 was set as the critical level of significance.

5.3 Results

5.3.1 ***Experiment 1***

Neither broccoli-supplemented diet significantly influenced the total counts of aerobic or anaerobic bacteria (Table 5.1A). An initial ANOVA revealed a significant effect of the diets on presumptive *E. coli* numbers (P=0.049), but a post hoc Dunnett's test produced differences of marginal statistical significance (raw broccoli versus control P=0.060; blanched-frozen broccoli versus control P=0.052). Further analysis, using linear contrasts produced substantive evidence that mean number of *E. coli* in the two groups of pigs fed the broccoli diets differed from the number of *E. coli* in pigs fed the control diet (P=0.017). No other significant changes were found in the composition of the flora, although, there was a trend towards a reduction in total coliforms (P=0.065) and an increase in the lactobacilli to coliform (L:C) ratio (P=0.065; Table 5.1A). The total concentration of SCFA and the ratio of individual SCFA were not significantly altered by either of the broccoli supplemented diets (Table 5.1B).

5.3.2 ***Experiment 2***

The raw broccoli and raw carrot supplemented diets failed to significantly alter the total counts of aerobic or anaerobic bacteria (Table 5.2A). However, both diets caused a significant reduction in total coliform and presumptive *E. coli* numbers and an increase in the L:C ratio; other bacterial groups were not significantly altered (Table 5.2A). The total concentration of SCFA and the ratio of individual SCFA were not significantly altered by either diet (Table 5.2B).

Table 5.1 Viable counts of bacteria (A) and concentrations of SCFA (B) in digesta from the mid colon of pigs fed cereal diets unsupplemented (control) or supplemented with raw broccoli (cv. Marathon) or blanched-frozen broccoli (cv. Marathon) for 12 d.

(A)

	Raw broccoli	Blanched-frozen	Control
Total aerobes	8.20 (0.45)	8.34 (0.19)	8.27 (0.37)
Total anaerobes	9.16 (0.20)	9.48 (0.26)	9.27 (0.22)
Bacteroides spp.	8.12 (0.20)	8.36 (0.59)	7.92 (0.56)
Total lactobacilli	8.49 (0.23)	8.44 (0.22)	8.21 (0.40)
Total coliforms	5.90 (0.80)	6.11 (0.72)	7.06 (0.43)
L:C	1.46 (0.22)	1.40 (0.18)	1.17 (0.06)
Presumptive E. coli	5.79 (0.83)	5.75 (0.98)	7.02 (0.50)
Aerotolerant lactobacilli	7.46 (0.76)	7.44 (0.46)	7.57 (0.51)

(B)

	Raw broccoli	Blanched-frozen broccoli	Control
Acetic acid	19.2 (3.0)	17.2 (3.7)	16.9 (2.5)
Propionic acid	8.4 (1.9)	8.8 (1.4)	7.9 (1.8)
n-butyric acid	4.6 (1.3)	4.3 (0.4)	4.7 (1.4)
Total SCFA	32.1 (5.9)	30.4 (3.9)	29.6 (4.7)

In table (A), data are the means (SD) of $\log_{10}$ colony forming units per gram of mid colon contents; L:C = lactobacilli to coliform ratio. In table (B) data represent the means (SD) for the concentrations of SCFA, expressed as mmol/kg wet weight of digesta. In tables A and B, two way ANOVA (for a randomised block design) was used to assess significance of differences; n=5 in each diet group in all cases.

Table 5.2 Viable counts of bacteria (A) and concentrations of SCFA (B) in digesta from the mid colon of pigs fed cereal diets unsupplemented (control) or supplemented with raw broccoli (cv. Monaco) and raw carrots (cv. Nairobi) for 12 d.

(A)

	Raw broccoli	Raw carrot	Control
Total aerobes	8.01 (0.72)	7.97 (0.56)	8.41 (0.40)
Total anaerobes	9.74 (0.18)	9.65 (0.17)	9.85 (0.17)
Bacteroides spp.	9.10 (0.61)	8.72 (0.51)	9.04 (0.25)
Total lactobacilli	8.80 (0.62)	8.65 (0.41)	8.75 (0.32)
Total coliforms	5.04 (0.67) *	5.38 (0.75) *	7.33 (0.92)
L:C	1.76 (0.21) *	1.63 (0.23) **	1.21 (0.12)
Presumptive E. coli	4.82 (0.58) *	5.33 (0.73) **	6.99 (1.30)
Aerotolerant lactobacilli	7.44 (0.71)	7.36 (0.44)	7.45 (0.36)

(B)

	Raw broccoli	Raw carrot	Control
Acetic acid	51.0 (17.4)	67.0 (21.0)	52.3 (13.4)
Propionic acid	28.7 (14.3)	30.5 (7.6)	30.8 (11.6)
n-butyric acid	10.7 (13.4)	11.8 (14.7)	9.3 (9.1)
Total SCFA	90.3 (42.5)	109.4 (32.0)	92.5 (31.4)

In table (A), data are the means (SD) of $\log_{10}$ colony forming units per gram of mid colon contents; L:C = lactobacilli to coliform ratio; *P<0.001 **P=0.002 versus control. In table (B) data represent the means (SD) for the concentrations of SCFA, expressed as mmol/kg wet weight of digesta. In tables A and B, n=5 in each diet group in all cases. Two way ANOVA (for a randomised block design) was used to assess significance of differences, followed where appropriate by post hoc Dunnett's test.

5.4 Discussion

In the first experiment presented here, adding raw broccoli and blanched-frozen broccoli to the diet, resulted in a trend towards an increase in the ratio of lactobacilli to coliforms; whereas, in the second experiment, raw broccoli and raw carrot supplemented diets both caused a significant increase in the ratio. These results are broadly in agreement with previous work of the group that showed a change in the ratio of lactobacilli to coliforms after the feeding of raw and microwaved broccoli (Kemble, 2000). In the present work, the smaller effect of the broccoli in the first experiment may have resulted from the feeding of a different cultivar, or could simply reflect insensitivity and measurement error inherent in the standard culture techniques used to quantify the microflora. An increase in the ratio of lactobacilli to coliforms is generally regarded as sign of improved colonic health (Muraldihara *et al.* 1977). Also, in animal studies, increases in lactobacilli have been shown to protect against chemically induced colon cancer (Arimochi *et al.* 1997; McIntosh *et al.* 1999). However, the observed increase in the ratio in the present experiments was mediated solely through a decrease in coliform number.

The relevance to CRC risk of the observed suppression in coliforms is uncertain. No studies have conclusively shown that a specific bacterial group increases the risk of CRC. However, coliform bacteria, such as *E.coli* can promote the conversion of proteins to toxic amines and are a possible source of nitroreductase, azoreductase and β-glucuronidase enzymes that appear to be able to activate various procarcinogens (Porter & Kennworthy 1969; Chen *et al.* 1999). So, a suppression of coliforms may reduce the exposure of the colonic epithelium to toxic and carcinogenic compounds. It has been suggested that the colonic microflora is a stable ecosystem that tends to slowly return to its normal composition after a change of diet (Tannock, 2005). Both experiments reported here were of short duration (12 d), and it is possible that the observed changes may have disappeared had the study continued for longer.

The mechanism(s) through which the vegetable supplements suppressed coliform bacteria was not investigated, but there are a number of possibilities. Carrots are a rich source of 6-methoxymellein and several acyclic and monocyclic terpimoids, whereas, broccoli contains GLS, which may form ITCs during processing or after consumption (Holst & Williamson 2004); These compounds all exhibit anti-microbial activity *in vitro* (Batt *et al.* 1983; Kurosaki & Nishi 1983; Amin *et al.* 1988; Delaquis & Sholberg 1997; Kyung & Fleming 1997, Ward *et al.* 1998; Fahey *et al.* 2002). Interestingly, AITC has been shown to suppress the growth of pathogenic bacteria including coliforms, but not beneficial species such as lactic acid producing bacteria (Ward *et al.* 1998). This appears to be consistent with the effect of broccoli in the present study. However, only small quantities of AITC are present in broccoli. It is unknown whether other ITC can exert similar effects, although an anti-bactericidal effect against *Helicobacter pylori* has been demonstrated for sulforaphane, the main ITC present in broccoli (Fahey *et al.* 2002).

The different anti-microbial compounds present in carrot and broccoli may have been responsible for the suppression of coliforms, however, the similarity in effect of both vegetables may indicate a common mechanism. Broccoli and carrots contain substantial amounts of fermentable fibre (Anderson & Bridges 1988). An increased supply of fermentable fibre could suppress microbial growth by decreasing the pH of the luminal contents. The pH of the colonic contents was not measured, but the lack of effect of either diet on SCFA concentrations appears to point against any large decrease in pH, at least in the mid-colon. Another possibility is that the vegetable supplements promoted the growth of a bacterial group that was not measured, and that group competitively suppressed the growth of coliforms.

Because the soluble fibre present in broccoli and carrots has been shown to be highly fermentable *in vitro* it was hypothesised that both types of vegetable supplement would increase SCFA concentrations *in vivo* (McBurney & Thompson 1990). However, neither supplement had a significant effect. No other studies appear to have investigated the effect of

broccoli consumption on colonic SCFA, however, one study has reported on the effect of another cruciferous vegetable, Brussels sprouts (Humblot *et al.* 2005). In agreement with the effect of the broccoli in the present study, Brussels sprouts failed to alter the total concentration of SCFA (Humblot *et al.* 2005). A statistically significant shift in SCFA profile in favour of aceteic acid was observed, but the magnitude of the effect was very small (an 8% increase relative to control group) and the amount of vegetables fed was proportionally greater than in the present study (Humblot *et al.* 2005).

Although no studies appear to have investigated the effect of carrot consumption on SCFA *in vivo*, Nyman and associates (1990 & 1994) have investigated the fermentative breakdown and faecal bulking capacity of blanched carrots in rats. In two separate studies they found large differences in fermentability, with one batch of carrots being relatively resistant to bacterial degradation and one batch being relatively easily fermented (47% and 14% of fibre being recovered in the faeces). They suggested that carrots might differ in fermentability, depending on storage time, harvest time, and growing conditions. They also suggested that differences in the size of the plant cell wall structure might determine fermentability, with larger cell walls being more resistant to bacterial degradation (Nyman *et al.* 1994).

The failure to detect an increase in SCFA in the present study may indicate that the fibre in broccoli and carrots is not easily fermented in the colon of pigs. However, weaknesses in the design of the present study may have masked an effect. SCFA concentrations were only determined in digesta sampled from the mid-colon. The fermentation of many undigested dietary substrates occurs predominantly in the caecum and proximal colon (Topping & Clifton 2001). If the broccoli and carrots were rapidly fermented in the caecum or proximal colon, then the SCFA produced might have been absorbed before the digesta reached the mid colon. A better experimental design would have been to measure SCFA concentrations in digesta collected from regions along the whole of the large intestine.

In general, studies investigating the effect of adding additional or different fermentable substrates to the diet of pigs have fed basal diets based on human foods. These have often included an additional fibre supplement of approximately 60 g/d to ensure laxation, but this is much less than that normally present in standard commercial pig diets (Topping & Clifton 2001). The basal diet fed in the present study was not analysed for its fibre content, but based on published values for the fibre content of its ingredients, it probably provided between 120-150 g of fibre per day (Norvite Feeds, 1995; NRC, 1998). Such a high intake of fibre may have masked a possible modest effect of the vegetable diets on fermentation, because these only supplied an additional 14-16 g of fibre per day (FSA, 2002).

In the two experiments reported here, the total concentrations of SCFA found in the digesta of the pigs were very different, the values in experiment 1 being three fold lower than those measured in experiment 2 and also much lower than published values (Cummings *et al.* 1987). This suggests an analytical problem may have occurred during experiment 1. A possible explanation is that the wrong concentration of internal standard was added to the digesta samples when they were being prepared for analysis by HPLC.

In summary, none of the vegetable diets altered the concentration of SCFA within the mid colon. In the first experiment, there was a trend towards an increase in the ratio of lactobacilli to coliforms after the feeding of raw and blanched-frozen broccoli (cv. Marathon). In the second experiment, there was a significant increase in the ratio of lactobacilli to coliforms in pigs fed supplemental raw carrots (cv. Nairobi) or raw broccoli (cv. Monaco). These changes were driven solely by a decrease in coliform number, rather than an increase in lactobacilli. Suppression of coliform bacteria is generally regarded as beneficial however, the relevance of such a change to protection against CRC is uncertain. It is also unclear how the raw vegetables exerted their effect.

Chapter 6

Effect of broccoli and carrot consumption on phase I enzymes, including an investigation into whether viable microsomes can be prepared from frozen tissue samples

6.1 Introduction

Previously it was shown that 600 g/d of raw broccoli (cv. Marathon and Monaco) and blanched-frozen broccoli (cv. Marathon) failed to induce phase II enzymes in the liver and colon of pigs (see Chapter 4). A number of explanations for this lack of effect were suggested including the level of broccoli fed, and the profile of GLS present in the two cultivars of broccoli. The Monaco cultivar was found to contain low levels of glucoraphanin and relatively high levels of indolyl GLS (see Chapter 4, pg 101). The GLS content of the Marathon cultivar was not determined, however, published data indicate that indolyl GLS typically predominate (Vallejo *et al.* 2002; Vallejo *et al.* 2003). Indolyl GLS and their breakdown products appear to be more potent inducers of phase I enzymes, than of phase II enzymes (Shertzer & Sainsbury 1991; Bonnessen *et al.* 1999). In fact, at low levels of intake, they have been shown to induce the hepatic phase I enzyme, CYP1A1, but have no effect on the hepatic phase II enzyme, GST (Bonnessen *et al.* 1999). Therefore, it is possible that cultivars of broccoli, which are rich in indolyl GLS, but low in glucoraphanin may induce certain phase I enzymes at a level of intake below that required to induce phase II enzymes. This possibility warrants investigation, because the selective induction of CYP1A enzymes without a reciprocal induction of detoxifying phase II enzymes may disturb the activation/deactivation balance leading to the increased activation of many chemicals to their carcinogenic or toxic form (Ioannides *et al.* 1995).

Few studies have investigated the effect of carrot consumption on phase I enzyme activity. In an early study, Bradfield *et al.* (1985) found that a 20% (w/w) carrot diet caused a modest

induction of hepatic ethoxycoumarin *O*-deethylase (ECOD; probably mainly reflective of CYP1A1 activity) in mice. However, no studies appear to have investigated the effect of lower intakes of carrots, in non-rodent animal models.

In animal experiments it is often logistically difficult to prepare microsomes on the day of tissue removal. Therefore, tissues are often frozen and stored for extended periods before microsomes are prepared. It is unclear, however, whether microsomal enzymes are stable during the freezing and storage of tissue. Some researchers have reported that CYP450 enzymes are particularly susceptible to degradation if liver tissue is frozen before microsomes are prepared, whereas, others have reported no major effect (Reinke & Stohs 1976; Tredger & Chabra 1976; Bartosek *et al.* 1980; Pearce *et al.* 1996). Also, no studies appear to have reported whether viable microsomes can be prepared from frozen colon tissue.

The first aim of this study was to investigate whether viable microsomes could be prepared from the frozen liver and colon tissue of pigs. Dependent on viable microsomes being prepared, the second aim of this study was to determine whether broccoli or carrot consumption alters the activity of CYP1A enzymes in the liver and colon of pigs. Because broccoli typically contains substantial quantities of indolyl GLS, which appear to be potent inducers of CYP1A, it was hypothesised that raw Marathon and Monaco broccoli would induce CYP1A enzymes in the liver and colon (Shertzer & Sainsbury 1991; Bonnessen *et al.* 1999; Vallejo *et al.* 2003). In chapter 2 it was found that blanching and freezing inactivated the myrosinase in broccoli. Plant-based myrosinase appears to be required for the optimal hydrolysis of GLS to indoles, so, it was hypothesised that blanched-frozen broccoli would have a lower ability to induce phase I enzymes than raw broccoli (Holst & Williams, 2004). High intakes of freeze-dried raw carrot cause a small induction of CYP1A1 in rat liver, but it was hypothesised that the much lower level of carrots fed in the current study would not induce CYP1A enzymes in pig liver or colon.

The objectives of this study were:

- To prepare microsomes from liver and colon tissue that had been stored frozen for 12 to 24 months.
- To determine whether freezing and long-term storage causes the conversion of CYP450 to its inactive CYP420 form.
- To measure ethoxyresorufin *O*-dealkylation (EROD) and methoxyresorufin *O*-dealkylation (MROD) activity in microsomes shown to contain active CYP450.
- To investigate the effect of cultivar and blanch-freezing on the ability of broccoli to modulate total CYP450 content and EROD and MROD activity.
- To investigate whether raw carrot, a non-GLS containing vegetable with a high carotenoid content, alters total CYP450 content or EROD and MROD activity.

6.2 Materials and Methods

6.2.1 ***Chemicals***

All chemicals were purchased from either, Sigma Aldrich Company Ltd, (St Louis, USA), Fisher Scientific Supplies (Loughborough, UK) or BDH Laboratory supplies (Poole, UK).

6.2.2 ***Animals and diets***

A detailed description of the pigs, diet and study design can be found in Chapters 2 and 3 (pgs, 51 to 53 and 76 to 77). Briefly, in both experiments a control group of pigs was fed a cereal diet. Treatment groups consumed the same cereal diet supplemented with 600 g/d of vegetables. In experiment 1, the supplemental vegetables consisted of raw broccoli (cv. Marathon) or blanched-frozen broccoli (cv. Marathon). In experiment 2, the supplemental

vegetables were raw broccoli (cv. Monaco) or raw carrot (cv. Nairobi). The experimental diets were fed for 12 d and then pigs were killed on the morning of d 13.

6.2.3 ***Tissue collection***

6.2.3.1 Collection of liver and colon tissue

Sections of liver and colon were removed and processed as described in Chapter 4, pg. 91-92.

6.2.3.2 Preparation of hepatic microsomes

All steps were conducted on ice or at 0-4^{o}C. Sections of liver from the middle of the left lobe (approximately 6 g) were allowed to defrost in ice-cold homogenising buffer (0.25 M sucrose, 20 mM Tris, 1 mM dithiothreitol (DTT), pH 7.4) to minimise degradation of CYP450 (Pearce *et al.* 1996). Defrosted liver segments were then rinsed with ice cold 1.15% (w/v) KCl buffer (pH 7.4), blotted dry, minced with scissors and rapidly transferred to homogenising buffer (4 ml/g of tissue). Liver segments were homogenised with one 45-60 sec burst of a rotor stator homogeniser (8000 rpm; Janke & Kunkel, Ultra-turrax T25, Germany). Homogenates were centrifuged at 10,000 **g** x 20 min (ALC PK121R, Cologno Monzese, Italy). Supernatants were removed and centrifuged at 100,000 **g** x 60 min (Beckman Coulter LE-70 ultracentrifuge, Fullerton, USA). The resulting supernatants were discarded and a quantity of homogenising buffer equal to the amount of discarded supernatant was added to the remaining pellet. The pellet was then re-suspended in the buffer with 6-8 strokes of a motor driven (Status R100C, CAT, Germany) Potter-Elvehjem Teflon/glass homogeniser (600 rpm). The resulting homogenate was centrifuged for a further 60 min x 100,000 **g**. Supernatants were removed and the remaining pellet was re-suspended in freezing buffer (10 mM Tris, 20% w/v glycerol, 1 mM EDTA, adjusted to pH 7.4 with acetic acid) with 6-8 strokes of the Potter-Elvehjem Teflon/glass homogeniser (600 rpm). The resulting homogenate was divided into 0.4 ml aliquots and rapidly snap frozen in liquid N_2 and stored at -80^{o}C until use.

6.2.3.3 Preparation of colonic microsomes

Colonic microsomes were prepared from sections of colon tissue excised from the mid-colon, using the method of Fang & Strobel (1978), with minor modifications. All procedures were conducted on ice or at 0-4^{o}C. Sections of frozen colon were allowed to defrost in ice-cold Tris-HCl buffer solution (10 mM Tris-HCl, pH 7.4 containing 0.15 M KCl, 10 mM EDTA and 1 mM DTT). Once defrosted, the colons were flushed with ice cold 1.15% (w/v) KCl, cut longitudinally and placed on a plastic tray sat on a bed of ice. Their lumens were rinsed with Tris-HCl buffer solution to remove any residual faecal material and patted dry with paper tissue. Mucosal cells were gently removed by scraping the exposed luminal surface with a glass microscope slide. Scrapings of mucosal cells were rapidly weighed and transferred to Tris-HCl buffer (1 g tissue per 4 ml of buffer). The mucosal cells were washed three times and recovered by low speed centrifugation (1200 **g** x 5 min, 4^{o}C). The washed mucosal cells were resuspended in Tris-HCl buffer solution with added phenylmethylsulfonyl fluoride (0.25 mM, a protease inhibitor) and homogenised with 10-12 strokes of a motor driven Potter-Elvehjem Teflon/glass homogeniser (600 rpm). Homogenates were centrifuged at 10,000 **g** x 20 min (ALC PK121R, Cologno Monzese, Italy). Supernatants were transferred to ultracentrifuge tubes and centrifuged for 60 min at 100,000 **g** (Beckman Coulter LE-70 ultracentrifuge, Fullerton, USA). Supernatants were discarded and the remaining pellet was resuspended in Tris-HCl homogenising buffer with 10-12 strokes of a motor driven (Status R100C, CAT, Germany) Potter-Elvehjem Teflon/glass homogeniser (600 rpm). Homogenates were transferred to ultracentrifuge tubes and centrifuged for a further 60 min x 100,000 **g** (Beckman Coulter LE-70 ultracentrifuge, Fullerton, USA). Supernatants were discarded and the remaining pellet was resuspended in 1.5 ml of freezing buffer (10 mM Tris, 20% w/v glycerol, 1 mM EDTA, adjusted to pH 7.4 with HCl) and homogenised with 6-8 strokes of a motor driven Potter-Elvehjem Teflon/glass homogeniser (600 rpm). The resulting homogenate was divided into 0.5 ml aliquots, snap frozen in liquid N_2 and stored at -80^{o}C until use. In addition,

one pooled colonic microsome was prepared by combining three pellets in 1.5 ml of freezing buffer.

6.2.4 Analytical methods

6.2.4.1 Measurement of cytochrome P450 and haemoglobin concentration

The CYP450 content of 15 microsomal samples was determined by the spectrophotometric methods of Omura & Sato (1964) and Matsubara *et al.* (1976). Subsequently, the method of Matsubara *et al.* (1976) was used to determine the CYP450 content of the remaining microsomal samples. Both methods rely on the fact that reduced CYP450 enzymes, when bound to CO have an absorption maximum at 450 nm (Fowler *et al.* 2001). The method of Omura & Sato (1964) measures the reduced-CO versus reduced difference spectra of the microsomal preparation, whereas the method of Matsubara *et al.* (1976) measures the reduced-CO versus the oxidised-CO difference spectra. The latter method compensates for contamination with haemoglobin. This is useful when microsomes are prepared from large animals and/or frozen tissue, because these are often contaminated with haemoglobin (Pearce *et al.* 1996; Guengerich, 2001).

6.2.4.2 Cytochrome P450 determination by the method of Omura & Sato (1964)

First, 0.3 ml of microsomal sample was diluted to a final volume of 2 ml with 0.1 M KH_2PO_4/K_2HPO_4 (pH 7.4) and divided into two quartz cuvettes (Hellma, CXA140 155). A baseline was recorded between 400 and 500 nm (Kontron, Uvikon 860, Kontron Instruments, Watford, UK). The sample cuvette was bubbled for 30-40 sec with CO. A few grains of sodium dithionite were added to both cuvettes (CYP450 is a ferric haemoprotein that requires reduction to its ferrous state before it can bind CO). Cuvettes were inverted and after approximately 60 sec the reduced-CO versus reduced difference spectra were recorded (400-500 nm). Total CYP450 content was calculated as the change in absorbance at 450 nm relative to the absorbance at 490 nm (isobestic point), converted to a concentration using the

extinction co-efficient of 91 mM/cm (see Appendix A.3.1, pg 156). The concentration of CYP420 was calculated as previously described by Guengerich (2001) using the extinction co-efficient of 110 mM/cm (see Appendix A.3.1.1, pg 156-157).

6.2.4.3 Haemoglobin measurement

The haemoglobin content of the microsomes was measured as described by Pearce *et al.* (1996). For this analysis, 0.2-0.3 ml of microsomal sample was diluted to a final volume of 2 ml in 0.1 M potassium phosphate buffer (KH_2PO_4/K_2HPO_4, pH 7.4) and divided into two quartz cuvettes. A baseline was recorded between 400 and 500 nm. The sample cuvette was bubbled with CO for 30-40 sec and spectra were recorded (400-500 nm; haemoglobin is a ferrous haemoprotein that can bind CO without the addition of sodium dithionite). The difference in absorbance between 420 nm and 405 nm was used to estimate the content of haemoglobin in the microsomes. The concentration of haemoglobin was estimated using the extinction co-efficient of 110 mM/cm to allow comparison with the CYP420 content (see Appendix A.3.3, pg 158).

6.2.4.4 Cytochrome P450 determination by the method of Matusubara et al. (1976)

The samples used to determine haemoglobin content were also used for this analysis. The reference cuvette was bubbled for 30-40 sec with CO and 5 µl of 20 mM NADH (prepared in potassium phosphate buffer) was added (to reduce cytochrome b_5). A few grains of sodium dithionite were added to the sample cuvette (to reduce CYP450 and cytochrome b_5), which was then bubbled for 30-40 sec with CO. Spectra were repeatedly recorded until the 450 nm peak reached a maximum. Total CYP450 content was calculated as the change in absorbance at 450 nm relative to the absorbance at 490 nm (isobestic point) converted to a concentration using the extinction co-efficient of 106 mM/cm (see Appendix A.3.2, pg 157-158).

6.2.4.5 Determination of CYP1A enzyme activity

The activity of CYP1A enzymes was determined by measuring the *O*-dealkylation of ethoxyresorufin and methoxyresorufin. CYP1A mediated *O*-dealkylation of these two phenoxazone ethers produces resorufin. The rate of production of resorufin can be determined by measuring the linear increase in fluorescence (Burke *et al.* 1985).

6.2.4.5.1 Determination of EROD activity in hepatic microsomes

EROD activity was determined fluorimetrically at 37^{o}C by the method of Burke *et al.* (1985). First, 0.2 mg of microsomal protein and 0.25 ml of an NADPH generating system (0.8 mg NADP, 1.2 mg isocitric acid, 50 µl 0.1M $MgSO_4$, 1 unit isocitrate dehydrogenase and 0.1 M Tris-HCl, pH 7.8 to make a volume of 0.25 ml) were added to a Perkin-Elmer fluorimeter cuvette (B06331107). Tris-HCl buffer (pH 7.8) was added to bring the volume to 1.99 ml and the cuvette was incubated for 2 min at 37^{o}C in a fluorimeter (Perkin-Elmer LS 55, Massachusetts USA). After 2 min, the reaction was initiated by adding 10 µl of ethoxyresorufin (10 µl of a 1mM solution in DMSO, final cuvette concentration 5 µM) to the cuvette and fluorescence was measured and recorded for 10 min. The fluorimeter settings were: excitation and emission slits 5 nm and excitation wavelengths 530 and 585 nm. After 10 min of linear reaction fluorescence was calibrated by the addition of a standard solution of resorufin (10 µM in DMSO; see Appendix A.3.4, pg 159 for sample calculation).

6.2.4.5.2 Determination of MROD activity, in hepatic microsomes

MROD activity was determined in the same way as EROD activity except that ethoxyresorufin was replaced with methoxyresorufin (final cuvette concentration 5 µM).

6.2.5 ***Statistical Analysis***

Data are presented as means (SD). The significance of differences between treatment means was then tested by two way ANOVA for a randomised experimental block design. Standardised residuals for each outcome variable were normality distributed (Shapiro-Wilk *W* test).

The haemoglobin and CYP420 data were tested for normality and then the relationship between haemoglobin and CYP420 content was determined by Pearson's product moment correlation co-efficient (*r*) (Altman, 1991). All statistical analyses were conducted on SPSS Release 17.0 (2008) and a value of $P<0.05$ was set as the critical level of significance.

6.3 **Results**

6.3.1 Stability of cytochrome P450 in frozen porcine liver samples

Hepatic microsomes initially scanned for total CYP450 content by the method of Omura & Sato (1964) contained a peak at 450 nm, but also a substantial, albeit variable second peak at 420 nm (Fig 6.1A). Replicate microsomes contained substantial quantities of haemoglobin and there was a strong correlation between the haemoglobin and CYP420 content in pairs of replicate samples ($r= 0.97$, $P<0.001$; Fig 6.1A, 6.1B & 6.1C). When microsomes were scanned for CYP450 content by the method of Matsubara *et al.* (1976), which compensates for the presence of haemoglobin, only a small shoulder was evident at 420 nm (Fig 6.1B).

6.3.2 Colonic Samples

No CYP450 or CYP420 could be detected in microsomes prepared from single sections of frozen colon tissue (Fig 6.3). When a pooled microsome was prepared from the colonic tissue

of three control pigs, no CYP450 was detected, but a small peak at CYP420 was observed (Fig 6.4). No EROD or MROD activity could be detected in the microsomes prepared from single sections of frozen colon tissue or in the pooled microsome.

6.3.3 Treatment effects

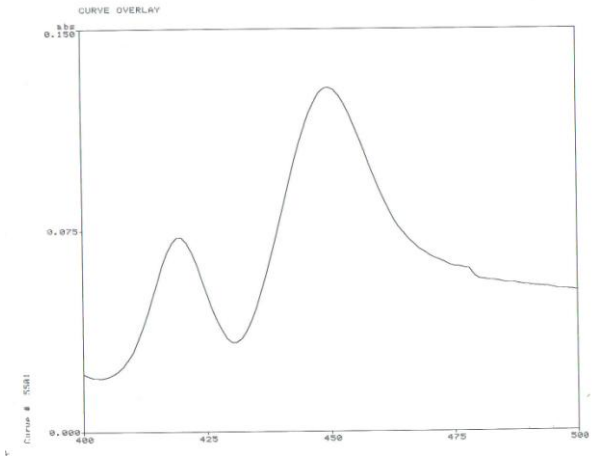
6.3.3.1 Experiment 1

The total hepatic content of CYP450 (pooled mean 0.61 (SD 0.099) nmol/mg protein; *P*=0.935) and the activities of EROD (pooled mean 107.0 (SD 27.46) pmol/min/mg of microsomal protein; *P*=0.683) and MROD (pooled mean 38.2 (SD 7.65) pmol/min/mg of microsomal protein; *P*=0.880) were unaffected by raw and blanched-frozen broccoli (cv. Marathon) consumption (Table 6.1).

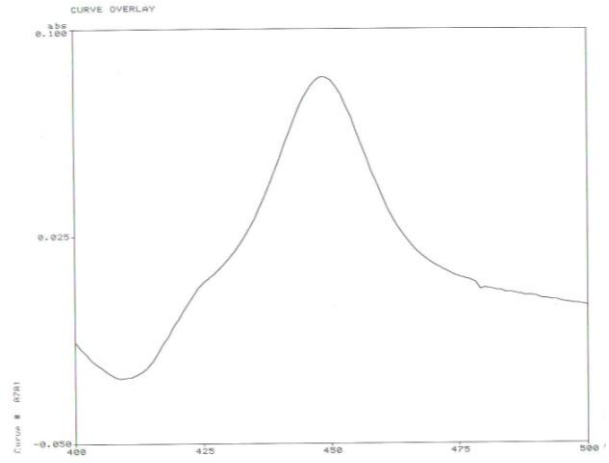
6.3.3.2 Experiment 2

Diets supplemented with raw Monaco broccoli and raw carrots (cv. Nairobi) also failed to alter the total hepatic CYP450 (pooled mean 0.55 (SD 0.081) nmol/mg protein; *P*=0.610) content or EROD (pooled mean 71.6 (SD 14.47) pmol/min/mg of microsomal protein; *P*=0.313) and MROD activities (pooled mean 25.5 (SD 4.41) pmol/min/mg of microsomal protein; *P*=0.349) (Table 6.1).

A



B



C

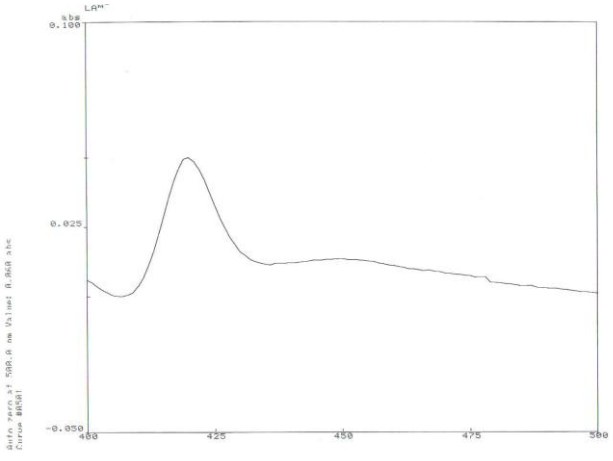


Fig 6.1 Cytochrome P450 spectra of a hepatic microsome determined by the method of (A) Omura & Sato (1964) and (B) Matsubara *et al.* (1978). (C) The haemoglobin spectra of the same hepatic microsome. Microsomes were prepared as outlined in the Methods section.

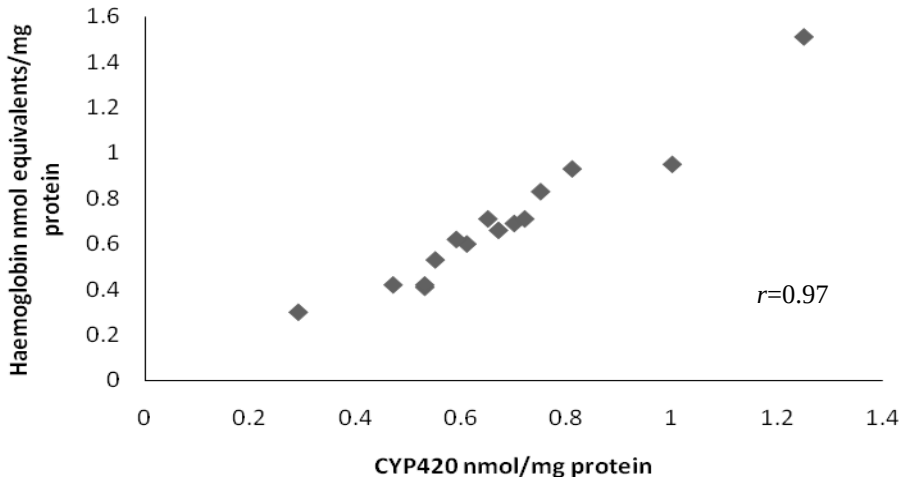


Fig 6.2 The correlation between CYP420 and haemoglobin content in hepatic microsomes prepared from young male pigs, *n*=15. Tissue was stored at -80^{o}C for at least 6 months before microsomes were prepared, see Methods section for more details.

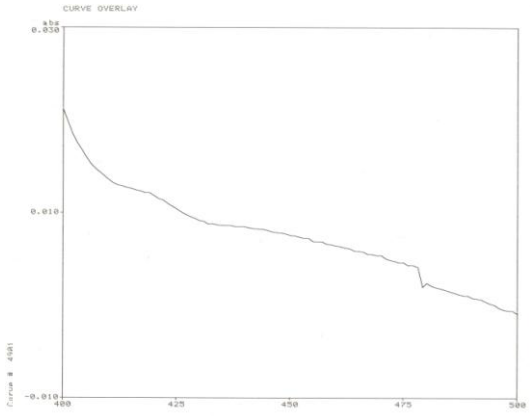


Fig 6.3 Cytochrome P450 difference spectra of a microsome prepared from a single section of pig colonic tissue. The colonic tissue was frozen and stored at -80^{o}C for at least 12 months before the microsome was prepared, see Methods section for more details.

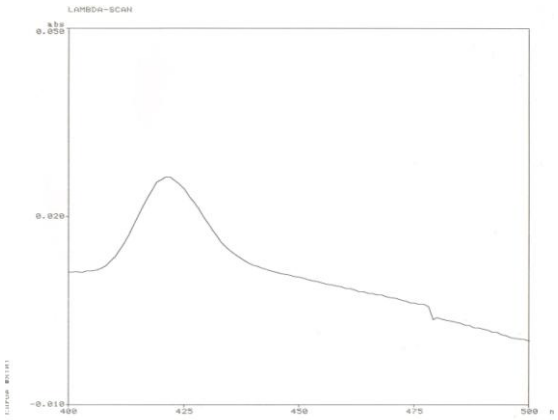


Fig 6.4 Cytochrome P450 spectra of a pooled colonic microsome prepared from the mucosa of three pigs. Sections of colonic tissue were frozen and stored for at least 12 months before microsomes were prepared, see Methods section for more details.

Table 6.1 Cytochrome P450 content and EROD and MROD activity in hepatic microsomes prepared from young male pigs fed cereal diets, unsupplemented (control) or supplemented with 600 g/d of broccoli or carrots for 12 d.

Experiment 1	Total CYP450 nmol/mg protein	EROD pmol/min per mg microsomal protein	MROD pmol/min per mg microsomal protein
Raw Broccoli (cv. Marathon)	0.61 (0.080)	106.1 (31.93)	38.3 (9.67)
Blanched-frozen Broccoli	0.60 (0.082)	111.6 (33.78)	39.1 (9.17)
Control	0.61 (0.144)	103.2 (20.82)	37.2 (4.98)
Experiment 2			
Raw Broccoli (cv. Monaco)	0.52 (0.068)	78.3 (20.17)	27.1 (5.85)
Raw Carrots (cv. Nairobi)	0.57 (0.099)	65.0 (13.18)	25.3 (2.84)
Control	0.53 (0.058)	71.4 (6.43)	24.0 (4.45)

Values are means (SD), in all cases n=5 in each diet groups. Total CYP 450 content and EROD and MROD activity were determined in triplicate. Two way ANOVA was used to assess significance of differences (P>0.05 in all cases).

6.4 Discussion

The first aim of this study was to determine if viable microsomes could be prepared from porcine liver and colon tissue that had been stored frozen for 12 to 24 months. Initial scans of 15 hepatic microsomes for CYP450 content by the method of Omura & Sato (1964) revealed substantial peaks at 420 nm, although the size of this peak was somewhat variable. A peak at 420 nm can reflect degradation of CYP450 to its inactive form, CYP420, and could be interpreted as evidence that freezing and long-term storage resulted in a substantial loss of CYP450. However, a peak at 420 nm may also result from contamination of microsomes with haemoglobin and other proteins, which absorb light at 420 nm (Pearce *et al.* 1996).

To investigate whether the peak at 420 nm represented a large degree of degradation of CYP450, or was caused by haemoglobin contamination, microsomes that had previously been scanned by the method of Omura and Sato (1964) were analysed for haemoglobin content. All the microsomes contained substantial quantities of haemoglobin. Furthermore, the magnitude of the haemoglobin peak correlated strongly with the previously measured peak at 420 nm. When the microsomes were scanned by the method of Matsubara *et al.* (1976), which compensates for haemoglobin contamination, there was only a very small shoulder at 420 nm rather than a substantial peak. This indicates that the microsomes contained little denatured CYP450, but were heavily contaminated with haemoglobin and possibly other proteins that absorb light at 420 nm. These findings are consistent with the work of Pearce *et al.* (1996) who found microsomes prepared from frozen human liver tissue contained high amounts of haemoglobin, but little degraded CYP450.

In contrast to liver tissue, it appears impossible to prepare viable microsomes from frozen colon tissue. When colonic microsomes prepared from single sections of colon tissue were scanned by carbon dioxide difference spectra, no CYP450 or CYP420 content could be detected. This was interpreted as indicating that the level of CYP450 or CYP420 in the

colonic microsomes was below the lower limit of detection of the assay. The assays for measuring EROD and MROD activity are able to detect very low levels of enzyme activity, so both assays were conducted on the colonic microsomes, but no activity towards either substrate was found. A comparison with the data of Fang & Strobel (1978) indicated that they used approximately 10 times greater amounts of microsomal protein in the sample and reference cuvettes to detect CYP450 in microsomes prepared from rat colonic tissue. Even then, they only detected a very small 0.002 increase in absorbance at 450 nm. From the amount of frozen colon tissue available, it was impossible to produce microsomes for each pig with such a high content of protein. In an attempt to confirm whether the frozen colon tissue contained any CYP450 or CYP420, the colon tissue from three pigs was pooled to produce a microsome with a higher content of protein. When the resulting microsome was scanned by the method of Matsubara *et al.* (1976) there was no peak at 450 nm but a small peak at 420 nm, seemingly indicating freezing and/or storage had resulted in the degradation of CYP450 to CYP420. The apparent lack of active CYP450 was further confirmed by analysing the pooled microsome for EROD and MROD activity; no activity towards either substrate was found. These results appear to indicate that porcine colonic CYP450s are unstable when colon tissue is frozen and stored for an extended period before microsomes are prepared. The contrast with liver CYP450 can probably be explained by the very low level of CYP450 which typically appears to be present in the colon of mammals (see for example Strobel *et al.* 1980).

All the vegetable diets failed to alter total CYP450 content and CYP1A enzyme activities in the liver. The failure of the carrot treatment to have any effect on total CYP450 content is consistent with the study of Bradfield *et al.* (1985), which found no effect of a 20% (w/w) carrot diet on the hepatic content of CYP450 in rats. However, in contrast to the present experiment, Bradfield *et al.* (1985) reported a small 1.2 fold induction of CYP1A1 (as measured using the *in vitro* probe ECOD). The present study fed proportionally 4 times lower amounts of carrots than the study of Bradfield *et al.* (1985). So, the failure to find any

induction of CYP1 enzymes may simply indicate that too low a level of inducing compounds was supplied by the carrot diet.

At least two rodent studies have reported on the effect of broccoli diets on the total CYP450 content of the liver, with contrasting results (Aspry & Bjeldanes 1983; Ramsdell & Eaton 1988). Aspry & Bjeldanes (1983) found a 1.5 and a 1.9 fold increase after the feeding of 10 and 25% (w/w) broccoli diets, whereas Ramsdell & Eaton (1988) found no increase after feeding a 25% (w/w) broccoli diet. In agreement with the latter study, the present study found no effect of any broccoli supplement on total CYP450 content. The lack of effect on total CYP450 content is not particularly surprising because total CYP450 content is not a sensitive measure of induction of individual CYP450 isoforms. Indeed, substantial induction of individual CYP1A enzymes may occur without any significant increase in total CYP450 content (Bradfield *et al.* 1985).

The failure of the broccoli diets to induce hepatic EROD and MROD was more unexpected because human studies have reported small inductions of hepatic CYP1A2 (typically 10-20%) after the consumption of proportionally 50-60% lower intakes of broccoli (500 g/d in 70-80 kg adults; Vistisen *et al.* 1992; Kall *et al.* 1996). Furthermore, one of the studies fed Marathon broccoli, which is the same cultivar as one of the broccolis fed in the present study (Kall *et al.* 1996). Whilst differences in growing conditions and post-harvest storage and processing can mean that the same cultivar of broccoli may vary substantially in its content of GLS, published data indicate that Marathon broccoli is typically rich in the relevant indolyl GLS required for CYP1A induction (Vallejo *et al.* 2002; Vallejo *et al.* 2003). It is possible that the disagreement between the present study and the human studies may reflect differences in the methods of measuring enzyme induction, or species differences. The human studies determined hepatic CYP1A2 activity by measuring the *in vivo* metabolism of a defined dose of caffeine. In humans, hepatic CYP1A2 is believed to be responsible for 95% of the metabolism of caffeine, therefore an increase in specific caffeine metabolites in the urine is

thought to reflect an increase in hepatic CYP1A2 activity (Kalow & Tang 1993). In contrast, the present study measured CYP1A1 and CYP1A2 *ex vivo*, in liver microsomes using the probes, ethoxyresorufin and methoxyresorufin. Using these probes in liver microsomes may not be directly comparable with the non-invasive measurement of the metabolism of caffeine. Also, although ethoxyresorufin and methoxyresorufin are thought to be fairly selective probes for the measurement of CYP1A1 and CYP1A2 in rats, selectivity for pig CYP1A1 and CYP1A2 has not been confirmed (Nerurkar *et al.* 1993; Van der Burght *et al.* 2000). It is possible that other CYP450 enzymes in pig liver may contribute to EROD and MROD activity, meaning that both probes may not have the specificity and sensitivity to detect a small dietary induction of CYP1A enzymes. Among all of the CYP450 enzymes, the CYP1A family are generally believed to be the most conserved across species (Lewis, 2001). However, some small differences in the inducibility of CYP1A have been reported in different species (Parkinson, 1996). It is possible the genes that encode pig CYP1A enzymes, are less sensitive to induction by GLS breakdown products than the corresponding human genes.

Other possible explanations for the present study failing to detect an induction of hepatic CYP1A include those discussed in Chapter 4 as reasons for finding no induction of phase II enzymes, i.e. the amount and form in which the broccoli was fed, the composition of the basal diet and the method used to kill the pigs. As many of the arguments are similar only additional points specifically relevant to this study will be discussed here. As already discussed, freezing liver tissue and storing it frozen prior to the preparation of microsomes appeared to result in substantial contamination with haemoglobin, but seemingly little inactivation of CYP450. Contamination with haemoglobin would be expected to decrease the specific activity for EROD and MROD because of its contribution to the protein content of the microsomes. Pearce *et al.* (1996) have previously reported a 20-40% decrease in the specific activity of a range of microsomal enzymes prepared from frozen/stored sections of human liver versus microsomes prepared from unfrozen sections of the same livers. The microsomes in which haemoglobin was measured in the current study showed variable levels of contamination. This

would be expected to lead to a variable decrease in specific enzyme activity, which may have masked a small induction by the vegetable diets.

In Chapter 4 it was acknowledged that the drugs used to sedate and anaesthetise the pigs before death may have masked a small induction of phase II enzymes by the vegetable treatments. It was argued that the short time lapse between administration of both drugs and removal of tissues probably provided too little time for any substantial degree of enzyme modulation. The same argument is relevant to this experiment, however, there is also evidence that Zoletil® administered at the dose used in the present study does not affect the total CYP450 content or CYP1A1/2 activities, even if administered 24 h before death. Wong *et al.* (1996) investigated the effect of increasing doses (20 to 120 mg/kg body weight) of Zoletil® (referred to by the US trade-name, Telazol®) on various cytochrome P450 enzymes in adult Long Evans male rats. Zoletil® had no effect on total CYP450 content, or on EROD activity, except for a modest 1.5 fold induction at the highest dose (120 mg/kg body weight). In this study, pigs were exposed to a 10 fold lower dose (mean dose 12.3 ± 1.35 mg/kg body weight) of Zoletil® than the level shown to induce EROD activity in rats, and much less time elapsed between administration and tissue removal (approximately 40 m versus 24 h). This suggests that if pigs and rats respond similarly, then the single dose of Zoletil® used in this study would be unlikely to have masked any effect of the vegetable supplements on the measured CYP enzymes. No studies have directly studied whether Somulose® modulates CYP450 enzymes. However, its main active ingredient, secobarbital, has been shown to cause a modest 1.5 fold induction of EROD activity when incorporated into the diets of rats at a concentration of 500 ppm for 15 d (Rice *et al.* 1994). However, given the relative modest induction after prolonged exposure, it seems unlikely that the single dose used in the present study approximately 10 min before the removal of the livers would have invoked any substantial effect. Although it is unlikely that drug administration resulted in any substantial enzyme induction, the presence of the drugs in the liver microsomes could have interfered with the enzyme assays. No investigation into this possibility was conducted. A possible approach

would have been to prepare some liver microsomes from pigs killed by a non-pharmaceutical method and to spike them with various concentrations of Zoletil® and Somulose® to investigate any interaction with the enzyme assays.

In summary, this study found that after freezing and long-term storage, viable microsomes could be prepared from porcine liver tissue, but not from colon tissue. The lower stability of CYP450 in the colon can probably be explained by the very low levels that are present. None of the vegetable treatments altered the content of CYP450 in the liver or the activity of enzymes measured by the EROD and MROD assays. This was despite the fact that one of the cultivars of raw broccoli (cv. Monaco) had previously being shown to contain relatively high levels of indolyl GLS (see Chapter 4), which are potent inducers of CYP1A activity in rodents. The most likely explanations for failing to detect an increase in CYP1A activity in the current work are that variable contamination of the microsomes with haemoglobin masked a modest induction, or that higher intakes of broccoli and indolyl GLS are required to induce CYP1A activity in pigs consuming a cereal based basal diet.

Chapter 7

General Discussion and Conclusion

7.1 General Discussion

Cruciferous vegetables and many of their bioactive constituents have been shown to inhibit chemically induced colon cancer in rodents. However, prospective cohort studies investigating the association between intakes of cruciferous vegetables and CRC have found little evidence of protection (see Chapter 1). In Chapter 1, it was speculated that one of the factors responsible for this disparity could be the failure of many epidemiological studies to consider the effect of processing. Processing can alter the content and bioavailability of bioactive compounds present in cruciferous vegetables (Price *et al.* 1998; Vallejo *et al.* 2002; Conaway *et al.* 2000). So, variation in the degree of processing that cruciferous vegetables undergo prior to consumption may mean that individuals with apparently similar levels of intake are exposed to different levels and types of bioactive compounds. Failing to consider this may misclassify a subject's exposure to bioactive compounds and attenuate any association between cruciferous vegetable intake and risk of CRC. Few experimental studies have investigated how processing alters the biological actions of cruciferous vegetables. However, blanching has been shown to remove the ability of raw Brussels sprouts to inhibit DMH induced ACF and homogenisation and microwave cooking have been shown to eliminate the ability of raw broccoli to protect colonocytes from DNA damage (Ratcliffe *et al.* 1999; Ratcliffe *et al.* 2000; Smith *et al.* 2003a).

The primary aim of the work described in this thesis was to further test the hypothesis that raw broccoli protects DNA in colonocytes from damage and that processing removes this protective effect. The secondary aims were to investigate possible mechanisms through which

raw broccoli confers protection and to determine whether a non-cruciferous raw vegetable also protects colonocyte DNA from damage. However, the studies produced surprising results. In two pig experiments, different cultivars of raw broccoli increased DNA damage in colonocytes, whereas, blanched-frozen broccoli and raw carrot exerted no significant effect (see Chapters 2 & 3). The reason for the disagreement with the previous work of the group is uncertain, but it is possible that the broccoli differed in some important aspect (see Chapter 2).

Despite the fact that the induction of phase II enzymes has previously been hypothesised to be a major mechanism through which broccoli confers protection against CRC, diets supplemented with raw and blanched-frozen broccoli failed to increase the activity of GST or QR in hepatic and colonic tissue (Keck *et al.* 2003; see Chapter 4). Although these results contrast with those of most animal studies, they are consistent with two studies in humans that failed to find any effect of consuming 200-300 g/d of steamed broccoli on the content of GST α in plasma or total GST activity in plasma and saliva (Steinkellner *et al.* 2000; Riso *et al.* 2009a). The disagreement between studies can probably be explained by the proportionally higher intakes of broccoli fed in the rodent studies (see Chapter 4). Collectively, these data appear to indicate that the substantial induction of detoxification enzymes is unlikely to occur at levels of broccoli consistent with habitual human intakes.

All the vegetable supplemented diets tended to suppress the growth of coliform bacteria in the colonic contents of the pigs, leading to an increase in the ratio of lactobacilli to coliforms (see Chapter 5). An increase in the ratio of lactobacilli to coliforms is normally considered beneficial to gut health (Muraldihara *et al.* 1977), but in the current work similar significant increases occurred after the consumption of raw broccoli (cv. Monaco), which increased DNA damage in colonocytes and raw carrots (cv. Nairobi), which had no significant effect on DNA damage. So, it appears that the changes in the colonic microflora were independent of the effects on DNA damage. Nevertheless, suppression of coliform bacteria may be expected to reduce the activity of certain bacterial XME such as β-glucuronidase that have been

implicated in the conversion of pro-carcinogens to carcinogens within the colonic lumen (Hughes & Rowland 2003). Therefore, it may be interest to explore whether the suppression of coliform bacteria by carrots and broccoli is associated with an inhibition of bacterial XME. In the current work, standard microbiological culture techniques were used to enumerate a limited number of bacterial groups. The use of 16s rRNA based molecular methods may be useful to provide a greater overview of the effect of vegetable consumption on the colonic microflora.

In the work described in Chapter 6, an attempt was made to identify a possible mechanism through which raw broccoli damaged colonocyte DNA. The broccoli fed in the current work appeared to be rich in indolyl GLS (see Chapter 4 and 5 for more details). On hydrolysis indolyl GLS form a range of breakdown products that are potent inducers of CYP450 enzymes (Shertzer & Sainsbury 1991; Bonnessen *et al.* 2001). In certain circumstances, the induction of CYP450 enzymes can increase the production of ROS and cause oxidative DNA damage (Park *et al.* 1996; Paolini *et al.* 2004). So it was decided to measure the activity of CYP1A enzymes in the colonic mucosa and liver. Raw broccoli failed to alter CYP1A activity (as measured by the probes ethoxyresorufin and methoxyresorufin) in liver microsomes. Unfortunately, attempts to measure CYP1A in the colonic mucosa proved unsuccessful because viable microsomes could not be prepared from the available frozen tissue samples (see Chapter 6). . The effect of raw broccoli consumption on the activity of colonic CYP1A enzymes could be clarified with further studies using microsomes prepared from non-frozen colonic tissue.

As discussed in Chapters 2 and 3, colonocytes isolated from the pigs on the control diets contained a substantial number of class 4 comets. This suggests that damage occurred during the isolation process. There is a possibility that raw broccoli consumption increased the susceptibility of colonocytes to DNA damage during isolation rather than increasing levels *in situ*. The implication of this is unclear, but as discussed in Chapter 3, putative dietary

anticarcinogens generally increase the resistance of isolated cells to damage by compounds such as H_2O_2, although it is uncertain whether this would translate into protection against other types of stress encountered during isolation. In retrospect, it may have been worth exploring alternative isolation techniques to see whether colonocytes could have been consistently isolated with less DNA damage. If such a method could be identified or developed, the current experiments should be repeated to confirm the effect of raw broccoli consumption. It may also be of interest to confirm the effects of blanched-frozen broccoli and raw carrot because it is possible that the high background level of DNA damage may have masked a modest genotoxic or protective effect. The standard comet assay used in the present study provides little information as to the cause of the strand breaks. However, various adaptations to the comet assay can increase its specificity. The inclusion of the lesion specific enzymes to detect oxidised purines (formamidopyrimidine glycosylase) and pyrimidines (endonuclease III) might be useful for exploring whether raw broccoli also increases the number of oxidised bases in colonocyte DNA (Collins, 2004). In the present work, DNA damage was measured in a section of tissue derived from the mid-colon. Because some epidemiological evidence indicates that vegetables only protect against distal colon cancer, future studies could establish whether the effect of raw broccoli varies in different regions of the colon (Koushik *et al.* 2007).

If the results of the current studies are accepted as evidence that raw broccoli damages DNA *in vivo*, the important questions to ask are, would a high intake of raw broccoli cause DNA damage in the colonocytes of humans? And does DNA damage measured by the comet assay indicate an increased the risk of CRC? It is difficult to answer either of these questions.

Pigs were chosen as the animal model for the current work because they have a number of advantages over the more commonly used rat model. Their larger size makes dissection and collection of tissues, digesta and body fluids easier. Other advantages include greater similarities with human digestive and absorptive capacities, comparable ingesta transit times

and the fact that they do not practise coprophagy (Kidder & Manners 1978; Millar & Ullray 1987; Topping & Clifton 2001). However, the pig also has several disadvantages that limit the direct extrapolation of the current findings to humans. Pigs tend to bolt rather than chew their food (Bird *et al.* 2000). The pig colon constitutes 48% of the total volume of their gastrointestinal tract, whereas in humans the colon accounts for approximately 17% (Topping & Clifton 2001). Also, the microflora of the pig colon is grossly different to humans and contains cellulolytic bacteria such as *C.herbivorans* that are highly effective at degrading plant cell walls (Yen, 2001). Such differences would be expected to have influenced the extent to which bioactive compounds were liberated from raw broccoli and possibly the type of metabolites formed in the colonic lumen. Because of these differences, direct testing in humans is needed to establish whether the consumption of high intakes of raw broccoli damages DNA in human colonocytes.

Direct replication of the current studies in humans would, however, be difficult. Recruiting healthy volunteers to undergo colonic biopsies at the start and end of a short intervention would be challenging and compliance may be expected to be poor. Other approaches may be of use in determining whether raw broccoli is genotoxic to human colonocytes. Juice prepared from raw broccoli and blanched-frozen broccoli could be incubated with human colon cell lines. Such studies could not however, account for the role that microbial metabolism may have played in mediating the genotoxic effect of raw broccoli in the current work. If the genotoxic component is present in the luminal contents, a possible *ex vivo* approach could be to test the genotoxicity of faecal water samples from humans consuming raw broccoli. Initially, a pig experiment duplicating the current studies would be needed to establish whether faecal water samples and digesta water samples collected from different sites along the colon are genotoxic to a colon cell line. The testing of both digesta from along the colon and excreted faeces would be necessary because any compound exerting a genotoxic effect in the mid-colon could be absorbed within the colon and therefore be absent from the faeces. If the experiment confirmed that faecal water from pigs consuming raw broccoli was genotoxic

then human studies could be conducted, although, designing a study to administer raw broccoli at quantities thought to be genotoxic may presents ethical concerns.

The comet assay as employed in the current work detects strand breaks and abasic sites that are converted to strand breaks under the alkali conditions of the assay. Strand breaks resulting from exposure to genotoxins may promote chromosomal aberrations and abasic sites could have mutagenic potential (Brendler-Schwaab *et al.* 2005). However, the comet assay has not yet been validated as a cancer biomarker and there no prospective data to indicate whether an increase in DNA damage detected by the standard comet assay translates into an increased risk of cancer in humans (Moller, 2006). The comet assay does appear to be able to discriminate between known carcinogens and non-carcinogens in rodents (Sasaki *et al.* 2000). It is also able to detect increased damage in humans exposed to antineoplastic alkylating agents, which are known carcinogens (Moller, 2006). Overall, such data are suggestive that increased DNA damage detected by the comet assay indicates potential carcinogenicity and possibly increased future risk of cancer. At the very least it is difficult to interpret the increase in DNA damage caused by raw broccoli in the current work as a beneficial effect.

Other than the work of the group before this project, no studies appear to have investigated the effect of raw broccoli consumption on basal levels of DNA damage in colonocytes. However, juice prepared from raw garden cress juice has been reported to decrease the background level of DNA damage in the colon of rats (Kassie *et al.* 2003). The different effect of garden cress juice and broccoli could reflect a species difference in response, or may be explained by variations in the content of bioactive compounds such as GLS (glucotropaeolin predominates in garden cress whereas glucobrassicin and glucoraphanin are the main GLS in broccoli) or the form of feeding, juice versus intact raw tissue (Kushad *et al.* 1999; Kassie *et al.* 2003). Although the current work appears to be the first report of cruciferous vegetables increasing DNA strand breakage in colonocytes, detrimental effects have been observed in a small number of other experimental studies (Kassie *et al.* 1996; Kassie & Knasmuller 2000; Murata

et al. 2000; Sorensen *et al.* 2001; Canistro *et al.* 2004; Paolini *et al.* 2004; Visanji *et al.* 2004). For example, AITC, PEITC and juices prepared from raw cruciferous vegetables have all been shown to produce positive results in a battery of genotoxicity and mutagenicity tests in bacterial and mammalian cells (Kassie *et al.* 1996; Kassie *et al.* 2000). Also, in rodents, an extract prepared from cooked Brussels sprouts has been shown to increase the levels of 8-oxo-dG in the liver, and ITC and indoles have occasionally been shown to promote or even initiate cancer, although the effects appear to be tissue specific (Owagi *et al.* 2001; Sorensen *et al.* 2001; see pgs 30-31 for a discussion of the possible tumour promoting effects of I3C).

In contrast to these negative findings, the majority of experimental studies have reported that cruciferous vegetables and various GLS breakdown products exert a range of anticarcinogenic actions such as, the induction of detoxification enzymes, cell cycle arrest, the promotion of apoptosis and protection against chemically induced colon cancer (See Chapter 1 or IARC, 2004 for reviews). It appears that whether cruciferous vegetables and GLS breakdown products exert beneficial or detrimental effects depends on the test system, dose, duration of exposure, and in the cases of *in vivo* studies possible how the vegetables are processed prior to feeding.

Most studies performed *in vitro* and in animal models have used unrealistically high doses of vegetables, GLS or GLS breakdown products. This makes extrapolation to humans difficult. Furthermore, work in cell culture cannot account for bioavailability or any metabolites produced by the colonic microflora. Arguably, an intriguing aspect of the current work is that it found a detrimental effect of raw broccoli consumption on colonocyte DNA at a level of intake that whilst greater than normal human intakes is substantially lower than levels commonly shown to induce chemoprotective effects in animal studies. To produce information that may be of more relevance to humans, future mechanistic work in animals needs to use realistic doses of GLS or vegetables.

The studies described in this thesis fed 600 g/d of raw broccoli. The original rationale was that this was consistent with public health recommendations to consume at least 5 portions of fruit and vegetables per day (a large portion being 120 g). However, it is unlikely that any human would habitually consume such quantities of raw broccoli. So, from a public health perspective it may not be particularly important that such quantities could be genotoxic to colonocytes. It is more important to know whether intakes of raw broccoli consistent with normal human intakes damage DNA in colonocytes. Future studies could feed graded intakes of broccoli encompassing levels consistent with habitual human intakes and levels shown to be genotoxic in the present study. This could include feeding broccoli on non-consecutive days because humans rarely consume the same vegetables each day. Such studies could provide information on the dose response relationship and help to clarify whether the consumption of raw broccoli at normal levels of intake is likely to be a public health concern. It is uncertain which constituent(s) of raw broccoli were responsible for its genotoxic effect however it could be common to all crucifers. Therefore, it would be of interest to test whether other raw cruciferous vegetables and combinations of cruciferous vegetables damage colonocyte DNA.

Although humans may be unlikely to habitually consume 600 g/d of raw broccoli, it is possible that some people in the population may be exposed to similar quantities of bioactive components through the consumption of either, supplements, concentrated food sources, or enriched food products. For example, broccoli sprouts containing 10 times greater amounts of glucoraphanin than standard broccoli are currently commercially available, as are a range of supplements containing substantial quantities of indole-3-carbinol, sulforaphane and quercetin (Fahey *et al.* 1997). The results of the present study indicate that relatively high intakes of phytochemicals may have unpredictable detrimental effects and suggest that the use of supplements and enriched food products may be unwise until we have a fuller understanding of their possible effects.

Ideally studies in humans are needed to clarify whether raw broccoli or other cruciferous vegetables influence risk of CRC. However, intervention studies are difficult because there are few reliable short-term biomarkers of risk and as discussed earlier, accessing tissue in healthy volunteers is challenging. Studies investigating the effect of cruciferous vegetables on tissue biomarkers in humans are sparse. Cooked Brussels sprouts (300 g/d) have been shown to cause a minor induction of GST isoenzymes in the rectal mucosa; however whether this would confer protection against CRC is uncertain (Nijhoff *et al.* 1995; see Chapter 1 pg 19). No studies appear to have measured the effect of cruciferous vegetable intake on DNA damage in human colonic DNA, however a small number of studies have assessed DNA damage in lymphocytes or measured the urinary excretion of 8-oxodG (a possible marker of whole body oxidative stress). Typically these studies have demonstrated that modest intakes of raw and cooked cruciferous vegetables (85-300 g/d) protect against DNA damage (Verhagen *et al.* 1995; Verhagen *et al.* 1997; Murashima *et al.* 2004; Gill *et al.* 2007; Hoelzl *et al.* 2008; Riso *et al.* 2009b). In a review of cruciferous vegetables and cancer it was suggested that protective effects seen in lymphocytes in these studies should translate into protection against DNA damage in the colon (Boyd *et al.* 2009). However, it is difficult to reconcile this argument with the knowledge that many ingested compounds accumulate to different extents in different tissues and that many compounds including ITC appear to exhibit organ specific genotoxicity and carcinogenicity (Deng *et al.* 1998; Owaga *et al.* 2001; Sorensen *et al.*2001). There is a need to clarify whether effects seen in lymphocytes reflect effects seen in tissues that are the site of major human diseases, such as the colon. Further studies in animals could measure DNA damage in lymphocytes and a number of organs to determine whether changes in lymphocytes are correlated with effects in other tissues. This would aid the interpretation of effects observed in lymphocytes and the design of future human intervention studies.

7.2 Conclusion

In conclusion, the work reported in this thesis appears to be the first to report that raw broccoli consumption increases DNA damage in colonocytes. Interestingly, this effect occurred at a level of intake below that normally shown to exert chemoprotective effects in other animal studies. The mechanism through which raw broccoli exerted its effect and the compounds responsible are unknown. Future studies are needed to confirm whether raw broccoli is genotoxic to human colonocytes and to identify the genotoxic component(s) and possible mechanisms of action.

It seems that whether cruciferous vegetables, GLS and their various breakdown products exert beneficial or detrimental effects may depend on the test system, dose, and duration and mode of feeding. In humans, the outcome of any exposure may also be modified by the genotype of the individual (see Chapter 1 pgs 34-38). Given the degree of uncertainty around the dose and frequency required to exert beneficial or detrimental effects, and the possibility that effects in different tissues may vary, it appears premature to make specific public health recommendations regarding the optimal intake of cruciferous vegetables to prevent CRC.

Standard curves and calculations

A.1 **Chapter 2**

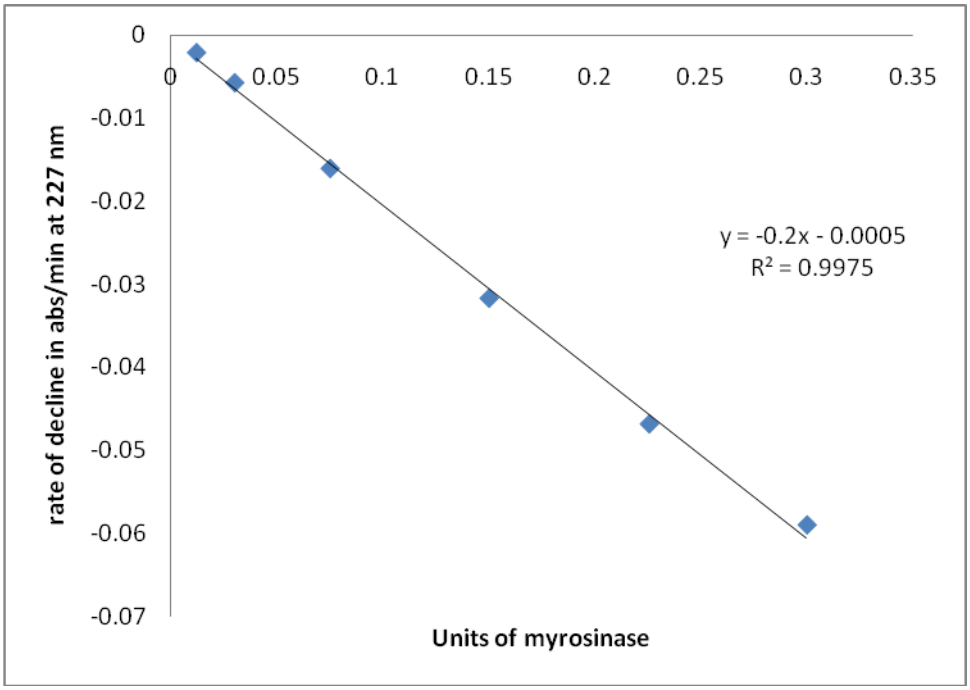


Fig A.1 Representative myrosinase standard curve. The curve was constructed by measuring the rate at which different concentrations of isolated myrosinase catalyse the decomposition of sinigrin, see Methods section of Chapter 2 (pg 51) for further details.

A1.1 ***Calculation of myrosinase activity in a sample of raw broccoli (Chapter 2, pg51)***

Mean rate of decline in absorbance = -0.03045/min

Unknown x= (y-b)/m

= (-0.03045-0.0005)/-0.2003

= (-0.02994)/-0.2003

= 0.149696 units per 100 µl of broccoli homogenate

= 0.149696 x 10 = 1.49696 units per ml of homogenate.

= 1.49696 x 5 (dilution factor) = **7.485** units of myrosinase/g of plant material.

A.2 **Chapter 4**

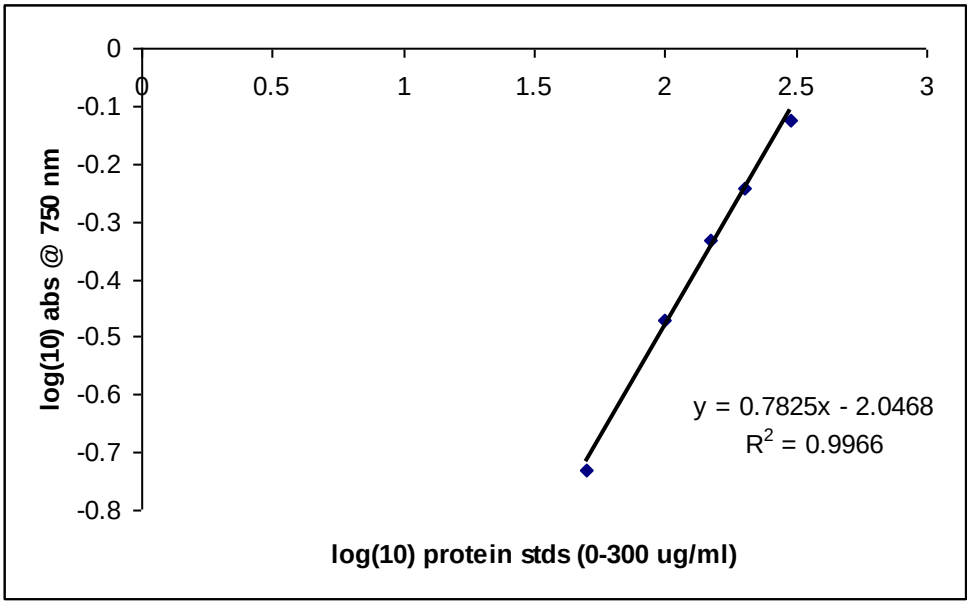


Fig A.2 Representative, protein standard curve. The curve was constructed from bovine serum albumin. The Lowry assay is inherently non-linear (see Chapter 4, pg 94) so standard curves were constructed from log transformed data, see Methods section, Chapter 4 (pgs 93-94).

A.2.1 ***Calculation of protein concentration*** **(Chapter 4, pgs 93-94)**

The absorbance values for each liver or colonic cytosolic preparation were log transformed and protein concentrations were determined by linear regression. The resulting values were back transformed.

Example:

Mean absorbance of a hepatic cytosolic preparation = 0.324

Log_{10} = -0.48945

Unknown = (y-b)/m

= (-0.48945- -2.0468)/0.7825

= 1.9902

= antilog 1.9902 = 97.77 µg/ml

= 97.77 x 200 (dilution factor) = 19554 µg/ml = **19.554** mg/ml

A.2.2 **Calculation of GST activity (Chapter 4, pg 95)**

GST activity was calculated in each hepatic and colonic cytosolic preparation using the following equation:

$$\frac{\Delta Abs/min\ sample - \Delta Abs/min\ blank}{9.6\ (ext.\ coefficient)} \times \frac{total\ cuvette\ volume}{sample\ volume} \times dilution\ factor = \textit{enzyme activity}$$

$$\frac{0.28300 - 0.01723}{9.6} \times \frac{1000}{100} \times 200 = 55.37\ \mu mol/ml/min$$

$$\textbf{\textit{Specific enzyme activity}} = \textit{enzyme activity} \times \frac{1}{mg\ of\ protein} = \mu mol/min/mg\ protein$$

$$= 55.37 \times \frac{1}{19.554} = 2.832\ \mu mol/min/mg/protein$$

Expressed as nmol/min/mg protein = 2.832 x 1000 = **2832** nmol/min/mg protein

A.2.3 **Calculation of QR activity (Chapter 4, pg 95)**

QR activity was determined in each hepatic and colonic cytosolic preparation in triplicate in the presence and absence of dicumoral. Dicumoral is a reasonably specific inhibitor of quinone reductase.

Mean Δabs/min without dicumorol - mean Δabs/min with dicumoral = dicumoral sensitive Δabs/min

$$= 0.1074 - 0.0172 = 0.0902/min$$

$$\frac{Dicumoral\ sensitive\ \Delta abs/min - \Delta abs/min\ blank}{21\ (molar\ ext.\ coefficient)} \times \frac{total\ cuvette\ volume}{sample\ volume} \times dilution\ factor = \textit{enzyme activity}$$

$$= \frac{0.0902 - 0.009}{21} \times \frac{3010}{300} \times 100 = 3.8795$$

Specific enzyme activity = *enzyme activity* X $\frac{1}{\text{mg of protein}}$ = μmol/min/mg protein

= 3.8795 X $\frac{1}{19.55}$ = 0.1984 μmol/min/mg protein

Expressed as nmol/min/mg protein = 0.1984 X 1000 = **198.4** nmol/min/mg protein

A.2.4. **Calculation of glucosinolates separated by reverse phase HPLC (Chapter 4, pgs 97-98)**

The concentration of each individual desulphated GLS in the broccoli extracts was calculated using the equation shown below. GLS concentrations are expressed as μmol/g DM. Benzyl GLS is the internal standard.

$$[\text{Individual GLS}] = \frac{\text{peak area of GSL} \times [\text{benzyl GLS}] \times \text{response factor of GLS relative to benzylGLS}}{\text{peak area of benzyl GLS} \times \text{weight of extracted sample}}$$

Worked example for glucoraphanin

$$[\text{glucoraphanin}] = \frac{730.9897 \times 4.9667 \times 1.13}{3716.729 \times 1.0012} = \frac{4102.585}{3721.189} = \mathbf{1.102\ \mu mol/g\ DM}$$

A.3 **Chapter 6**

A3.1 **Calculation of total CYP450 in hepatic microsomes by the method of Omura & Sato (1964; Chapter 6, pgs 128-129)**

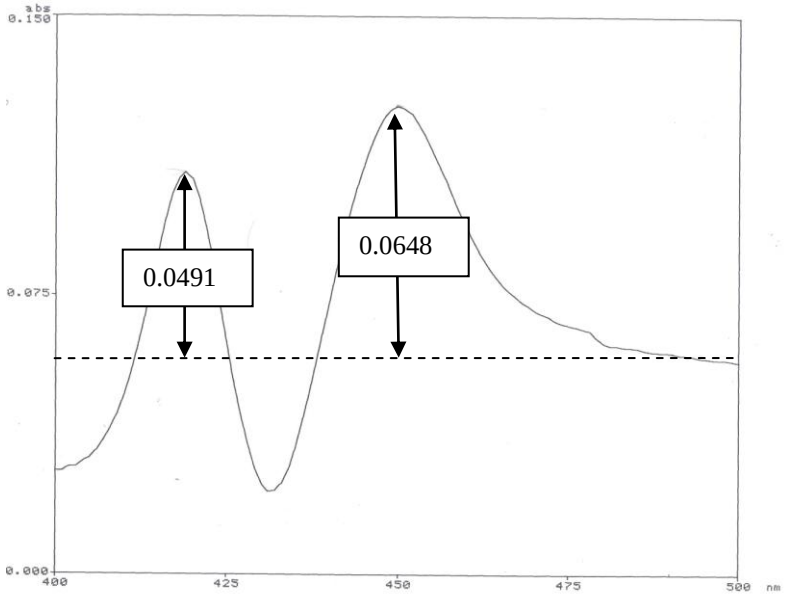


Fig A.3 Representative CYP450 spectra determined by the method of Omura & Sato (1964; see Chapter 6, pgs 128-129)

Total CYP450 content was determined by calculating the change in absorbance at 450 nm relative to 490 nm. The peak was measured with a ruler and converted into an absorbance value by adjusting for the scale of the y axis.

$$\text{CYP450 content} = \frac{(A450 - A490)}{0.091} \times \text{dilution factor} \times \frac{1}{\text{protein}} = \text{nmol/mg protein}$$

$$= \frac{0.0648}{0.091} \times 6.667 \times \frac{1}{8.91} = \mathbf{0.533} \text{ nmol/mg protein}$$

A.3.1.1 **Calculation of CYP420 content in hepatic microsomes (Chapter 6, pg 129)**

The change in absorbance at 420 nm relative to 490 nm was determined for each spectra as shown in Fig A.3. Total CYP420 content was then estimated using the following formula:

nmol of P450 per ml x (-0.041) = $(\Delta A_{420} - A_{490})$ *theoretical*

= 0.7121 x (-0.041) = -0.0292

$(\Delta A_{420} - A_{490})$ *Observed - (*$\Delta A_{420} - A_{490}$*) theoretical = nmol of P420*

= 0.0491 - (-0.0292) = 0.0783

= 0.0783 /0.110 = 0.7118 nmol P420 per ml

x dilution factor (6.667) x (1/mg protein)

= 4.745 x (1/8.91) = **0.532** mg CYP420/mg protein

(Guengerich, 2001)

A.3.2. **Calculation of total hepatic microsomal CYP450 measured by the method of Matsubara *et al.* (1976; Chapter 6, pg 129)**

Total CYP450 content measured using the method of Matsubara *et al.* (1976) was calculated as described in A.3.1 for the method of Omura & Sato (1964) except that a different extinction co-efficient was used.

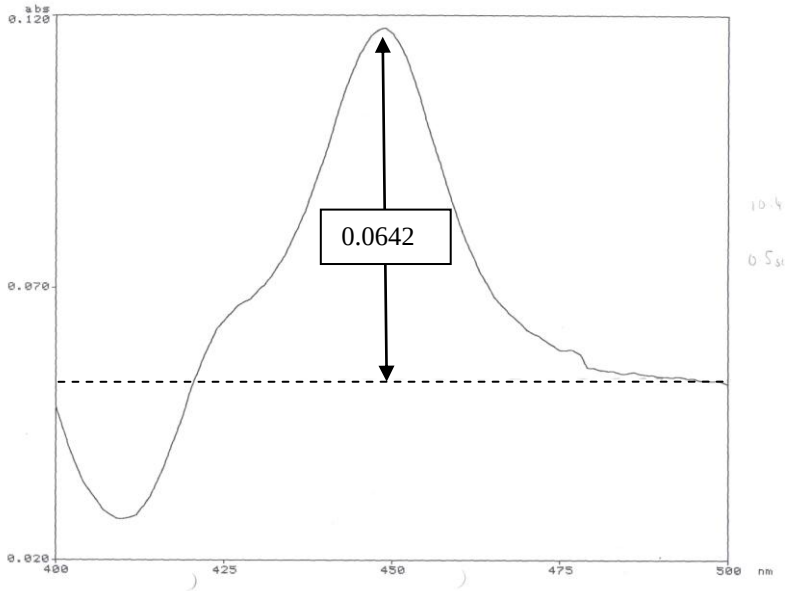


Fig A.3.1 Representative CYP450 spectra determined by the method of Matsubara *et al.* (1976; see Chapter 6, pg 129)

$$\text{CYP450 content} = \frac{(A450 - A490)}{0.106} \times \text{dilution factor} \times \frac{1}{\text{mg of protein}} = \text{nmol/mg protein}$$

$$= \frac{0.0642}{0.106} \times 6.667 \times \frac{1}{8.91} = \mathbf{0.453} \text{ nmol/mg protein}$$

A.3.3 **Estimation of haemoglobin content of a microsome (Chapter 6, pg 129)**

Haemoglobin was determined as described by Pearce *et al.* (1996) by calculating the change in absorbance from 405 to 420 nm. This was derived from each haemoglobin spectra as shown below. The peak was measured with a ruler and converted into an absorbance value by adjusting for the scale of the y axis.

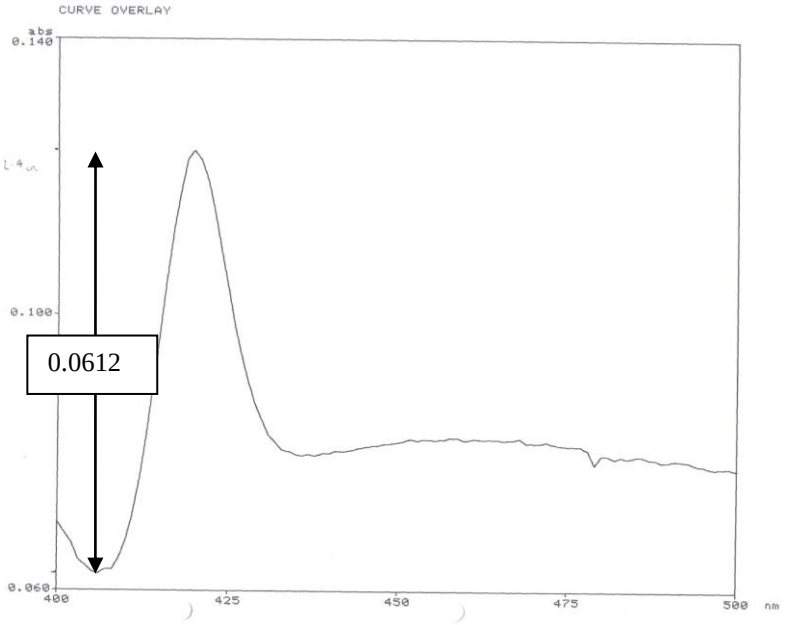


Fig A.3.2 Representative haemoglobin spectra (see Chapter 6, pg 129)

$$\text{Haemoglobin content} = \frac{(A420 - A405)}{0.110} \times \text{dilution factor} \times \frac{1}{\text{mg of protein}} = \text{nmol equivalents/mg protein}$$

$$= \frac{0.0612}{0.110} \times 6.667 \times \frac{1}{8.91} = \mathbf{0.416} \text{ nmol equivalents/mg protein}$$

A.3.4 **Calculation of EROD and MROD activity in hepatic microsomes (Chapter 6, pg 130)**

EROD and MROD activities were calculated using the same formula, shown below:

Rate of increase in fluorescence = 8.831/min

Y nM of resorufin = Z fluorescence units

(0.1 nM of resorufin = 65.811 fluorescence units)

$$\text{nM/unit} = \frac{\text{Ynm resorufin}}{\text{Z fluorescence units}} = \frac{0.1}{65.811} = 0.00152 \text{ nM/unit}$$

Fluorescence units in sample/min x nM/unit = nM/min

8.831 x 0.00152 = 0.01342 nM/min

$$\textbf{\textit{Specific enzyme activity}} = \textit{enzyme activity} \times \frac{1}{\text{mg of protein}} = \text{nM/min/mg protein}$$

$$0.01342 \times \frac{1}{0.2} = \mathbf{0.0671} \text{ nM/min/mg protein}$$

Results are expressed as pmol/min/mg protein i.e. 0.0671 x 1000 = **67.1 pmol/min/mg prot**

(Fowler *et al.* 2001)

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