THE PHYSIOLOGICAL EFFECTS OF GLUCOSINOLATES AND S-METHYL CYSTEINE SULPHOXIDE ON SHEEP CONSUMING FORAGE BRASSICA CROPS

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Declaration

I hereby declare that I composed this thesis myself and that, unless otherwise stated, the work contained herein is my own.

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List of Abbreviations

AATCC	N-acetyl (allylthiocarbamoyl) cysteine
ACN	Allyl cyanide
ADF	Acid detergent fibre
AITC	Allyl isothiocyanate
ALP	Alkaline phosphatase
ARC	Agricultural Research Council
BBTU	Benzyl butyl thiourea
C	Centigrade
CHB	S-1-cyano-2-hydroxy-3-butene
CHEB	1-cyano-3,4-epithiobutane
CN	Cyanide ion
CP	Crude protein
d	day
DM	Dry matter
DMDS	Dimethyl disulphide
DTNB	5,5'-dithiobis-2-(nitrobenzoic) acid
ESP	Epithiospecifier protein
FTI	Free thyroxine index
g	Gram
GGTP	Gamma glutamyl transpeptidase
GLC	Gas liquid chromatography
GSH	Reduced glutathione
GSH-px	Glutathione peroxidase
h	Hour
HPLC	High performance liquid chromatography
J	Joule
kg	Kilogram
l	Litre
M	Molar
ME	Metabolisable energy
MFO	Mixed function oxidase
min	Minute
MLURI	Macaulay Land Use Research Institute
mol	Mole
NDF	Neutral detergent fibre
O.D.	Outer diameter
PCV	Packed cell volume
OM	Organic matter
5-OZT	5-Oxazolidine thione
RBC	Red blood cell
RIA	Radio-immuno assay
rpm	Revolutions per minute
RSM	Rapeseed meal

SCN	Thiocyanate ion
SE	Standard error of mean
SED	Standard error of difference
SOD	Superoxide dismutase
SMCO	S-Methyl cysteine sulphoxide
T3	Tri-iodothyronine
T4	Thyroxine
UV	Ultra-violet
VFI	Voluntary food intake
W	Weight

ABSTRACT

The growth of sheep fed forage brassica crops is lower than would be predicted from the chemical composition of the crops, which are generally highly digestible and contain moderate concentrations of carbohydrate and protein. The problem has been attributed to low voluntary food intake (VFI) and among potential reasons for this is the presence, in the herbage, of secondary plant metabolites. The fate and physiological effects of two groups of compounds, the glucosinolates and S-methyl cysteine sulphoxide (SMCO) were studied in a series of *in vivo* and *in vitro* experiments.

The glucosinolate breakdown products allyl cyanide (ACN) and allyl isothiocyanate (AITC) were continuously infused for 21 days into the rumen of sheep fed either fresh forage rape or dried grass pellets. The VFI of forage rape by ACN-infused sheep (2.4 mmol / d) was reduced, although not significantly, while AITC (2.4 mmol /d) caused no VFI reduction. Neither compound affected VFI when infused (4.8 mmol / d) into sheep fed the dried grass pellet diet. Thyroid hormone concentrations were unaffected by treatment on the dried grass diet but plasma T3 concentrations were reduced by AITC on the forage rape diet.

In a further experiment, 3 levels of ACN (0, 4.8 and 9.6 mmol / d) were infused intra-ruminally into dried grass-fed sheep for 63 days. Voluntary food intake was again reduced by treatment and liver damage was indicated by elevated plasma gamma glutamyl transpeptidase (GGTP) concentrations. Clinical indicators of kidney function (plasma creatinine, plasma urea) indicated no renal effects. Hepatic cytochrome oxidase activity was significantly depressed at the highest rate of ACN infusion at the end of the treatment period indicating chronic cyanide toxicity.

Rumen degradation of glucosinolate breakdown products was examined by measuring the stability of ACN and AITC in rumen fluid *in vitro*. ACN was degraded by rumen fluid from cabbage-adapted sheep but not when the donor had been offered dried grass pellets. In a further experiment, rumen fluid samples taken at intervals from sheep consuming cabbage for 30 days had variable ACN-degrading activity with little evidence for a cumulative increase in activity over time.

The digestive fate of sinigrin was examined by dosing sheep with either sinigrin, AITC or ACN and analysing for urinary metabolites of AITC in the urine. Preliminary results indicated the proportion of sinigrin broken down to AITC *in vivo* to be 0.53.

In a final experiment potential interactions between AITC, ACN (0 or 10 mmol / d) and the rumen product of SMCO, dimethyl disulphide (DMDS) (0 or 25 mmol / d) were studied by dosing sheep with combinations of compounds for 35 days. Combined administration did not increase the effects of individual compounds. The dual presence of DMDS and ACN reduced overall effects and this was attributed to changes in metabolic fate of the compounds emphasizing the importance of the composition of the whole diet in determining the ultimate effects of individual constituents.

CHAPTER 1 - INTRODUCTION

Forage brassica crops are widely used in temperate agricultural systems as a feed for ruminant livestock. Traditionally, kale has been used, *in situ*, for feeding dairy cows while rape and swedes have more often been used in sheep systems as a finishing feed for growing lambs. Swedes have also been used in beef cow and ewe rations. The primary current use of forage brassica crops in the UK is in the finishing of growing lambs. As such, they provide a source of fresh herbage in the autumn when grass pasture is unavailable or of poor nutritional quality. Although root crops are prevalent in the North East of Scotland, forage rape and "catch" crops such as stubble turnips and fodder radish are more commonly used in the rest of the country.

Brassica species, including the forage brassica crops used in agricultural systems, contain representatives of two groups of plant secondary compounds, which have potentially harmful effects on the animal. Glucosinolates are a group of plant thioglucosides, which undergo hydrolysis when the vegetative parts of the plant are damaged, to yield a range of potentially toxic compounds. These include nitriles, isothiocyanates, 5-vinyl oxazolidine-2-thione and thiocyanate ions. The potential toxic effects of these compounds are manifold but include effects on thyroid function and liver and kidney integrity as well as more fundamental effects on cellular metabolic processes such as oxidative phosphorylation. The presence of glucosinolates in rapeseed meal (RSM) has spawned a large body of work on the detrimental effects of the compounds to farm livestock including pigs, poultry, cattle and sheep. Much of this work has been directed towards quantifying the effects of various concentrations of the parent compounds in RSM on animal production variables, such as liveweight gain and voluntary food intake (VFI), without determining the underlying biochemical effects of specific compounds. More detailed toxicological experiments have been conducted on the effects of glucosinolate-derived compounds and analogous compounds on the physiology of laboratory animals such as rats and mice. However, information on the specific physiological effects of individual glucosinolates in ruminant animals is lacking.

S-Methyl cysteine sulphoxide is the other main secondary plant compound found in the vegetative parts of Brassica species. This non-protein amino acid has been shown to undergo hydrolysis in the ruminant digestive tract to yield dimethyl disulphide. Following absorption and entry into the peripheral circulation, dimethyl disulphide causes lysis of erythrocytes and haemolytic anaemia. The toxicology of SMCO and the epidemiology of haemolytic anaemia have been studied to a limited extent in ruminants. However, although haemolytic anaemia has been unequivocally linked to the presence of SMCO in the grazed plant, the intermediate biochemical processes leading to cell lysis are uncertain. In addition, the severity of the syndrome and the extent of its occurrence in brassica-fed sheep has not been clearly established.

Feeding experiments, in which the performance of sheep fed on forage brassica crops has been quantified, have indicated animal performance to be low in relation to the chemical composition of the feed. This shortfall in performance appears to be due largely to the poor VFI of sheep offered forage brassica crops. The following series of experiments was conducted to establish the extent to which secondary compounds present in brassica herbage are responsible for limitations in VFI through their physiological effects in the animal. Both the individual and the interactive effects of glucosinolates and SMCO in sheep were considered.

CHAPTER 2 - REVIEW OF LITERATURE

THE USE OF FORAGE BRASSICA CROPS IN THE FEEDING OF RUMINANT ANIMALS.

Introduction to forage brassica crops

Plants of the genus *Brassicae* are important agriculturally because of their use as vegetables, as oilseed crops and as feeds for livestock. Species used for the latter purpose include *Brassica campestris* (turnips), *Brassica oleracea* (kales and cabbages) and *Brassica napus* (rapes and swedes). This taxonomic distinction is of little practical relevance however and a more useful division, is into root and leafy crops. Crops used primarily for their roots include turnips and swedes, while the leafy crops comprise the kales, rapes and stubble turnips.

Root crops can be fed either *in situ* mainly to sheep, or harvested and fed indoors to both sheep and cattle. Root crops are generally sown as main crops in the spring and utilized in autumn and winter. Leafy crops are usually fed *in situ* with kale being sown predominantly as a main crop and traditionally fed to cattle, while the rapes and stubble turnips may be sown as a catch crop after, for example, a winter cereal crop, and are utilized principally by sheep in the autumn.

Fitzgerald (1983) documented the decline in the use of forage brassicas in the UK this century and reported a drop in the area sown to turnips and swedes of 86% in the period from 1901-1976. A similar drop in the use of leafy crops was evident with a slight increase in the 1960's being attributed to the use of kale for feeding to dairy cows. Hodgson, Milne and Armstrong (1986) also noted a decline in the use of forage brassica crops but pointed out that increasing use of rape and stubble turnips as a catch crop was not reflected in conventional statistical returns because of their late sowing dates. Among reasons for the decreasing popularity of forage brassica crops are the high labour requirements for successful husbandry, the increasing use of silage as a winter forage and the increasing use of artificial herbicides and fungicides obviating the need to use forage crops as "cleaning crops". There is evidence that the

century-long decline in forage brassica use is slowing down because of the increasing costs of silage production and the need for lower input agricultural systems (Fitzgerald, 1983). In the future, forage brassicas could have an increasing role to play in the UK sheep industry in spreading the supply of lamb onto the market particularly as seasonal government support for sheep-meat is removed (Milne,1990). The recent development of winter-hardy varieties of forage rape at the Scottish Crop Research Institute should aid this objective. In addition, new initiatives by bodies such as the Highlands and Islands Development Board which aim to promote the natural image of lamb and which, of necessity, rely on low input husbandry practices, such as the finishing of lambs on forage brassica crops, should help to improve the public perception of lamb as an environmentally acceptable food (P. Brown, pers. comm.)

Nutritive composition

Major nutrients

Selected studies of the chemical composition and nutritive value of forage brassica crops are summarised in Table 2.1 in order to highlight important features of herbage composition. The dry matter content of these crops is low with typical values falling between 80 and 150 g/kg DM.

Characteristic of the carbohydrate component of both root and shoot tissues of forage brassica species are the high levels of water soluble carbohydrates and low structural carbohydrate component of the dry matter. Soluble carbohydrate concentrations are particularly high in roots and values in excess of 400 g/kg DM are common. *In vitro* estimates of organic matter digestibility are uniformly high among forage brassica crops and may exceed 0.90 in young plants. High digestibility values together with high concentrations of water soluble carbohydrates make forage brassicas an excellent source of metabolisable energy and ME values of 11-14 MJ /kg OM are typical.

Crude protein concentrations tend to be higher in leaves than in roots of all species with the result that leafy crops such as rape have higher concentrations of crude protein (typically 200 g/kg DM) than root crops such as swedes (typically 100 g/kg

Table 2.1 Composition of forage brassica crops
Units : g/kg DM unless otherwise stated

Crop	DM (g/kg)	CP	WSC	OMD (no units)	ME (MJ / kg DM)	ASH	CF	ADF	NDF	Reference
Kale	154	204	NS	NS	NS	NS	147	NS	NS	Jones, 1959a
Rape	179	223	NS	NS	NS	NS	145	NS	NS	Jones, 1959b
Kale	135	143	302	0.889	14.28	118	123	NS	10.01	Wainman et al., 1984
Turnip	85	194	418	0.910	NS	81	NS	NS	6.50	Livingstone, Jones and Mennie, 1977
Swede	105	91	NS	0.876	14.00	60	82	NS	NS	Wainman et al., 1984
Kale	NS	164	279	0.881	12.82	122	NS	NS	12.79	Barry et al, 1984b
Swede	95	96	NS	0.932	14.00	66	NS	130	NS	Dewey and Wainman, 1984

Key CP = Crude protein
WSC = Water soluble carbohydrate
OMD = Organic matter digestibility
ME = Metabolisable energy
CF = Crude fibre
ADF = Acid detergent fibre
NDF = Neutral detergent fibre
NS = Not stated

DM). Crude protein concentrations at the lower end of this range may be insufficient to support optimum rumen microbial protein yields as pointed out by Milne (1990) and Burnett (1988). Experiments in which lambs grazing kale showed improved growth rates in certain circumstances when supplemented with intra-peritoneal methionine (Barry and Drew,1978; Barry, McDonald and Reid, 1981a) lend support to this hypothesis.

Consideration of whole plant changes in nutrient composition throughout the season is also important with respect to better prediction of nutrient supply to grazing animals over the season and a number of studies have addressed this question (Bradshaw and Borzucki, 1981; Bradshaw and Borzucki, 1983; Wainman, Dewey and Brewer, 1984; Jung, Byers, Panciera and Shaffer, 1986; Kunelius, Halliday, Sanderson and Gupta, 1989). Aside from a consistent increase in dry matter content over the season, no clear trends are evident with regard to changes in nutrient composition during the phase of plant growth at which the crop is most likely to be grazed. Indeed, the maintenance of nitrogen and energy levels, together with only a minor decline in digestibility characteristics throughout the season, are traditionally one of the attractive features of forage brassica crops. In one study, *in vivo* ME determinations showed ME values of a spring-sown kale crop to increase slightly through the growing season (11-12 MJ ME/kg DM) and this was associated with a steady increase in concentrations of water soluble carbohydrates (Wainman et al., 1984). Jung et al. (1986) examined changes in nutrient composition in a range of brassica forages over the season and found crude protein contents to increase slightly with plant maturity, although this was less true of roots than of leafy crops. In only one crop (kale) was there any significant decline in digestibility over the season and in all crops *in vitro* digestibility values remained above 0.80 throughout the season.

Mineral and trace element composition

The mineral and trace element composition of a particular forage crop is dependent on the soil upon which it is grown and general consideration of the adequacy of micro-nutrients of individual species would be unrealistic (Cornforth, Stephen, Barry and Baird, 1978). Studies which compare the composition of brassica species and

pasture grown on the same soils do, however, provide some indication of elemental deficiencies which may occur. Thus Cornforth *et al.* (1978) in a study involving swedes , turnips and kale concluded that brassicas contained higher sulphur concentrations and calcium:phosphorus ratios than concurrently grown grass pasture, while sodium, phosphorous, molybdenum, manganese and copper concentrations were lower than pasture values. In a similar comparison Barry *et al.* (1981a) found kale to have a calcium:phosphorus ratio of 6.9 compared with 1.2 for autumn pasture while sulphur concentrations in kale were almost double those of pasture values. In a later study, iodine concentrations in kale were found to be less than 0.10 those of pasture values and the diet was judged to be iodine deficient (Barry, Duncan, Sadler, Millar and Sheppard, 1983a). The major potential problem with regard to trace element composition of brassica herbage, however, relates to copper, whose concentration in brassica herbage is generally low (<0.5 mg/kg DM). High concentrations of sulphur in the herbage may further reduce copper availability and lead to deficiency symptoms (Barry *et al.*, 1981a; Barry, Millar, Bond and Duncan, 1983b). Because sheep maintain considerable copper reserves in the liver, copper deficiency is only likely to be a problem if animals consume brassica forage for longer than the typical 6-8 week finishing period.

Consideration of the composition of forage brassicas has shown that they have a potentially high nutritive value by virtue of their high ME values, high digestibility and moderate crude protein concentrations; furthermore declining digestibility analogous to that observed with grass pasture is not a prominent feature of the ontogeny of forage brassica species and nutritive value is maintained at a high level as the plants approach mature size. The mineral and trace element composition of forage brassica crops appear, in general, to be sufficient to satisfy requirements in most circumstances, although copper and iodine deficiency may become problems in long-term grazing situations. The extent to which the nutritive value of the feed is reflected in animal performance will now be considered.

Performance of sheep fed forage brassica crops.

A number of recent papers which address nutritional aspects of forage brassica crops make reference to the poor or variable performance of animals consuming the crops relative to the apparently high nutritive value of the grazed material. Consideration of live-weight gain data shows that performance of animals is indeed variable with growth rates varying from 0 to 250 g/d. Average growth rates also appear to be low and are generally in the range 100-150 g/d (Table 2.2). This sub-optimal performance has been attributed to low levels of VFI among animals grazing forage brassica crops.

Recent reviews (Fitzgerald, 1983; Milne, 1990) have pointed out the need to consider the limitations associated with field measurements of liveweight gain and food intake when evaluating these studies. Estimates of food intake made in outdoor studies are high relative to those measured in indoor, *ad libitum* feeding experiments and may reflect the problems of food intake measurement in the field (Barry et al., 1981a; Armstrong, Maxwell and Sibbald, 1984; c.f. Table 2.3). The errors involved in food intake measurement in the field are potentially large with estimates of herbage off-take by "before and after" cuts (e.g. Barry et al., 1981a) being at best approximate, and at worst misleading due to factors such as soiling and trampling. Estimation of food intake by faecal output methods employing markers (e.g. Young, Austin and Orr, 1982) must be treated with caution since the extremely high digestibility of brassica herbage will tend to magnify any error. Measurements of growth rates also have associated difficulties; grazing studies are often conducted in poor weather conditions in which soiling and wetting of the fleece can lead to significant error. Changes in gut-fill following movement of animals from grass pasture to brassica forage may also be important. Studies which detail the effects of feeding on forage brassicas on carcass composition have been conducted (e.g. Barry, Manley, Millar and Smith, 1984a) but tend to be the exception.

A further limitation in interpreting performance data relates to the failure to adequately quantify herbage allowance in many studies. In reviewing work on the effect of herbage allowance on lamb performance, Fitzgerald (1983) concluded that crop allowances below 6-7% W/d resulted in depressed food intake and performance.

Table 2.2 Performance of lambs grazing forage brassica crops

Crop	Allowance	Intake		LWG	Carcass gain	Regime	Reference
	% W/head/d	g OM/d	g DM/d	g/d	g/d		
Rape	7.0	770		100		Strip gr.	Armstrong et al, 1984a
	4.0	746		40		Strip gr.	
	7.0	856		130		Set st.	
	4.0	673		86		Set st.	
Kale	17.0		1430	132	94	Strip gr.	Barry et al, 1981a
	11.5		1375	131	93	Strip gr.	
	5.8		1045	87	59	Strip gr.	
Kale	12.7			108		Strip gr.	Barry et al, 1983b
Kale	9.3			104	65	Strip gr.	Barry et al, 1984a
				145	91	Strip gr.	
				148	86	Strip gr.	
				179	128	Strip gr.	
Rape			1080	152	71	Strip gr.	Fitzgerald and Black, 1984
Kale			1261	112	52	Strip gr.	
Fodder radish			948	108	37	Strip gr.	
Tyfon				249		Set st.	Koch et al, 1987
Cabbage				138		Set st.	Rutherford and Dover, 1987
Swede/turnip				65		Set st.	
Stubble turnip	4.0			3		Strip gr.	Young, Austin and Orr, 1982
	6.0			37		Strip gr.	
Fodder radish/rape	8.0			56		Strip gr.	
	4.0			52		Strip gr.	
	6.0			84		Strip gr.	
	8.0			89		Strip gr.	

Strip gr. = Strip grazed
Set st. = Set stocked

Admittedly, most performance studies with forage brassicas probably would have satisfied this level of allowance and certainly this is the case with the studies summarised in Table 2.2, but at least some of the variation in animal performance observed can be attributed to variation in herbage allowance.

Although of questionable application to the field situation, indoor measurements of VFI do provide reliable estimates of the food intake potential of forage brassicas and, as such, are valuable in assessing whether certain intrinsic properties of the crop cause food intake limitation. Data from three studies in which VFI of a number of crops has been determined indoors are summarised in Table 2.3; food intakes are scaled to metabolic live-weight for comparative purposes. Despite the range of material fed, VFI in these studies is fairly consistent and mean values between the three studies are similar at approximately 50 g DM/ $W^{0.75}$/d. Calculation of expected VFI using ARC (1980) data for a coarse forage with an ME value of 13.0 MJ ME/kg DM yields a predicted value of 70 g DM/kg $W^{0.75}$/d for a 30-40 kg growing sheep. Thus from the limited work conducted, the food intake potential of sheep consuming forage brassicas does appear to fall short of observed VFI's of a range of other forages surveyed on which the ARC (1980) equations were based. Furthermore the true potential VFI of these crops as grazed in the field may be much lower than those suggested by indoor food intake studies due to factors such as the physical structure of the crop and the environmental conditions in which the crop is consumed.

Thus, despite the fact that some of the observed variability in performance of animals grazing brassica crops may be attributed to variation in herbage allowance together with limitations in the methods used to assess performance, the "intake potential" of forage brassica crops does appear to be less than that of similar non-brassica forages.

Potential reasons for sub-optimal performance

Previous reviews of the use of forage brassica crops in livestock production systems (Nicol and Barry, 1980; Fitzgerald, 1983; Burnett, 1988) have examined potential reasons for the sub-optimal performance recorded in practical grazing

Table 2.3 Voluntary food intake of sheep offered brassica crops indoors.

Crop	Voluntary food intake g DM/ kg W $^{0.75}$/d	Mean voluntary food intake g DM/ kg W $^{0.75}$/d	Reference
Rape leaf	53.3		Armstrong et al., 1984b
Rape leaf	49.4		
Rape stem	47.5		
Rape stem	36.3	46.6	
Swede root	66.1		Barry, Drew and Duncan, 1971
Turnip root	47.5	56.8	
Cabbage leaf	46.6		Burnett, 1988
Whole hybrid turnip	61.5		
Stubble turnip leaf	60.9		
Rape leaf	56.6		
Kale leaf	46.4		
Rape stem	47.1		
Kale stem	49.5		
Stubble turnip root	62.8		
Swede root	52.1	53.7	

situations. Among suggested factors involved are amino acid deficiencies, mineral imbalances, insufficient herbage allowance, energy costs of harvesting feed, nitrate poisoning, tooth loss and sub-clinical health effects due to plant secondary compounds. A number of experiments have been conducted aimed at assessing the importance of one or more of these factors in depressing VFI of forage brassicas. In many cases, the results have been equivocal; for example, copper supplementation of kale diets resulted in improved performance in one study (Barry, Reid, Millar and Sadler, 1981b) but not in another (Barry et al., 1983b). Similarly, supplementation of kale-fed sheep with intra-peritoneal methionine improved growth rates in one experiment (Barry and Drew,1978), but not in another (Barry et al., 1981a). Furthermore, addition of S-methyl cysteine sulphoxide (SMCO) to non-brassica diets caused VFI depression, but only when added at levels higher than those typical of brassica forages (Barry, Manley and Millar, 1982). It would appear, therefore, that many of these factors are indeed important in the nutrition of ruminants fed on forage brassicas but no factor, in isolation, can explain the sub-optimal performance observed among animals grazing forage brassica crops. The remainder of this review will consider the importance of the two main groups of brassica anti-metabolites, the glucosinolates and S-methyl cysteine sulphoxide and their effects on animal physiology and health. Other factors will be discussed in as much as they are important in either enhancing or alleviating the potential toxicity of the secondary plant compounds occurring in brassica herbage.

PHYSIOLOGICAL EFFECTS OF BRASSICA SECONDARY METABOLITES - GLUCOSINOLATES

Glucosinolates have been the subject of a host of reviews which have placed varying emphasis on their distribution (e.g. Kjaer, 1960; Fenwick, Heaney and Mullin, 1983), chemistry (Benn, 1977), biosynthesis (Underhill and Wetter, 1973; Kjaer, 1976; Underhill, 1980) and physiological effects. (Heaney and Fenwick, 1987; Duncan and Milne, 1990). Areas which have been inadequately reviewed and which have the most relevance to the current work include studies of glucosinolate toxicity and metabolic fate. Therefore, aspects relating to the distribution, structure and biosynthesis of

glucosinolates in the plant will only be briefly discussed before describing in detail, studies concerned with their toxicity and metabolism in the animal.

Distribution, Biosynthesis and Structure of Glucosinolates

Glucosinolates are a group of plant thioglucosides found in a wide range of dicotyledonous plant families including the *Capparaceae, Cruciferae, Moringaceae, Resedaceae* and *Tovariaceae*. However, it is their presence in the *Cruciferae* and more specifically in the genus, *Brassica*, which is of interest here. Their chemical structure was ultimately elucidated in 1956 as consisting of a B-thioglucose group, linked by a side chain, R-, to a sulphonated oxime moiety (Ettlinger and Lundeen, 1956) (Figure 2.1). Over 90 different R-groups have been described and, although some plant species have been found to contain as many as 30 different glucosinolates (Grob and Matile, 1980), in general, fewer than five are prominent in any one species. The R-group varies widely in structure and may be either aliphatic or aromatic. Of the aliphatic glucosinolates found in the forage brassica crops, a terminal unsaturated bond is a common feature, the simplest case being prop-2-enyl glucosinolate or sinigrin which is ubiquitous in the kales and cabbages (*Brassica oleracea*). Methyl, sulphonyl and hydroxyl groups may also be present; for example, 2-hydroxy-3-butenyl glucosinolate is found in high concentrations in the swedes and rapes (*Brassica napus*). Of the aromatic glucosinolates, those with an indolyl group are the most important with respect to forage brassica species and concentrations of indole glucosinolates are especially high in the vegetative tissue of a number of *Brassica* species. The structures of glucosinolates prominent in cabbage leaves are depicted in Figure 2.1 to illustrate the diversity of the R-group of the glucosinolates.

Glucosinolates are found throughout the plant material with concentrations in seeds being typically an order of magnitude greater than concentrations in the vegetative tissue. Seeds of rape (*Brassica napus*), for example, may contain 100 mmol/kg DM of total glucosinolates, while concentrations in the vegetative tissue may be approximately 10 mmol/kg DM. In a survey of the glucosinolate content of a range of forage brassicas (Bradshaw, Heaney, MacFarlane Smith, Gowers, Gemmel and Fenwick,

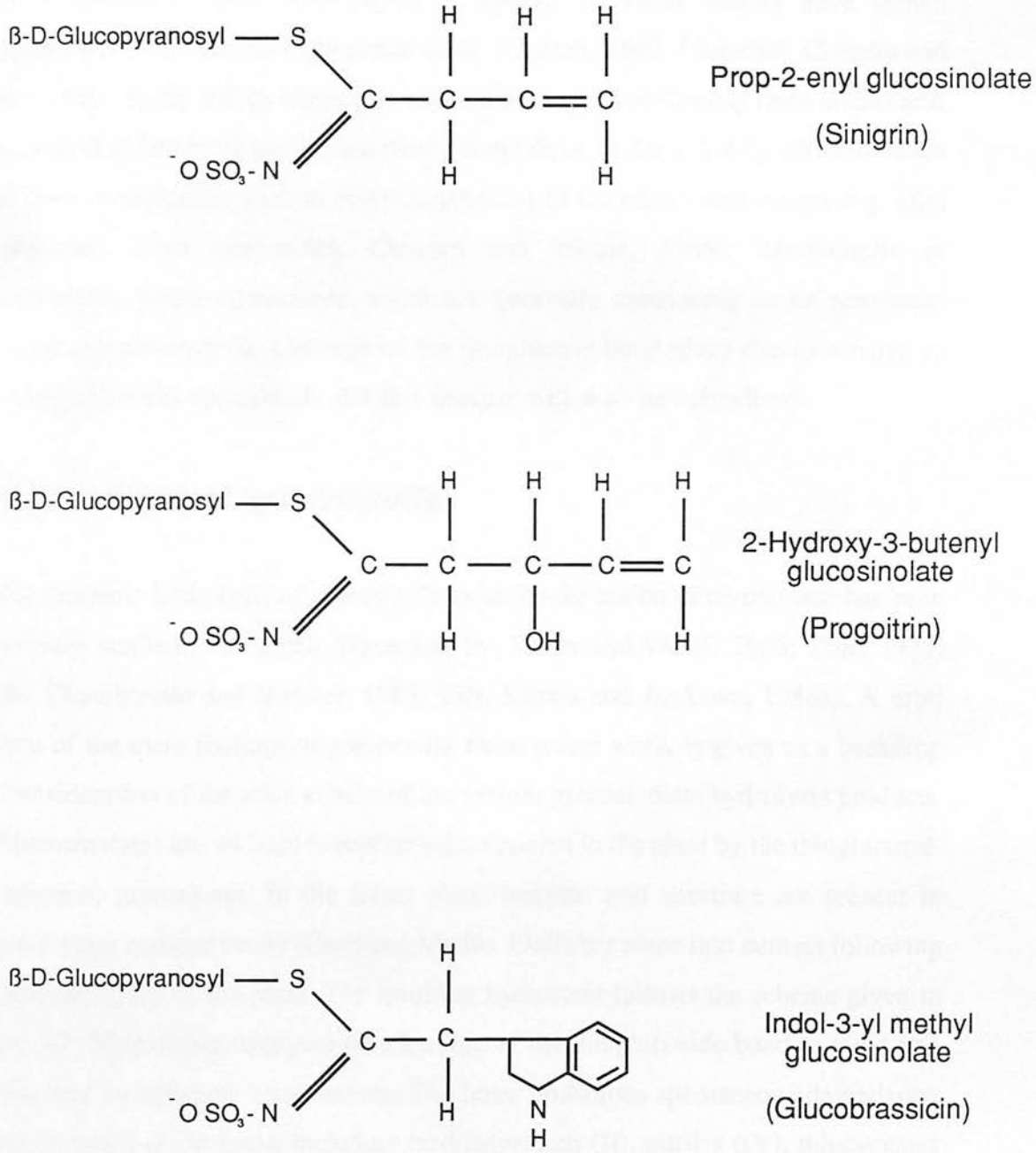


Figure 2.1 Structures of prominent glucosinolates found in cabbage (*B. oleracea*)

1984), total glucosinolate concentrations ranged from approximately 10 mmol/kg DM in swede bulb to approaching 30 mmol/kg DM in cabbage leaves, while individual concentrations of as high as 22 mmol/kg DM were recorded in rape stem (2-hydroxy but-3-enyl glucosinolate).

With respect to their biosynthesis, a number of tracer studies have shown glucosinolates to be derived from amino acids (Kutacek, 1962; Underhill, Chisolm and Wetter, 1962; Benn, 1962). Some glucosinolates are derived directly from amino acid precursors (e.g. benzyl glucosinolate from phenyl alanine; Benn, 1962), while in others some prior modification such as chain lengthening of the amino acid occurs (e.g. allyl glucosinolate from methionine; Chisolm and Wetter, 1964). Biosynthesis of glucosinolates yields compounds which are generally considered to be non-toxic. However, simple enzymic cleavage of the thioglucose bond gives rise to a range of potentially harmful compounds and this process will now be considered.

Enzymic hydrolysis to toxic metabolites

The enzymic hydrolysis of glucosinolates under the action of myrosinase has been extensively studied (Van Etten, Daxenbichler, Peters and Wolff, 1966; Cole, 1975; Searle, Chamberlain and Butcher, 1983; Uda, Kurata and Arakawa, 1986a). A brief account of the main findings, together with more recent work, is given as a backdrop to a consideration of the toxic effects of the various glucosinolate hydrolysis products.

Glucosinolates are without exception accompanied in the plant by the thioglucosidase enzyme, myrosinase. In the intact plant, enzyme and substrate are present in different plant compartments (Grob and Matile, 1980) but come into contact following mechanical injury of the plant. The resulting hydrolysis follows the scheme given in Figure 2.2. Myrosinase catalyses the cleavage of the thioglucoside bond to yield free glucose and an aglucone intermediate. The latter undergoes spontaneous degradation to yield a range of products, including isothiocyanates (II), nitriles ((V), thiocyanates (III) and epithioalkanes. In the case of the glucosinolate, 2-hydroxy-3-butenyl glucosinolate, a β-OH group on the R-chain of the aglucone facilitates cyclisation of the isothiocyanate to produce 5-vinyl oxazolidinethione (5-OZT). Of particular interest

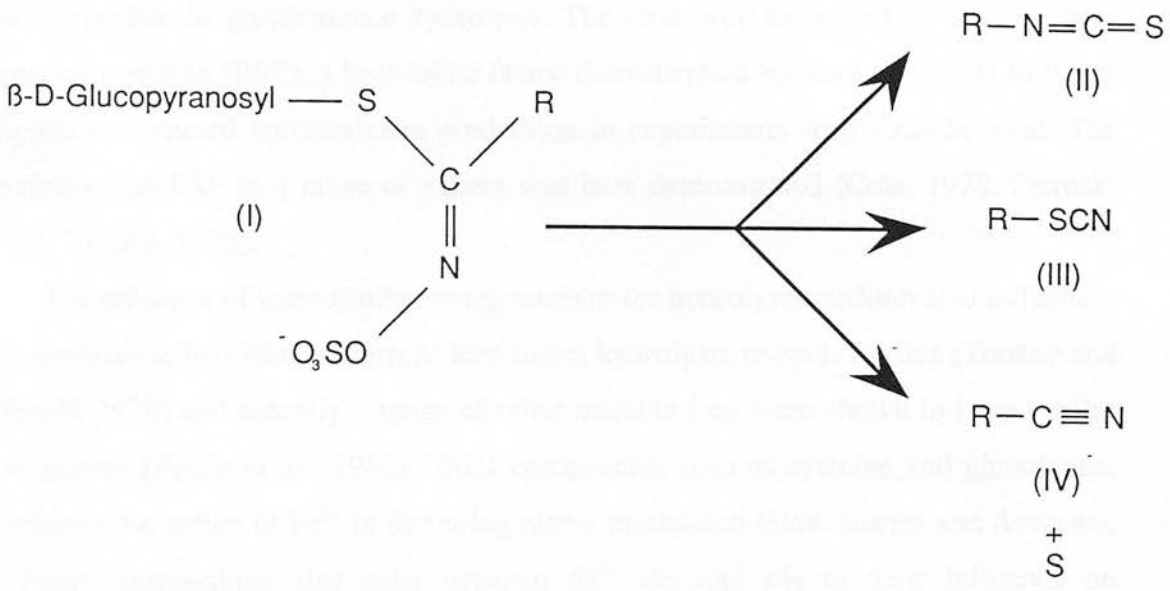


Figure 2.2 Enzymic hydrolysis of glucosinolates

in relation to the toxicology of glucosinolates is the observation that the products arising following aglucone degradation are influenced by features of the hydrolysis environment such as pH, temperature, Fe^{2+} and Fe^{3+} concentrations and the presence of various protein co-factors. These interact in a complex way to determine the proportions of the various hydrolysis products arising following glucosinolate hydrolysis but certain general conclusions can be drawn.

Low pH tends to favour nitrile production while neutral and high pH leads to isothiocyanate production (Van Etten et al., 1966). For example, Uda et al.(1986a) included an assessment of pH effects in a study of sinigrin hydrolysis *in vitro*. Increas-

ing pH was found to increase the rate of glucose release up to pH 6.5 as well as favouring formation of allyl isothiocyanate at the expense of allyl cyanide. Thus, pH was shown to influence both enzymic aglucone formation as well as the non-enzymic rearrangement of the aglucone to give the final products.

Temperature appears to have little direct effect on the proportions of glucosinolate hydrolysis products (Gil and MacLeod, 1980); however, indirect temperature effects have been noted, associated with the denaturation of certain heat-labile factors which are important in glucosinolate hydrolysis. The most well known of these is epithiospecifier protein (ESP), a heat-labile factor demonstrated by Tookey (1973) to direct hydrolysis toward epithioalkane production in experiments with *Crambe* seed. The existence of ESP in a range of genera was later demonstrated (Cole, 1978; Petroski and Tookey, 1982).

The presence of various other compounds in the hydrolysis medium also influences glucosinolate breakdown. Ferrous ions direct hydrolysis towards nitriles (Tookey and Wolff, 1970) and recently a range of other metallic ions were shown to have similar properties (Searle et al., 1983); Thiol compounds, such as cysteine and glutathione, enhance the action of Fe^{2+} in favouring nitrile production (Uda, Kurata and Arakawa, 1986b). Interactions also exist between Fe^{2+} ion and pH in their influence on glucosinolate hydrolysis (Uda et al., 1986a).

Glucosinolate hydrolysis is clearly a complex process and the range of hydrolysis products arising is highly dependent on the nature of the medium. This is important in consideration of the likely toxic effects of glucosinolates. Conditions in the digestive tract are likely to greatly influence which toxic products are produced following plant ingestion. An obvious example is the effect of gastric pH on glucosinolate hydrolysis; the acid conditions characteristic of the monogastric stomach provide a very different chemical environment to that of the ruminant fore-stomach. Comparative studies are likely to lead to an improvement in our understanding of glucosinolate toxicology.

Anti-nutritional effects of consuming glucosinolate-containing material

The general phenomenon of impaired performance amongst animals consuming glucosinolate-containing material is well known, particularly in relation to the effects of rapeseed glucosinolates on production characteristics of poultry and pigs. There is a vast literature relating glucosinolate content of rapeseed meals with animal production variables such as live-weight gain and egg production (e.g. Bell, Young and Downey,1971; Lo and Bell, 1972; Wight, Scougall, Sharman and Wells, 1987a)

Growth and performance of pigs and poultry is impaired when rapeseed meal is included in the diet. High glucosinolate rapeseed meals may be included at levels of up to 50 g/kg of the diet without significantly impairing performance, while low glucosinolate meals can constitute around 10% of the ration without significant performance depression (Rundgren, 1983; Fenwick and Curtis, 1980). Performance experiments indicate that ruminants are more tolerant to the inclusion of rapeseed meal in the diet than mono-gastric animals (Thomke, 1981); inclusion levels of low glucosinolate rapeseed meal of up to 25% have been reported before significant effects on performance have been noted. The greater tolerance of ruminants to rapeseed meal in the diet may be a result of detoxification of deleterious compounds in the rumen. Inclusion levels for both high and low glucosinolate rapeseed meals in rations for livestock recommended by the Canola Council of Canada illustrate the greater tolerance of ruminants than mono-gastrics to rapeseed meal (Fenwick, 1982). These recommendations are based on a considerable body of work conducted in Canada using both traditional and newer, low glucosinolate varieties such as Candle and Tower rapeseed.

It is worth pointing out that inclusion of rapeseed meal within recommended levels does not necessarily imply a lack of detrimental effects; recommendations rather indicate acceptable inclusion rates for commercial purposes and represent one point along a continuum of toxicity. The inclusion levels chosen for the purposes of national recommendations reflect the sensitivity of experimental procedures along with economic considerations more than the biology of the situation.

Work on the effects of glucosinolates present in forage brassicas on animal performance is scarce. Preliminary work in which the breakdown products of sinigrin, allyl isothiocyanate and allyl cyanide were infused into the rumen of lambs, showed only transitory depression of VFI in response to the compounds when animals were offered a diet of cabbage (Burnett, 1988). No such VFI depression occurred when the compounds were infused against a dried grass diet. Plasma thyroid hormone concentrations were unaffected by glucosinolate administration regardless of diet. The infusion rates in these experiments were conservative, however, being approximately 10% of potential natural ingestion rates and further studies employing higher concentrations are required.

The reasons for the depressed and variable performance of farm livestock on diets containing glucosinolates are not well established but have been attributed to their goitrogenic properties, palatability effects and generalised metabolic responses. Current understanding of these effects comes primarily from studies with mono-gastrics with only limited data being available for ruminants and this is reflected in the range of work reviewed in the following paragraphs.

A common experimental approach has been to process rapeseed meal in various ways in order to adjust both absolute concentrations of parent compounds and also likely toxic hydrolysis products in the feed, and to compare resulting toxicity. For example, Srivastava, Philbrick and Hill (1975) controlled hydrolysis of glucosinolates in rapeseed meal to produce meals rich in either nitrile hydrolysis products or goitrin. The resulting meals fed to rats or chicks both caused depression in live-weight gain but the toxic mechanisms appeared to be different; the goitrin-rich meal caused increased thyroid weight, whilst the nitrile-rich meal resulted in kidney enlargement. More recently, Wight et al. (1987a), in attempting to determine the agent responsible for liver haemorrhage of laying hens, manipulated rapeseed diets in order to produce a nitrile-rich meal. However, the incidence of liver haemorrhage was not increased among animals consuming the nitrile-rich meal. Campbell (1987) also fed variously treated rapeseed meals to laying hens and related the concentrations of intact glucosinolates and their various breakdown products to the incidence of liver haemorrhage. Liver haemorrhage appeared to be related to the presence of intact

glucosinolates in the diet but there were no obvious effects of level of breakdown products. Another approach has been to subject seed meals to various extraction procedures and to add the extracts to glucosinolate-free diets. Lee, Pittam and Hill (1984) added intact glucosinolate extracts to soyabean meal and demonstrated a depression in the VFI of growing pigs consuming the diet. This approach has also allowed toxic effects to be attributed to more specific groups of glucosinolate hydrolysis products. Van Etten, Gagne, Robbins, Booth, Daxenbichler and Wolff (1969) fed isolates of *Crambe* seed meal and showed goitrin to cause thyroid enlargement while a nitrile fraction affected the liver and kidney tissues of dosed rats. A similar approach was adopted by Lo and Bell (1972), who prepared a rapeseed isolate composed largely of butenyl isothiocyanate and butenyl cyanide. Rats dosed with this mixture showed depressed live-weight gain and VFI and, although thyroid weight was unaffected, tracer studies with ^{125}I suggested that biosynthesis of thyroid hormones was affected by the treatment.

The development of improved methods of isolating intact glucosinolates from plant material led to a series of experiments in which individual pure glucosinolates were fed to animals to determine their anti-nutritional effects (Bille, Eggum, Jacobsen, Olsen and Sorensen, 1983; Eggum, Olsen and Sorensen, 1983; Eggum, Olsen and Sorensen, 1985; Vermorel, Heaney and Fenwick, 1987). The emphasis of this work was more on performance aspects, although organ weights were recorded as crude measures of toxicological effect. In a recent experiment, Vermorel et al., (1987) showed that administration of a range of intact, isolated glucosinolates to rats over a period of 29 days at levels representative of those in low glucosinolate rapeseed had little effect on VFI or live-weight gain; indeed there were trends towards increased VFIs on the glucosinolate diets. The results of this experiment emphasize the difficulties involved in assessing the toxicity of compounds, which enter the body in benign form and yield a range of potentially toxic metabolites, the release of which is dependent on the presence of myrosinase and the proportions of which are influenced by features of the hydrolysis environment, such as pH and metallic ion concentration. In the above experiment, knowledge of the extent of glucosinolate breakdown or the proportions of toxic products produced in the alimentary tract could

have aided its interpretation. In order to understand the anti-nutritional effects of glucosinolates more clearly, consideration must be given to the toxic properties of the breakdown products.

Toxic properties of breakdown products

Nitriles

The toxicity of glucosinolate-derived nitriles is generally attributed to their effects on the liver and kidney and increased weights of these organs in animals exposed to nitriles have regularly been observed (Van Etten et al., 1969; Srivastava et al., 1975; Nishie and Daxenbichler, 1980). The biochemical mechanisms underlying nitrile toxicity remain obscure though a number of histological studies have been conducted.

Histological examination of kidney tissue from rats dosed with a crude nitrile extract of *Crambe* seed meal revealed hypertrophy and enlarged nuclei of the epithelial cells lining the convoluted tubules (Van Etten et al., 1969). A similar phenomenon was observed by Gould, Gumbmann and Daxenbichler (1980) in rats receiving the hydroxylated nitrile, 1-cyano-3,4-epithiobutane (CHEB), in a chronic toxicity experiment. Acute doses of S-1-cyano-2-hydroxy-3-butene (CHB) to rats also produced karyomegaly and hypertrophy of the kidney tubule epithelial cells (Nishie and Daxenbichler, 1980) and Gould, Fettman, Daxenbichler and Bartuska (1985) likewise noted the rapid production of kidney lesions in rats receiving CHEB; in addition, elevated levels of plasma urea nitrogen and creatinine suggested functional alterations in the kidneys of rats receiving CHEB.

Liver lesions, both gross and histological, have also been attributed to nitrile toxicity. Van Etten et al. (1969) reported disruption of the normal lobular structure of the livers of nitrile-fed rats along with irregular bile duct proliferation. Bile duct proliferation was a prominent feature of the histological lesions observed by Gould et al. (1980) in CHEB-dosed rats. Other lesions included hepatocyte necrosis associated with elevated levels of serum alkaline phophatase, gamma glutamyl transpeptidase (GGTP) and other plasma enzymes, indicating hepatocyte damage and cholestasis.

Nitriles have also been implicated in the aetiology of liver haemorrhage in laying hens (Campbell, 1987).

Although rarely considered in the context of glucosinolate-derived nitriles, *in vivo* release of free cyanide in the tissues may be an important route of nitrile toxicity. The acute toxicity of a number of aliphatic nitriles, both saturated and unsaturated, has been attributed to their biotransformation to cyanide (Ohkawa, Ohkawa, Yamamoto and Casida, 1972; Willhite and Smith, 1981) and a similar phenomenon may occur in the case of chronic exposure to naturally derived nitriles. Toxicity appears to be related to chemical structure, presumably due to differences in metabolism and extent of free cyanide release (Ahmed and Farooqui, 1982). Willhite and Smith (1981) showed the liver microsomes to be a site of biotransformation using *in vitro* incubation techniques.

The capacity of nitriles to bind sulphydryl groups and so lower tissue glutathione levels was suggested by Szabo, Bailey, Boor and Jaeger (1977) and Ahmed and Farooqui (1982) as being responsible, in part, for their toxicity but neither set of authors proposed specific mechanisms. Further general toxic effects of nitriles include haemorrhage and necrosis in the adrenal cortex of rats dosed with acrylonitrile (Szabo, Huttner, Kovacs, Horvath, Szabo and Horner, 1980) and ulcerogenic effects (Szabo and Reynolds,1975).

There is considerable evidence for an association between the levels of rapeseed in the diet and the incidence of liver haemorrhage (Yamashiro, Umemura, Bhatnagar, David, Sadiq and Slinger, 1977; Papas, Cansfield and Campbell, 1979) but attempts to correlate pathology with specific toxic agents, including nitriles, have been unfruitful. Campbell (1987) fed rapeseed meal diets containing varying concentrations of intact glucosinolates and breakdown products to laying hens; there appeared to be no clear association between the concentrations of intact glucosinolate, goitrin or CHB and the occurrence of liver haemorrhage. Wight, McCorquordale and Scougall (1987b) attempted to elucidate the causative mechanism of liver haemorrhage by supplementing rapeseed meal and soyabean meal diets with various additives with known effects on liver metabolism and determining their influence on the incidence of liver haemorrhage. For example, it was suggested that liver haemorrhage might be caused

by weakening of hepatic blood vessels, due to inhibited collagen synthesis caused by hypothyroidism, or more directly by lathyrogenic compounds such as nitriles. These possibilities were tested by adding the goitrogen, thiouracil, and the lathyrogen, β-aminopropionitrile, to RSM diets and looking histologically at effects on liver haemorrhage. However, none of the agents produced liver haemorrhages characteristic of those produced on rapeseed meal diets and the aetiology of the syndrome remains obscure.

Isothiocyanates

A small number of studies suggest that nitriles predominate in the digestive tract of monogastrics (Smith and Campbell, 1976) but, pending more detailed work, isothiocyanates must also be considered as potential toxic agents. There is a lack of information on the intestinal metabolism of glucosinolates and the concentrations of various breakdown products produced in the animal.

The reported gross physiological effects of the isothiocyanates are diverse and the underlying biochemical mechanisms are often unclear. Early workers found allyl isothiocyanate (AITC) to be goitrogenic; daily dosing of rats with the compound led to increases in thyroid weight (Langer, 1964; Langer and Stolc, 1965), although the goitrogenic properties of the compound were found to be weak, compared to more conventional goitrogens such as thiouracil (Ahmad and Muztar, 1971). Possibly related to effects on metabolic rate and fuel metabolism through depressed thyroid function were the increased plasma phospholipid levels noted by Idris and Ahmad (1975) in rats dosed with AITC. These authors also observed AITC to decrease blood clotting time, a phenomenon which they attributed to elevated plasma phospholipid levels. Further effects on metabolism were reported by Muztar, Ahmad, Huque and Slinger (1979a), who administered AITC to rats and observed depressed levels of plasma glucose and uric acid. Hypothyroidism was again suggested as being responsible for altered carbohydrate metabolism, although more direct effects on enzymes involved in carbohydrate metabolism may have been a factor (Ahmad, Rahman, Rahman and Begum, 1967). The goitrogenic effects of the isothiocyanates may in part be associated with their metabolism to thiocyanate ion. Thiocyanate ion, along with other

monovalent anions, depresses uptake of iodine by the thyroid glands by simple, competitive inhibition. This may be the explanation for reduced uptake of ^{131}I by the thyroids of rats given isothiocyanates in a number of studies (Langer and Stolc, 1965; Langer and Greer, 1968).

In addition, isothiocyanates may have more direct toxic effects associated with their extreme electrophilic nature and their capacity to bind the sulphydryl groups of biologically important molecules (Bjorkmann, 1973; Tang, 1974; Kawakishi and Namiki, 1982; Kawakishi, 1983). For example, the formation of complexes between isothiocyanates and the sulphydryl moiety of tyrosine may be responsible for depressed biosynthesis of thyroid hormones, as reported in a number of studies (Langer and Greer, 1968; Muztar, Huque, Ahmad and Slinger, 1979b). Analogous mechanisms can be conceived for a host of metabolic processes; for example, inactivation of anti-diuretic hormone (Muztar et al., 1979a) and the denaturation of insulin following cleavage of the disulphide bond (Kawakishi, Goto and Namiki, 1983). Complex formation may be a reversible process, however, as was found for glutathione-AITC complexes *in vitro* by Bruggemann, Temmink and van Bladeren (1986). They showed glutathione conjugation to be an important excretion route for isothiocyanates but pointed out that dissociation in certain chemically favourable sites may lead to localization of the toxic effects of the isothiocyanates.

Goitrin

The gross goitrogenic effects of 5-vinyl oxazolidinethione (5-OZT) are well documented with many studies demonstrating the potent effects of the compound on the thyroid weight of rats (e.g. Krusius and Peltola, 1966; Langer and Michajlovskij, 1969; Akiba and Matsumoto, 1976) even at low dose rates (approximately 1-2 µg/rat/d). The mechanism of action of the compound is less clear with some studies reporting depression of thyroidal iodine uptake (Langer and Michajlovskij, 1969), some reporting increases (Akiba and Matsumoto, 1976) and others reporting no change in iodine uptake following 5-OZT administration (Elfving, 1980). The variable results may be associated with variation in the iodine content of experimental diets (Elfving, 1980). Detailed studies on the incorporation of radio-labelled iodine into thyroid

hormones and their precursors have shown 5-OZT to interfere with organic iodination of thyroxine in the thyroid with resultant accumulation of thyroxine precursors in the thyroid (Akiba and Matsumoto, 1976; Elfving, 1980). The effects of 5-OZT on peripheral T3 and T4 concentrations are less obvious and higher doses (>100 g/rat/ d) are required to elicit depressive effects (Elfving, 1980); this may be due to compensatory enlargement of the thyroid in response to 5-OZT administration. 5-OZT may also have peripheral effects on the conversion of T4 to T3 in the liver, analogous to the action on propylthiouracil; this was recently tested in rats but without conclusive results (Langer, Foldes and Gschwendlova, 1984).

The practical importance of any of the possible toxic routes described is dependent on the digestive fate of the parent compounds and the rate and extent to which the compounds can be modified or excreted before exerting their toxic effects in the animal.

Metabolic fate and detoxification of glucosinolates

Information on the metabolic fate and possible routes of detoxification of glucosinolates following their ingestion from plant sources is potentially extremely useful when assessing the likely toxicity of the compounds and also in the planning of the experiments aimed at determining specific toxic effects. Research in this area has been rather limited both with respect to animal species studied and compounds investigated. In the latter case work on related compounds can provide insight into likely metabolic routes and detoxification processes. The following brings together work relating to the fate of glucosinolates and their likely breakdown products, both in the digestive tract and following their absorption.

Digestive Metabolism

The extent of glucosinolate breakdown in the gut is likely to depend on the nature of the food source and particularly on the presence or absence of plant myrosinase. Thus, extensive breakdown is probable following ingestion of fresh brassica herbage, while with heat-treated rapeseed meal, minimal enzymic breakdown might be

expected. However, actual experimental data are sparse and the only significant attempt to determine sites of breakdown and the identity of the products has been with poultry consuming rapeseed meal. Smith and Campbell (1976) determined the proportions of progoitrin hydrolysis products in various portions of the intestinal tract of laying hens offered rapeseed, and found nitrile products to predominate throughout. However, absolute concentrations were not determined and the extent of glucosinolate hydrolysis was thus unknown. More recent balance trials showed proportional glucosinolate recovery in intact hens to be in the range 0.15 - 0.50, with the remainder presumably undergoing enzymic hydrolysis in the intestinal tract (Slominski, Campbell and Stanger, 1987; Freig, Campbell and Stanger, 1987; Slominski, Campbell and Stanger, 1988; see also Marangos and Hill, 1974). Surgical removal of the caecum or the inclusion of antibiotics in poultry diets was found to greatly increase proportional faecal recovery of glucosinolates to approximately 0.80 (Slominski et al., 1987), indicating the involvement of hindgut micro-organisms in glucosinolate breakdown. This was later corroborated by work which showed a caecal fraction from hens to have significant activity with respect to glucosinolate hydrolysis (Freig et al., 1987), a finding which had also been noted by Marangos and Hill (1974).

Bacterial myrosinase activity was first demonstrated by Oginsky, Stein and Greer, (1965) in humans. Detailed work on intestinal glucosinolate breakdown in other species is scarce, although Lo and Hill (1971) reported minimal (1-2%) faecal recovery of glucosinolates in rats fed rapeseed. The only significant work in this area with ruminants was conducted by Forss and Barry (1983), who examined the fate of the glucosinolates present in kale and swedes by incubating plant material with rumen fluid and monitoring nitrile production. As a proportion, between 0.30 and 0.50 of parent glucosinolates were hydrolysed to nitriles, although information on individual compounds was lacking because of the non-specific nature of the analysis. In addition, marked differences in production rates were found between swedes and kale and work with isolated compounds along with more specific assay techniques would improve understanding in this area.

Further metabolism of glucosinolate breakdown products in the digestive tract has rarely been considered, although, in the light of the reactive nature of the compounds arising in the gut, this is a strong possibility.

Systemic Metabolism

The post-absorptive metabolism of these compounds is more comprehensively documented and a series of studies on the metabolic fate of isothiocyanates have shown glutathione conjugation to be the major excretory pathway in most species studied (Brusewitz, Cameron, Chasseaud, Gorler, Hawkins, Koch and Mennicke, 1977; Mennicke, Gorler and Krumbiegel, 1983). However, significant species differences have been reported; thus, in dogs, hippuric acid is the major urinary metabolite (Brusewitz et al., 1977), while in guinea pigs and rabbits a cyclic mercapturic derivative appears to predominate (Gorler, Krumbiegel, Mennicke and Siehl, 1982). In mice a range of unidentified urinary metabolites were detected as well as the expected mercapturic acid (Ioannou, Burka and Matthews, 1984). Information on isothiocyanate metabolism in ruminants is lacking. In recent work with rats, conjugation of glutathione with isothiocyanates was shown to be reversible (Bruggemann et al., 1986) and even after initial conjugation, local release of free isothiocyanates cannot be discounted. In support of glutathione conjugation as a detoxification route for isothiocyanates, Sparnins, Venegas and Wattenberg (1982) found enhanced activity of glutathione-S-transferase in the intestinal mucosa and liver of mice following benzyl isothiocyanate consumption. Enhanced glutathione-S-transferase activity was also observed in animals consuming brussels sprouts (Godlewski, Sharman, Anderson and Stoewsand, 1985) and cabbage (Stoewsand, Anderson and Lisk, 1986) and this may have been due, in part, to isothiocyanate release.

On the fate of aliphatic nitriles there is less information. A number of workers have observed increased urinary excretion of thiocyanate ion following nitrile dosing (Contessa and Santi, 1973; Langvardt et al., 1980; Silver, Kuttab, Hasan and Hassan, 1982) and this has been taken as evidence for the *in vivo* release of free cyanide in the tissues. Although, in the light of the reactive nature of the C≡N bond, release of free

cyanide intact might appear unlikely, Willhite and Smith (1981) conducted incubations of liver slices and microsomal fractions with aliphatic nitriles and demonstrated HCN production. In common with isothiocyanates, glutathione conjugation with nitriles was suggested by the identification of a mercapturic acid derivative in the urine of rats dosed with acrylonitrile (Langvardt, Putzig, Braun and Young, 1980). Although lacking experimental evidence, Silver et al. (1982) also indicated glutathione as being important in nitrile catabolism, either through direct conjugation or by conjugation with an epoxide intermediate. Indeed, significant clearance of glutathione from the tissues has been noted in rats dosed with the unsaturated nitriles, allyl cyanide (Ahmed and Farooqui, 1982), and acrylonitrile (Szabo et al., 1977) and this may be taken as further evidence for glutathione conjugation as a major route of nitrile metabolism. Glutathione conjugation was also found to be an important excretory route for 1-cyano-3,4-epithiobutane, a breakdown product of butenyl glucosinolate (Brocker, Benn, Luthy and von Danicken, 1984); 1-cyano-3,4-epithiobutane was excreted rapidly as a mercapturic acid derivative in the urine with excretion being substantially complete within 12 hours. Again, information on nitrile metabolism in ruminants is lacking.

Work on the excretion and metabolism of other glucosinolate metabolites is rather fragmentary with most work concentrating on 5-vinyl oxazolidinethione (5-OZT). Elfving (1980) extended the earlier work of Peltola and Krusius (1969) and Langer and Michajlovskij (1969) on the metabolism of 5-OZT, to show that 5-OZT is concentrated in the thyroid following intra-peritoneal injection. 5-OZT was identified, intact, in the thyroid along with a 4-OH derivative and inorganic sulphate. These compounds were subsequently excreted fairly rapidly in the urine with sulphate showing the most persistence. Excretion of a labelled dose of 5-OZT was substantially complete (85%) within 24 hours.

Finally, the complexity of xenobiotic metabolism is highlighted by studies which followed the finding that consumption of certain cruciferous vegetables was beneficial in the inhibition of tumour formation (Wattenberg, 1977). Examination of the activity of various enzymes of the mixed function oxidase (MFO) system indicated the involvement of glucosinolate metabolites, including benzyl isothiocyanate, indole-3-

carbinol and indole acetonitrile in non-specific induction of the MFO system and cytoplasmic glutathione-S-transferase, with accompanying protection against potential carcinogens (Shertzer, 1982; Sparnins et al., 1982; Stoewsand et al., 1986). Similar interactions in the metabolism of other xenobiotics doubtless exist and may be significant in relation to the overall toxic effects of the secondary metabolites of the *Cruciferae*.

PHYSIOLOGICAL EFFECTS OF BRASSICA SECONDARY METABOLITES-S-METHYL CYSTEINE SULPHOXIDE

S-Methyl cysteine sulphoxide (SMCO) is a non-protein sulphur-containing amino acid which is present in the vegetative material and flowers of *Brassica* species at concentrations of up to 20 g/kg DM. Its toxicity to ruminants was discovered at the Rowett Research Institute in 1973 following feeding experiments of the isolated compound to goats (Smith, 1974). Subsequent work showed that production of a secondary haemolysin, dimethyl disulphide, under the action of rumen micro-organisms, was responsible for the haemolytic anaemia caused by brassica feeding.

Distribution

S-Methyl cysteine sulphoxide was first isolated from turnip (Morris and Thompson, 1956) and cabbage (Synge and Wood, 1956) but was subsequently found to be widely present among the *Cruciferae* and the *Liliaceae*. Its presence in the *Liliaceae* is accompanied by that of the homologous compounds S-allyl cysteine sulphoxide and S-propyl cysteine sulphoxide, but among the *Cruciferae*, the methyl derivative, SMCO, alone appears to be present (Virtanen, 1964). A number of surveys of the occurrence of SMCO in different species and plant tissues have been conducted and results are summarised in Table 2.4; (results expressed as mg/kg fresh weight and mg/100g DM are broadly comparative, assuming DM content of approximately 100 g/kg DM). Clearly, wide variation exists in the concentration of SMCO between species and plant

Table 2.4 SMCO concentrations in a range of plant species and plant parts.

Plant	Young leaf	Leaf	Stem	FLowers	Root	Units	Reference
Turnip	167	79			202	mg/kg fresh	Morris and Thompson, 1956
Cabbage		396					
Cauliflower				2380			
Kohlrabi		558	1069				
Radish					117		
Mustard		297					
Broccoli		1770	851	2406			
Turnip		234			283	mg/100g DM	Whittle et al., 1976
Swedes		1140			640		
Chinese cabbage	549	107	174		94	mg/100g DM	Mae et al., 1971a
Rape: Bonar		510	570			mg/100g DM	Griffiths et al., 1989
Hobson		520	490				
84411		510	670				

tissues, with little consistency in distribution between leaves, stems and roots. Concentrations in flowers and young leaves, however, do appear to be markedly higher than those in the rest of the plant.

Several studies have shown SMCO concentrations to vary throughout the life of the plant and, in general, concentrations appear to increase with time (Smith, 1974; Whittle, Smith and McIntosh, 1976). However, frequent-sampling experiments have demonstrated dramatic fluctuations in SMCO concentrations through the growing season (Mae, Ohira and Fujiwara, 1971; Griffiths and MacFarlane Smith, 1989); in one study parallel changes in concentrations were observed in 3 varieties of forage rape, indicating the involvement of environmental factors in determining SMCO content (Griffiths and MacFarlane Smith, 1989). Certainly, soil sulphate concentrations are positively related to SMCO concentrations (Barry et al., 1984a). Reduction in soil sulphate levels has been proposed as a possible means of reducing SMCO concentrations in herbage and so alleviating the problems of haemolytic anaemia. Anecdotal evidence also exists for the effects of temperature on kale toxicity (Rees Evans, 1951) and declining temperature may be one reason for increases in SMCO concentrations as the season progresses. Further experimentation is needed in order to validate these suggestions.

Hydrolysis and Metabolic Fate

The fate of SMCO following its ingestion by animals has not been rigorously studied and there is no quantitative information on its rate of breakdown in the rumen and subsequent absorption of its breakdown products. Following the identification of SMCO as the primary toxin causing haemolytic anaemia, experiments in which SMCO was incubated with rumen fluid showed dimethyl disulphide (DMDS) to be a prominent component of the headspace of these incubations (1-3 hours) with methane thiol appearing in later samples (>3 hours) (Smith, 1974). A survey of rumen micro-organisms for SMCO-lyase activity showed *Lactobacillus*, *Veillonella alcalescens*, *Peptostreptococcus elsdenii* and *Anaerovibrio lipolytica* to have significant activity (Smith, 1974). Furthermore, cultures of micro-organisms from the stomach and

caecum of SMCO-fed rats also showed SMCO-lyase activity. Detection of DMDS in the blood of kale-fed steers provided further evidence for conversion of SMCO to DMDS in the digestive tract (Earl and Smith, 1982). Barry, Manley and Duncan (1984b) cited the failure to detect SMCO in the digestive tract of kale-fed cattle as evidence for its rapid conversion to DMDS; further experiments showed conversion of SMCO to DMDS to be more rapid in rumen fluid from kale-fed cows than in that from animals fed lucerne. It was suggested that this was due to the higher concentrations of readily fermentable carbohydrate in the kale diet providing a ready substrate for rumen micro-organisms and this was presented as an explanation for the greater toxicity of synthetic SMCO to kale-fed compared with lucerne-fed sheep (Barry et al., 1982).

Further work is needed on the rate of breakdown of SMCO in the rumen and the subsequent absorption of DMDS into the blood. Information on factors affecting rumen hydrolysis of SMCO would also prove helpful in improving our understanding of the occurrence of haemolytic anaemia in the field.

Toxic Effects

Reports of the occurrence of haemolytic anaemia among livestock fed forage brassica crops go back to those of Rosenberger (1943) and Rees Evans (1951). Early reports documented a steady decrease in blood haemoglobin concentrations and packed cell volumes together with the appearance in the blood of characteristic, darkly staining inclusions known as Heinz bodies (Clegg and Evans, 1962; Grant, Holtenius and Jonsson, 1968). In response to these reports, work was initiated at the Rowett Research Institute aimed at characterising the syndrome and discovering its cause. In feeding experiments with a number of animal species, characteristic features of the disease were quantified; the anaemia was found to develop over a period of weeks with haemoglobin concentrations typically dropping to 4-6 g/100ml RBC with PCV's following a similar decline and reaching levels as low as 12-15 %. The rapid appearance of Heinz bodies in the erythrocytes was noted, and when the anaemia was most severe, as many as 50% of cells were found to be affected (Greenhalgh, Sharman

and Aitken, 1969). Comparative studies with sheep, goats, cattle, rabbits and guinea pigs showed cattle and goats to be more susceptible than sheep, while rabbits and guinea pigs appeared to be unaffected (Greenhalgh et al., 1969). In addition young animals were found to be more severely affected than more mature animals, and it was suggested that this was due, simply, to their greater food intake relative to their metabolic body weight. A further observation was the apparent recovery of kale-fed animals after the initial haemolytic crisis, followed by further bouts of anaemia which were attributed to the appearance in the peripheral circulation of new, resistant populations of erythrocytes (Tucker and Kilgour, 1973). Attempts to elucidate the cause of the anaemia involved the infusion of various extracts of kale and the toxic compound was ultimately identified as S-methyl cysteine sulphoxide, a non-protein amino acid, which had been isolated at the institute 15 years before-hand (Synge and Wood, 1956). The secondary toxin was later found to be dimethyl disulphide, the rumen transformation product of SMCO.

The mechanism underlying the haemolysis remains unclear and a number of factors are clearly important. The involvement of glutathione was suggested by the finding that sheep which were genetically deficient in glutathione showed a more severe anaemia than normal sheep (Tucker and Kilgour, 1973). Glutathione is involved in protection of the red blood cell from oxidative injury caused by release of oxygen radicals and peroxides in the normal metabolism of the erythrocyte (Gordon-Smith and White, 1974). Recent work using spin-trapping techniques and electron resonance spectroscopy has demonstrated a dramatic free-radical burst following addition of dimethyl disulphide to red blood cell suspensions (Macphail and Sibbald, in press). As well as direct conjugation with the sulphydryl group of glutathione, dimethyl disulphide may reduce erythrocyte concentrations of reduced glutathione by causing inhibition of glutathione reductase activity. In a series of experiments, Steven, Griffin and Smith (1981) showed DMDS to significantly inhibit activity of papain, presumably following disulphide exchange reactions with the disulphide bond of the enzyme. Analogous inhibition of glutathione reductase was suggested as a factor involved in the aetiology of DMDS -induced haemolytic anaemia.

The low concentrations of copper and selenium, typical of forage brassicas, have been suggested as contributing to a more severe haemolytic anaemia because of their role as constituents of the enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GSH-px), which are involved in the removal of potentially damaging radicals from the cellular environment. There is conflicting evidence on this aspect of SMCO toxicity in the literature, with one study showing copper supplementation of kale-fed cows to reduce anaemic symptoms and improve growth rates (Barry et al., 1981b) and another showing the copper and selenium status of the animals to have little effect on the severity of anaemia among sheep fed on rape (Suttle, Jones, Woolliams and Woolliams, 1987); The importance of the trace element status of animals in the aetiology of brassica-induced anaemia may depend on the length of the grazing period since ruminants maintain considerable hepatic reserves of copper. There may also be species differences in the degree to which SOD and GSH-px are important in maintaining the integrity of the erythrocyte.

Dimethyl disulphide may also bring about cell lysis by its effects on lipid metabolism; normal lipid metabolism is obviously important in maintaining the integrity of cell membranes. Studies in which rodents have been fed on garlic and onion diets, which are rich in S-alkyl cysteine sulphoxides, showed reduced plasma cholesterol concentrations and depressed hepatic lipid synthesis to be features of the toxicity of these compounds (Itokawa, Inove, Sasagawa and Fujiwara, 1973 ; Augusti and Matthew, 1974; Chang and Johnson, 1980). The mechanism of toxicity is unclear but may involve disulphide exchange reactions between alkyl disulphides and key enzymes such as fatty acid synthetase (Steven et al., 1981). It appears, therefore, that DMDS may cause anaemia through its action on two distinct areas of erythrocyte metabolism; firstly, protective mechanisms within the erythrocyte such as reduced glutathione and glutathione reductase activity may be depressed leading to membrane injury by lipid peroxidation and free-radical release and secondly, the actual physical integrity of the red cell membrane may be weakened following reduced biosynthesis of lipids through the action of dimethyl disulphide.

Effects on Animal Performance

Although a considerable volume of experimental work has been conducted on the haematological and biochemical changes associated with brassica-induced haemolytic anaemia, the extent to which the syndrome affects animal productivity has rarely been quantified. Isolated studies in which SMCO or derived compounds were incorporated into rodent diets indicated growth and VFI to be affected (Itokawa et al., 1973; Benevenga, Yeu and Lalich, 1976) but the degree of anaemia in these experiments was slight and their applicability to the more severe anaemia seen in ruminants consuming brassica crops is questionable. The only notable work with ruminants in this area was conducted by Barry and associates. In a grazing experiment in which SMCO concentrations in kale crops were manipulated using differing S-fertilizer regimens, low SMCO-kale was found to support significantly higher growth rates (152 vs. 75 g/d) among growing lambs and to cause a milder anaemic response (Barry et al., 1984a). The results of this experiment are open to interpretation, however, since the low-sulphate fertilizer regimen also led to low levels of glucosinolates in the herbage. Attempts were made to diminish the effects of glucosinolates overall by supplementing animals with iodine; however, this would only have reduced goitrogenicity associated with thiocyanate ion production and the toxicity of glucosinolates may be associated with a whole range of other biochemical mechanisms, as previously described. An earlier experiment in which artificial SMCO was incorporated into both kale and lucerne diets was, in principle, a more rigorous approach (Barry et al., 1982). Significantly, however, marked effects on VFI were found only on the kale diet and this may have been associated with the presence of glucosinolates in the background diet. Incorporation of SMCO into a lucerne diet precipitated only a mild haemolytic anaemia and growth rates and VFI were only affected at the highest levels of inclusion. These experiments indicate an important role for SMCO in depressing performance of animals consuming brassica forages. However, they do not provide a complete explanation for the depressed performance observed among brassica-fed livestock. In particular, the fact that the effects of SMCO were much larger when incorporated into a kale diet than when fed as part of a non-brassica diet, suggest that

other features of the brassica diet may be significant. As suggested by Barry et al. (1982), this may be related to differences in the rumen fate of SMCO on different diets but, equally, physiological interactions between SMCO and other components of the brassica diet, notably the glucosinolates, may be important. The importance of interactions between the effects of glucosinolates and SMCO has never been tested experimentally.

CONCLUSIONS

Examination of the literature has shown that (i) growth of weaned lambs grazing forage brassicas is low in relation to the nutrient composition of the herbage with sub-optimal VFI's being implicated as the most important contributing factor, (ii) one of the most likely causes of the low VFI observed among ruminants grazing brassica forage is the presence in the herbage of the sulphur-based secondary metabolites, namely the glucosinolates and S-methyl cysteine sulphoxide, (iii) while the phenomenon of impaired performance among animals consuming glucosinolate-containing diets is well documented, the detailed physiological mechanisms underlying these findings are unclear, (iv) although the nutrition of animals consuming glucosinolate-containing rapeseed has been extensively studied, this work has focused primarily on mono-gastric animals and the toxicity of the glucosinolates present in forage brassica vegetative tissue to ruminant animals has been inadequately investigated, (v) understanding of the metabolic fate of glucosinolates from both the seed and vegetative tissue of brassica species is poor, particularly with regard to ruminants, and improvements in analytical methodology should facilitate experimentation in this area, (vi) the mechanisms underlying the syndrome of haemolytic anaemia caused by S-methyl cysteine sulphoxide have been partly identified but the way in which dimethyl disulphide affects erythrocyte metabolism and, in particular, the mechanism by which erythrocyte membrane integrity is disrupted is not clearly understood and (vii) although the toxicity of individual secondary compounds found in brassica herbage has been investigated to a certain extent, the way in which these compounds interact to either enhance or suppress overall toxicity has not been studied.

Three areas stand out in which work is required: (i) the digestive fate of parent glucosinolates and their breakdown products, (ii) the effects of individual glucosinolate breakdown products on the physiology and productivity of ruminants and (iii) the interactive effects of brassica secondary metabolites on overall brassica toxicity. These areas will be addressed in the experimental work.

Chapter 3 - RUMEN HYDROLYSIS OF SINIGRIN

Experiment 1 - Detection of the mercapturic acid metabolite of allyl isothiocyanate in the urine of sheep as a means of characterising the rumen hydrolysis of sinigrin.

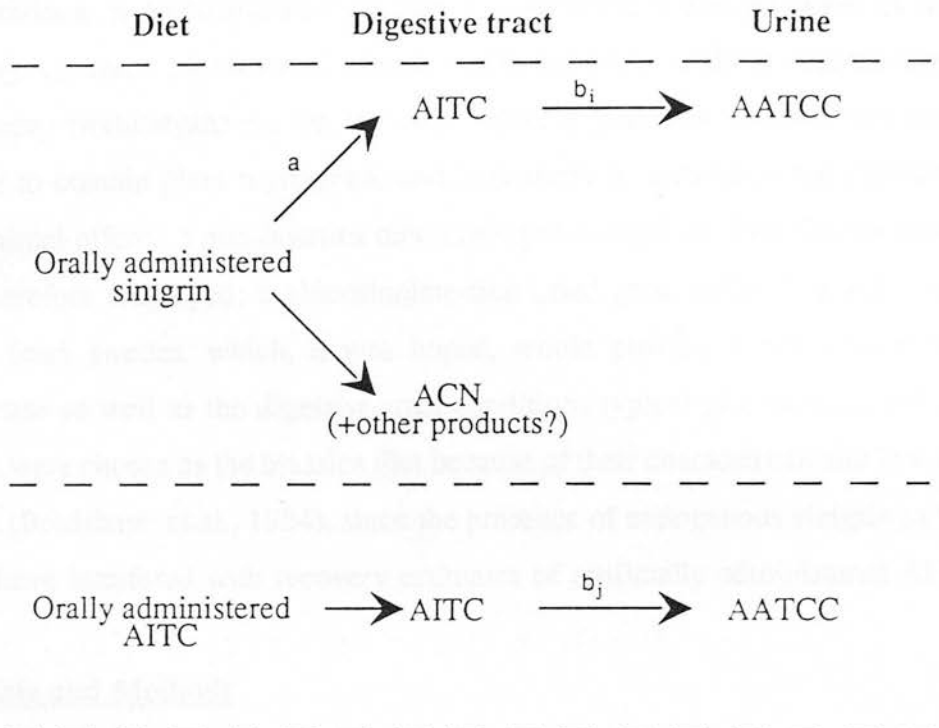
Introduction

Investigations into the toxicity of glucosinolates are complicated by the fact that the compounds exist in benign form in the intact plant and only release toxic products following disruption of plant tissue such as occurs during ingestion by herbivores. The range of toxic compounds released during glucosinolate hydrolysis is dependent on the chemical environment in which the hydrolysis occurs, with features such as pH and metallic ion concentration being particularly important (see Literature Review). Although the chemistry of glucosinolate hydrolysis has been extensively studied in plant systems and in purified *in vitro* systems, the fate of glucosinolates and their toxic products in digestive fluids is almost unknown. This is surprising given the extensive literature on glucosinolate toxicity and the fact that toxicity to animal tissues is wholly dependent on the digestive fate of glucosinolates.

The reasons for the lack of attention to this important area of glucosinolate toxicology are probably related mainly to the practical difficulties inherent in both the chemical analyses required and the biological interpretation of resulting data. Glucosinolate breakdown products, such as the aliphatic isothiocyanates and nitriles, are very reactive chemically, while digestive fluids tend to be relatively complicated chemical media in which to quantify the presence of such compounds. In addition, because transitory concentrations of breakdown products represent the instantaneous balance between rates of hydrolysis, absorption, passage and further chemical modification, it is difficult to draw conclusions as to the production rates of the various compounds in the digestive tract from measurements of concentrations. However, despite the potential difficulties inherent in this area of investigation, a knowledge of

the digestive fate of glucosinolates is of primary importance in assessing the toxicity of these compounds and in understanding their mode of action.

To set subsequent infusion experiments in context with respect to infusion rates of different sinigrin breakdown products, this experiment was designed to characterise the digestive fate of sinigrin. A preliminary approach to studying the digestive fate of glucosinolates, involving detection of glucosinolate metabolites following addition of sinigrin to rumen fluid *in vitro*, proved unfruitful due to analytical difficulties. The reactive nature of aliphatic isothiocyanates and nitriles together with the complex chemical nature of the rumen fluid incubation medium made detection of metabolites difficult (unpublished data). To avoid the analytical difficulties associated with measuring concentrations of glucosinolate breakdown products in rumen fluid, an alternative approach is to analyse for urinary metabolites of such compounds. The post-absorptive metabolic fate of aliphatic isothiocyanates is well characterised in various monogastric species with glutathione conjugation and urinary excretion as the corresponding mercapturic acid being a prominent excretory route (Mennicke et al., 1983). This information was used to construct an experiment in which urinary outputs of N-acetyl-S-(allylthiocarbamoyl)-L-cysteine (AATCC), the mercapturic acid metabolite of AITC, were compared following dosing of sheep with AITC or its glucosinolate precursor, sinigrin. The primary aim of the experiment was to test whether AITC would be recovered in the urine as AATCC when introduced into the rumen by direct oral administration and when it arose in the digestive tract following sinigrin hydrolysis. If this proved to be the case, the urinary output of AATCC following administration of sinigrin as a proportion of that following direct administration of AITC could be used to give an estimate of *in vivo* production of AITC from sinigrin, if it was assumed that the conversion of AITC to AATCC was the same in both circumstances (Figure 3.1). This approach also relies on the assumption that urinary AATCC exclusively originates from AITC. In order to exclude the possibility that urinary AATCC arose from other sources, treatments were included in which animals were given the other major sinigrin hydrolysis product, ACN, or blank capsules (control).



$$a.b_i = \frac{\text{Urinary output of AATCC (moles) on sinigrin treatment.}}{\text{Amount of sinigrin administered (moles)}}$$

$$b_j = \frac{\text{Urinary output of AATCC (moles) on AITC treatment.}}{\text{Amount of AITC administered (moles)}}$$

$$\text{If } b_i = b_j \text{ then } a = \frac{a.b_i}{b_j}$$

Where a = proportion of sinigrin hydrolysed to AITC in the digestive tract.
b_j = recovery of AATCC in urine following oral AITC administration.
b_i = recovery of AATCC in the urine following hydrolysis of sinigrin to AITC in the digestive tract.
AITC = Allyl isothiocyanate
ACN = Allyl cyanide
AATCC= N- Acetyl - S - (allyl thiocarbamoyl) L - cysteine

Figure 3.1 Assumed digestive fate of orally administered sinigrin and method of calculating *in vivo* AITC production from dietary sinigrin.

The choice of the most appropriate diet for the experiment was subject to various considerations; a glucosinolate-free diet was desirable from the point of view of obtaining accurate estimates of dosed AITC recovery without interference from endogenous isothiocyanates. On the other hand, a glucosinolate-free diet would be unlikely to contain plant myrosinase and hydrolysis of sinigrin in the digestive tract of an animal offered a non-brassica diet might prove atypical. Two dietary treatments were therefore employed: a glucosinolate-free dried grass pellet diet and a brassica diet of fresh swedes, which, it was hoped, would provide a rich source of plant myrosinase as well as the digestive tract conditions typical of a brassica-fed animal. Swedes were chosen as the brassica diet because of their characteristically low sinigrin content (Bradshaw et al., 1984), since the presence of endogenous sinigrin in the diet would have interfered with recovery estimates of artificially administered AITC.

Materials and Methods

Animals

At the beginning of December, 1989, eight Scottish Blackface wethers aged 3-4 years (mean weight 58.9 kg; range 49.0 - 61.0 kg), were housed, weighed and ranked by liveweight. Animals were then divided into two dietary groups balanced for liveweight and offered experimental diets for seven days prior to the start of experimental treatments.

Feeds

Feeds consisted of dried grass pellets or freshly chopped swedes. Dried grass pellets (N, 32.4 g/kg DM; NDF, 482 g/kg DM; ADF, 280 g/kg DM; Ash, 108 g/kg DM) were fed without further preparation; swedes (N, 24.1 g/kg DM; NDF, 144 g/kg DM; ADF, 117 g/kg DM; Ash, 54 g/kg DM) were purchased locally and were washed, topped and chopped by a turnip chipper immediately prior to feeding. Feeds were offered at a fixed rate of 720 g DM/ d in two equal feeds at 0800 h and 1600 h.

Treatments and experimental design

The experiment involved oral administration of discrete doses of sinigrin or its breakdown products, AITC or ACN, to four sheep fed on dried grass and four sheep fed fresh swedes followed by total collection of urine for subsequent detection of urinary metabolites. The four treatments consisted of a single dose at 0900 h on day 1 of each experimental period of either 4 mmol of sinigrin, AITC or ACN administered orally in gelatin capsules. There was also a control treatment whereby sheep received an empty gelatin capsule.

The experimental design was a 4 x 4 Latin Square design with one square for each of the two experimental diets. Each experimental period was of 72 hours duration. Rows and columns, which corresponded to the four periods and eight animals respectively, were randomized following construction of the Latin Squares. Details of the allocation of animals to treatments are given in Appendix 3b.

Procedures

Animals were confined to standard metabolism cages equipped with chutes and separators throughout the experiment to allow total urine collection. Experimental periods began at 0900 h on day 1. Initial pre-treatment urine samples were collected in the 24 hours preceding the start of each period. Compounds were then orally administered to animals at 0900 h and urine samples were collected at 1, 2, 4, 8, 24 and 48 hours thereafter. Urine volume was recorded at each sampling and aliquots (20 ml) were filtered through filter paper (Whatman no. 1) and immediately frozen pending analysis. The remaining urine was discarded. A final sample was collected 72 hours after oral administration which acted as the pre-treatment sample for the next period.

Allyl isothiocyanate (AITC) (Aldrich, Dorset, UK), allyl cyanide (ACN) (Aldrich, Dorset, UK) and sinigrin (Sigma, Dorset, UK) were used as supplied by the manufacturers. Capsules were prepared on the morning of administration by accurate pipetting of ACN or AITC into 3 ml capacity gelatin capsules (Davcaps, Monmouth, UK) which were then sealed with a small strip of cellulose tape. Sinigrin was weighed into capsules which were sealed in the same way. Identical empty capsules were

sealed with tape for administration to control animals. Capsules were administered orally using a dosing gun. Capsule breakdown was presumed to be rapid (<10 minutes) since the capsules were observed to disintegrate quickly when placed in water.

Analysis

Urine samples were analyzed for the urinary metabolite of AITC, N-acetyl-S-(N-allylthiocarbamoyl)-L- cysteine, by an HPLC method based on that of Mennicke, Kral, Krumbiegel and Rittman (1987). AATCC was synthesised for use as a quality control standard according to Mennicke et al. (1983) (melting point 141-145°C; quoted range: 143-145°C). Benzyl butyl thiourea (BBTU) was synthesized as described by Mennicke et al. (1987) for use as an internal standard.

Sample preparation was as follows. One millilitre of urine was added to a thick-walled, screw-top test tube (150 x 20 mm O.D., Philip Harris Scientific, London, UK) along with 50 µl n-butylamine (Sigma, Dorset, UK) and 100 µl BBTU internal standard in ethanol (40 µg BBTU / ml ethanol). Tubes were tightly capped with PTFE-lined screw tops and incubated in a water bath at 60°C for 30 minutes. When cool, 300 µl of 25% sulphuric acid was added to remove the excess amine. Following brief agitation, the sample was extracted with 5 ml diethyl ether (Aldrich, Dorset, UK). The ether extract was then washed sequentially with 1 ml 1 M sodium hydroxide and two washes with 1 ml of distilled water. A vacuum was then applied directly to the tube at room temperature using a water aspirator to rapidly evaporate the ether. The residue was dissolved in 1 ml of mobile phase, transferred to a glass HPLC vial and capped with a PTFE-lined crimp top.

HPLC separation was carried out on a Gilson modular HPLC system run in isocratic mode with a 25 cm Spherisorb S5ODS2 reverse-phase C18 column (Phase Separations, Ltd., Clwyd, UK). The mobile phase was 1:1 water/acetonitrile (Aldrich, Dorset, UK) delivered at a flow rate of 1 ml/min. Injection volume was 20 µl and detection was by UV absorbance at 240 nm. Data was collected and peaks were integrated using standard "Gilson 714" software on an IBM PC-AT personal computer. N-acetyl-S-(N-allylthiocarbamoyl)-L-cysteine concentration was calculated by

comparing its peak area with that of the internal standard (BBTU) following calibration with synthetic AATCC standards. Quality control standards were analyzed at 10 sample intervals throughout the analysis. These were prepared by dissolving 10 mg AATCC in 10 ml ethanol; 20 µl of this solution was then added to 1 ml distilled water and the resulting standards were treated identically to test urine samples in the analysis.

Feed analyses were conducted by the methods described in Chapter 7.

Statistical analysis

Because of the large spread in the 24-hour cumulative excretion data, a log transformation was applied prior to analysis. Data were then analysed by analysis of variance using the GENSTAT 5 statistical package (Lawes Agricultural Trust , Release 1.3). The analysis of variance took into account the effects of treatment, diet, period and animal and a sample ANOVA table is presented in Appendix 3a.

Results

Oral administration of either sinigrin or AITC resulted in a rapid increase and steady decrease in the urinary concentration of AATCC (Figures 3.2a and 3.2b and 3.3a and 3.3b) over 24 hours on both grass and swede diets. The quantity excreted over 24 hours represented 0.716 of the total excreted over the whole 72 hour period. Twent-four hour excretion totals were used in subsequent calculations since the low concentrations in later samples together with large sample volumes would have resulted in unacceptable magnification of error. The large standard errors associated with the mean values for urinary AATCC concentration were the result of large variation in total urinary AATCC outputs and, to a lesser extent, differences in the rate of AATCC excretion between animals and periods. Administration of ACN or empty gelatin capsules (control) resulted in negligible excretion of AATCC (Figure 3.2c and 3.2d and Figure 3.3c and 3.3d)

Cumulative excretion values over the first 24 hours were calculated using urinary concentrations of AATCC together with urine output (Table 3.1). For the AITC

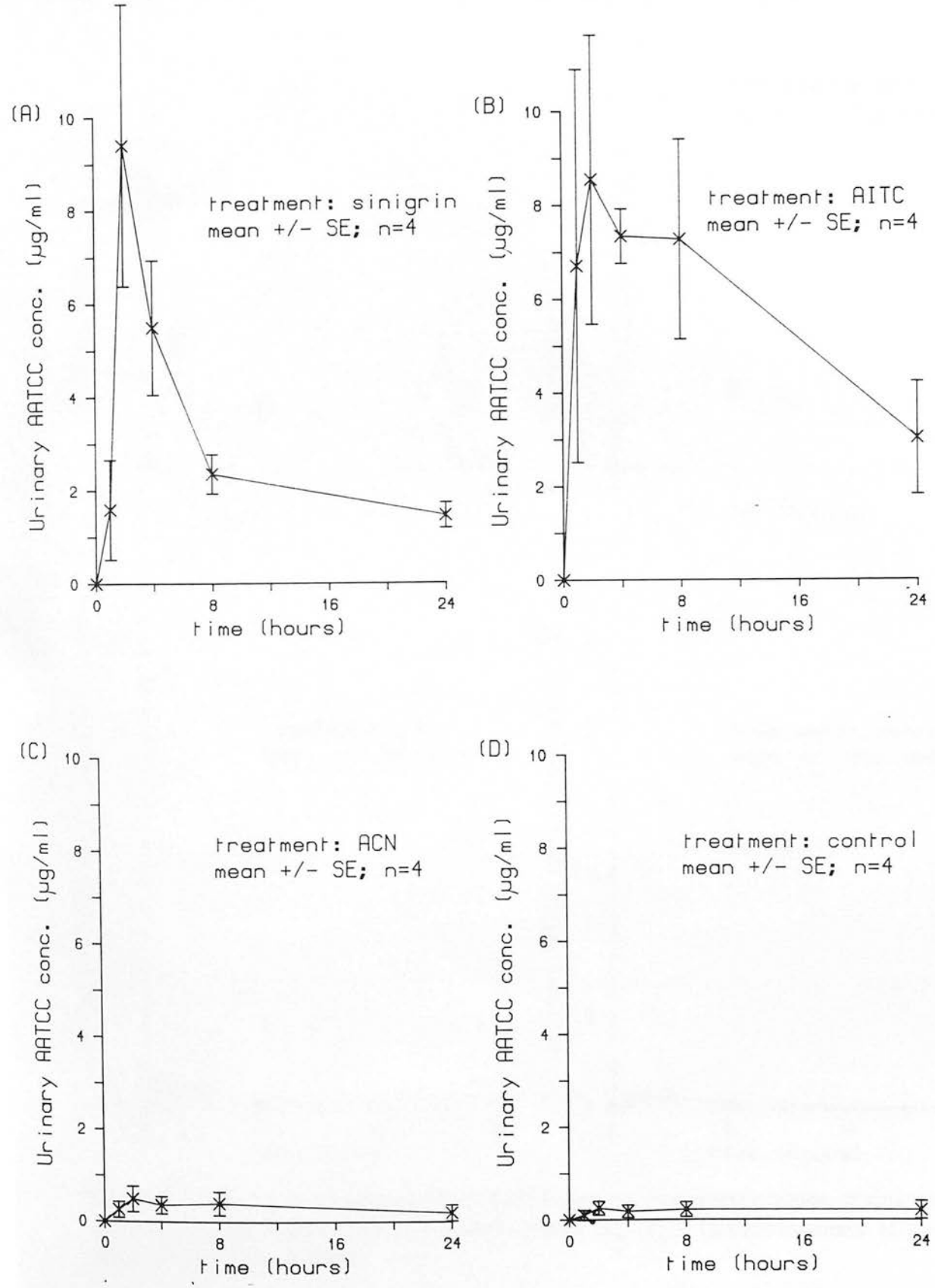


Figure 3.2 Urinary concentration of AATCC following oral administration of sinigrin (A), allyl isothiocyanate (AITC) (B), allyl cyanide (ACN) (C) or the control treatment (D) to sheep offered dried grass pellets.

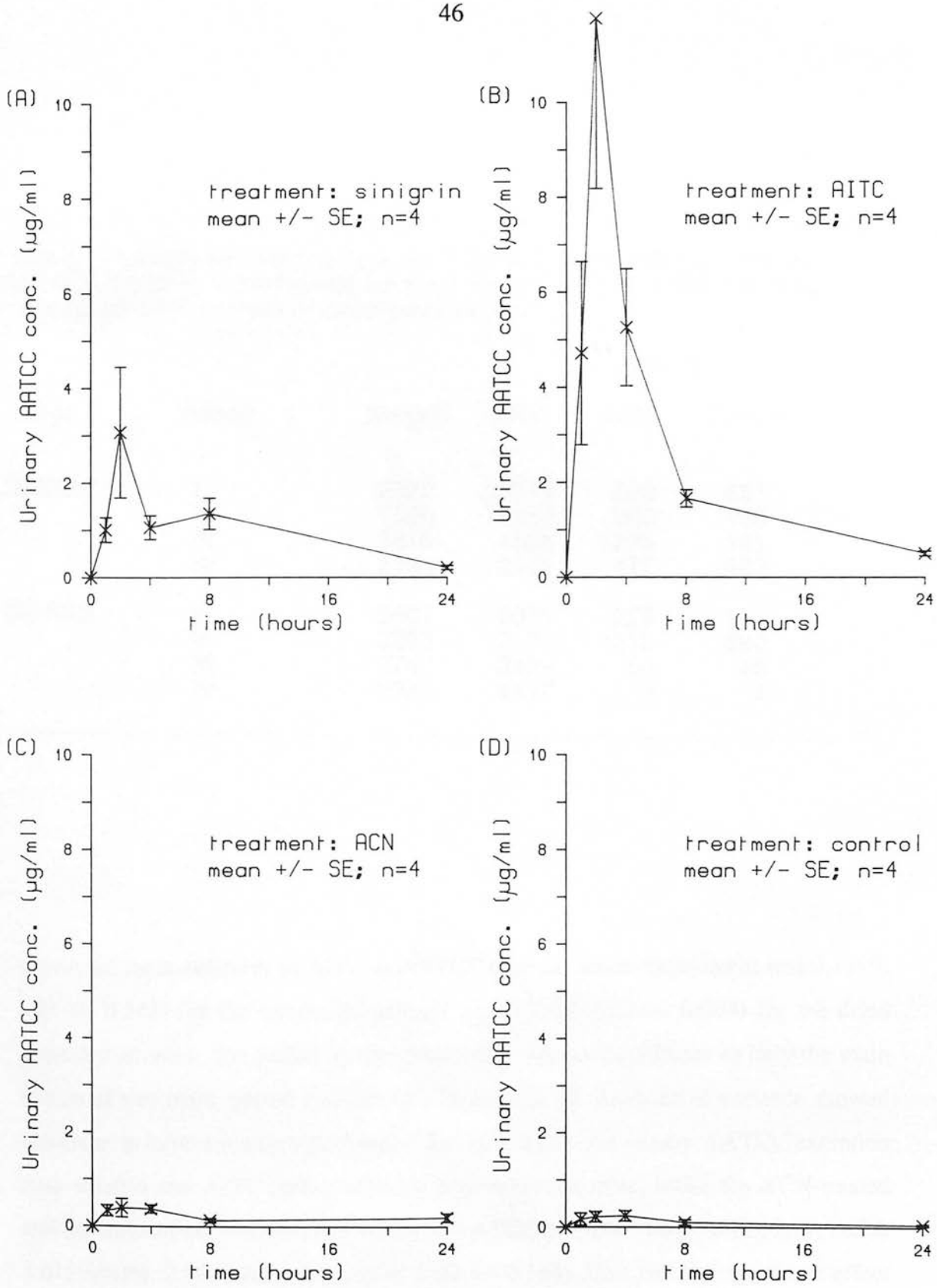


Figure 3.3 Urinary concentration of AATCC following oral administration of sinigrin (A), allyl isothiocyanate (AITC) (B), allyl cyanide (C) or the control treatment (D) to sheep offered fresh swedes

Table 3.1 - Urinary excretion of N-acetyl-S-(allyl thiocarbamoyl)-L-cysteine (AATCC) (µg/24 h) following oral administration of sinigrin, AITC and ACN to sheep fed fresh swedes or dried grass pellets.

		Treatment			
Diet	Period	Sinigrin	AITC	ACN	Control
SWEDE	I	2376	13515	824	651
	II	1508	10859	983	433
	III	3815	4528	1233	141
	IV	2190	2471	477	603
GRASS	I	5801	8071	228	151
	II	3298	7171	1610	590
	III	2747	3439	88	45
	IV	2382	4147	0	4

treatment, mean recovery of AITC as AATCC over the whole experiment was 0.439% (SE +/- 0.145) for the swede-fed animals and 0.322% (SE +/- 0.064) for the dried grass-fed animals. The period by diet interaction was not significant so only the main effects of treatment, period and diet will be considered. Analysis of variance showed treatment to have a highly significant effect ($p < 0.001$) on urinary AATCC excretion with sinigrin and AITC both producing high excretion rates, while the ACN-treated and control animals excreted low levels of AATCC (sinigrin, 3.45; AITC, 3.77; ACN, 2.61; control, 2.34; units = log(µg/d); SED +/- 0.164). Diet had no significant effect on the amount of AATCC excreted but there was a significant period effect ($p < 0.05$) with AATCC excretion rates being lower in periods III and IV than in periods I and

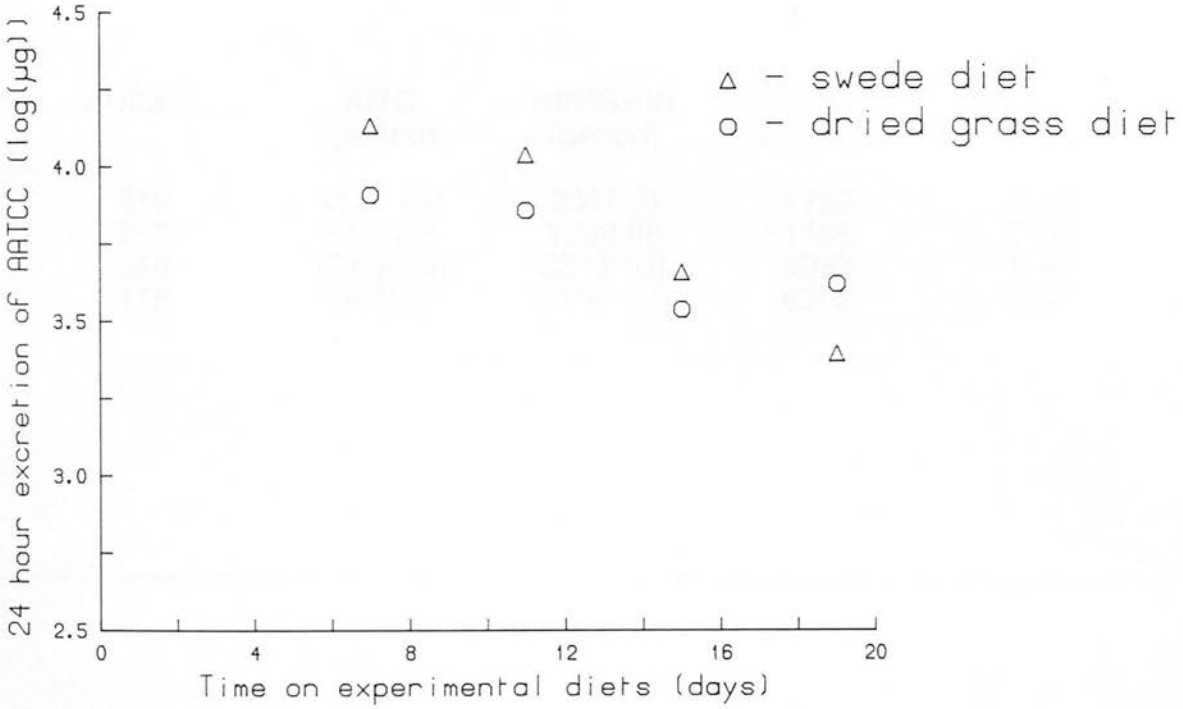


Figure 3.4 Effect of length of time on experimental diets on AITC recovery as AATCC in the urine of AITC-dosed sheep.

II (period I, 3.18; period II, 3.27; period III, 2.88; period IV, 2.82; units = log(µg/d); SED +/- 0.164). The effect of period on the decline in AITC recovery as AATCC among the AITC-treatment animals only is presented in Figure 3.4. There was a significant linear relationship ($p < 0.05$) between AATCC excretion by AITC-dosed

Table 3.2 - Urinary N-acetyl-S-(allylthiocarbamoyl)-L-cysteine (AATCC) excretion by sinigrin and AITC-dosed sheep (μg/24 hour) fed swedes with adjustment for period effects.

ANIMAL	AITC (period)	SINIGRIN (period)	ADJUSTED SINIGRIN	SINIGRIN: AITC ratio
219	4529 (iii)	2367 (i)	1153	0.25
203	2471(iv)	1508 (ii)	1186	0.48
320	10859 (ii)	3815 (iii)	5093	0.47
115	13515 (i)	2190 (iv)	6352	0.47

animals and the length of time on the swede diet; AATCC excretion by AITC-dosed animals was dependent on the length of time on the swede diet according to the following relationship:

$$a = -0.06484d + 4.647 \qquad \text{eq. 3.1}$$

where a = log(AATCC output over 24 hours)

d = number of days on turnip diet
SE of slope estimate = 0.00972
Percentage variance accounted for = 93.5%

The AATCC concentrations in the urine of swede-fed sheep dosed with sinigrin, were adjusted for the effects of time on AITC recovery as AATCC using equation 3.1 (Table 3.2). The sinigrin:AITC ratio of AATCC concentrations following adjustment

for recovery gives an indication of the extent to which sinigrin is broken down to AITC in the rumen (i.e. recovery "a" in Figure 3.1). A mean ratio of 0.539 (95 % confidence limits were 0.366 to 0.712) was calculated indicating the conversion ratio of sinigrin to AITC *in vivo*.

Discussion

This experiment was conducted to quantify the metabolic fate of sinigrin in sheep both in the digestive tract and following absorption of its digestive metabolites. This information is important in placing infusion studies involving breakdown products, such as AITC, in context. For example, it would be futile to conduct extensive toxicological investigation on aliphatic isothiocyanates without establishing the extent to which they arise *in vivo* following ingestion of glucosinolates.

An indirect experimental approach was adopted based on identification and quantification of urinary AATCC, a known metabolite of AITC following dosing with a parent glucosinolate, sinigrin. Urinary output of the AATCC following both sinigrin and AITC administration was measured to allow estimation of *in vivo* production of AITC.

The precision of the estimates of AITC production from sinigrin *in vivo* was reduced by two main sources of error. Firstly, between-animal variation in the extent of AITC metabolism to AATCC in the urine proved to be substantial. Studies on the metabolic fate of isothiocyanates in rodents indicated mercapturic acids to be important excretion products of isothiocyanates, with renal recoveries of AATCC following AITC administration in the range 10 - 20 % being reported (Mennicke et al., 1983). Results presented here however, indicate this route to be of minor importance in sheep. Twenty-four hour outputs of AATCC following AITC administration represented less than 1% of the administered dose. Theoretically, this should have made no difference to estimates of the proportion of sinigrin being hydrolysed to AITC *in vivo*, provided that recoveries of AITC as AATCC were identical whether the AITC was administered directly or arose following sinigrin hydrolysis. In practice, the low recovery levels meant that minor differences in abso-

lute urinary output of AATCC caused substantial errors to be introduced into calculations of the yield of AITC from sinigrin *in vivo*. The low urinary concentrations of AATCC also increased the importance of the analytical error associated with the measurements.

A further source of error which reduced the precision of sinigrin degradation estimates was the declining recovery of AITC as AATCC over the course of the experiment. This phenomenon appeared more marked on the swede diet than on the grass diet and may have been the result of adaptation of the rumen microflora to the greater presence of glucosinolates in the diet (see Experiment 4 for fuller discussion). Whatever the cause, the nature of the cross-over design meant that individual animals were dosed with sinigrin and AITC in different periods and thus were subject to different recoveries of AITC as AATCC. Thus, the numerator and denominator in the proportion calculations were not based on equivalent recoveries of AITC. An attempt was made to correct for declining recovery of AITC as AATCC over the experiment by calculating recovery estimates for each period based on the AATCC outputs of AITC- dosed animals and adjusting the AATCC outputs of sinigrin-dosed animals accordingly. However, even following this adjustment for recovery variation the confidence interval associated with the final estimate of the proportion of sinigrin hydrolysis to AITC was large (0.539 +/- 0.173 at 95 % confidence limits).

The precision of the experiment could potentially have been markedly improved by employing a "recovery internal standard". A homologous isothiocyanate, for example benzyl isothiocyanate, could have been given at a fixed rate to every animal at each dosing in order to provide an estimate of isothiocyanate recovery, which was independent of the period in which the dose was administered and the animal to which it was given. Urinary output of the corresponding mercapturic acid of benzyl isothiocyanate would thus have provided a good estimate of isothiocyanate recovery associated with each individual dose, assuming that recoveries of different isothiocyanates as mercapturic acids are subject to the same constraints. This is a reasonable assumption since excretion *via* the mercapturic acid pathway is likely to depend on the availability of common substrates, such as glutathione, and enzymes, such as glutathione transferase.

The low recoveries of AITC as AATCC suggest that conjugation with glutathione and subsequent excretion of the corresponding mercapturic acid is not the main route of isothiocyanate excretion in sheep. Significant species differences have previously been reported; for example in rats and man, the mercapturic acid appears to be the predominant metabolite (Mennicke et al., 1983; Mennicke, 1988), while in guinea pigs and rabbits a cyclic mercapto-pyruvic acid conjugate predominates (Gorler et al., 1982). Dogs on the other hand excreted benzyl isothiocyanate as the corresponding hippuric acid (Brusewitz et al., 1977). The metabolic fate of isothiocyanates in ruminants has not been studied; presumably the higher pH conditions characteristic of the rumen will affect the digestive fate of glucosinolates. The effects of pH on the equilibrium between sulphydryl conjugates of isothiocyanates and the free compounds have been demonstrated in *in vivo* experiments (Bruggemann et al., 1986) and this may influence their subsequent systemic fate. Urinary excretion profiles in the current experiment indicated some delay in the excretion of mercapturic acids following administration of AITC to sheep fed dried grass pellets (Figure 3.2b). This may have been due to conjugation of AITC with proteins and sulphydryl compounds in the digestive tract retarding absorption and hence excretion. On the other hand excretion of AATCC, following AITC administration to swede-fed sheep, was more rapid with very low residual urinary concentrations 24 hours after dosing.

Excretion of AATCC following administration of ACN was minimal confirming the assumption that urinary AATCC arose exclusively from AITC introduced into the digestive tract by direct administration or following hydrolysis of sinigrin. Swede-fed control animals also excreted minimal amounts of AATCC as expected since swedes typically contain low concentrations of sinigrin (Bradshaw et al., 1984).

The substantial excretion of AATCC following dosing of grass-fed sheep with sinigrin indicates that hydrolysis of sinigrin in the gut occurred despite the absence of plant myrosinase. Myrosinase-type activity has previously been demonstrated in the gut flora of monogastrics (Oginsky et al., 1965) and recent work has identified an intestinal *Lactobacillus* found in rats capable of hydrolysing sinigrin (Nugon-Baudon, Rabot, Wal and Szylit, 1990). The present results indicate similar activity to occur in micro-organisms found in the ruminant digestive tract. The appearance of AATCC in

the urine of grass-fed animals given sinigrin alternatively may have been due to simple chemical hydrolysis of sinigrin to AITC. Whether the hydrolysis was chemical or bacterial, the hydrolysis of sinigrin in the digestive tract in the absence of plant myrosinase demonstrated here is of interest in relation to the detrimental effects known to occur in livestock fed on heat-treated rapeseed meal.

Although the indirect experimental approach adopted in this experiment resulted in unforeseen error due to variation in the extent to which AITC arising in the digestive tract was ultimately excreted in the urine as its corresponding mercapturic acid, the results of this experiment are among very few successful attempts to characterise the digestive fate of glucosinolates in either mono-gastrics or ruminants and the first time such a question has been addressed using *in vivo* techniques. Results indicate that a significant proportion of sinigrin breaks down to AITC following its ingestion by sheep. The remainder presumably breaks down to ACN or other nitrile products but this would have to be verified by conducting analysis for urinary nitrile metabolites. The use of a "recovery internal standard" to improve precision of recovery estimates together with determination of urinary metabolites of ACN in order to quantify *in vivo* nitrile production would represent useful extensions to this work.

Chapter 4 - EFFECTS OF SHORT-TERM INTRA-RUMINAL INFUSION OF SINIGRIN BREAKDOWN PRODUCTS ON THE APPETITE AND THYROID AND ANAEMIC STATUS OF LAMBS.

Introduction

Glucosinolate hydrolysis under the catalytic action of plant myrosinase yields a number of potentially toxic compounds. Although information on the digestive fate of glucosinolates is scarce, isothiocyanates and nitriles are known to be prominent products following autolysis of glucosinolates in brassica herbage (see Literature Review). The results of a previous experiment (Experiment 1) indicated that AITC was produced in significant quantities in the digestive tract of animals following oral administration of isolated sinigrin. The remaining sinigrin was presumably hydrolysed to ACN (or possibly 1-cyano-3,4-epithiopropane), an assumption supported by the *in vitro* work of Forss and Barry (1983) which showed nitriles to be prominent hydrolysis products when the vegetative material of swedes of kale was macerated in ovine rumen fluid.

To determine the relative potency of glucosinolate- derived nitriles and isothiocyanates as VFI depressants, representatives of these two classes of glucosinolate breakdown products were infused into the rumen of sheep. The compounds tested were ACN and AITC, which are both readily available hydrolysis products of sinigrin, a simple glucosinolate found in significant quantities in the kales and cabbages (*Brassica oleracea*). The compounds were infused for a relatively short period (3 weeks) to allow an assessment of their short term effects.

Voluntary food intake can be important in limiting performance of lambs grazing forage brassica crops (see Literature Review) and for this reason, in these and subsequent experiments, VFI was used as a gross measure of the overall effects of *Brassica* secondary compounds in the animal. Thyroid status was assessed because of the known goitrogenic effects of isothiocyanates (Langer and Stolc, 1965); nitriles may also have anti-thyroid effects following their metabolism to goitrogenic thiocyanate

ions (Paxman and Hill, 1974). Haematological measurements were also taken to assess the contribution, if any, of nitriles and isothiocyanates to the haemolytic anaemia caused by S-methyl cysteine sulphoxide present in the basal diet of forage rape.

The compounds were infused continuously over the treatment period in order to mimic the release of toxins from parent glucosinolates which presumably occurs steadily over time in the digestive tract of ruminants grazing brassicas continuously. Actual concentrations of breakdown products must vary to some extent in the rumen of grazing animals due to cyclical feeding patterns, but continuous infusion was used as a reasonably realistic experimental method of administering the compounds. The sinigrin breakdown products, ACN and AITC were infused as separate treatments so that observed effects could be attributed to specific toxins. Two parallel experiments were conducted which differed with respect to experimental diet and infusion rate; in Experiment 2b (dried grass pellet diet) the glucosinolate breakdown compounds were infused at relatively high levels which were based on typical concentrations of sinigrin in fresh cabbage (Bradshaw et al., 1984) together with likely ingestion rates of forage brassica herbage. In the Experiment 2a (forage rape diet) animals were already consuming glucosinolates in their diet and the applied treatments therefore supplemented dietary glucosinolate ingestion. In order to avoid supra-physiological concentrations of glucosinolate breakdown products, infusion levels in this experiment were half those in Experiment 2b.

Experiment 2b (dried grass pellet diet) was conducted as a definitive test of toxicity based on administration of isolated compounds to animals consuming a diet free of the potential toxins in question. However, preliminary experiments at this laboratory (Burnett, 1988) have indicated the potential importance of interactions between glucosinolate breakdown products and other components of the brassica diet in VFI depression. It was for this reason that Experiment 2a (forage rape diet) was conducted.

Materials and Methods

Experiment 2.a - Effects of 21-day continuous infusion of allyl cyanide or allyl isothiocyanate on thyroid function, haematocrit and the voluntary food intake of forage rape (*Brassica napus*) by lambs.

Animals

Nine Greyface x Suffolk wether lambs (25 - 35 kg W) were surgically prepared with "Trocar" type rumen cannulae (1 cm diameter, Arnolds Veterinary Products, Essex, UK) four weeks prior to the start of the experiment. Surgery was conducted under general anaesthetic following administration of long-acting penicillin. After a recovery period of two weeks, animals were gradually introduced over three days to the experimental diet of fresh forage rape.

Feed

Forage rape (*Brassica napus*; variety Nevin) was sown at a site in Midlothian in late July and was harvested from mid-September until mid-December, 1987. Rape plants were cut to ground level and chopped in a chaff cutter daily before being fed *ad libitum* in two approximately equal feeds at 0930 h and 1600 h.[1] Refusals were collected before feeding each morning and the current day's feed allowance was calculated as 1.15 that of the previous day's food intake.

Treatments and experimental design

Treatments consisted of continuous intra-ruminal infusion of ACN (2.4 mmol/d; treatment ACN) or AITC (2.4 mmol/d; treatment AITC); CONTROL animals were infused with the infusion vehicle (propylene glycol) only. Infusion periods lasted 21 days and were separated by 7-day periods. Forage rape contains low concentrations of sinigrin but moderate levels of the homologous compound, but-3-enyl glucosinolate (Bradshaw et al., 1984). Infusion rates were designed to supplement natural glucosin-

[1] Details of the chemical composition of the diet are unavailable as samples were destroyed by fire before they could be analysed.

olate ingestion in the rape such that infusion rates were equivalent to levels of sinigrin in 0.5 kg DM cabbage (in which sinigrin is the predominant glucosinolate) assuming 0.50 hydrolysis to ACN and 0.50 hydrolysis to AITC.

A Latin Square design was used; treatments were arranged in three 3 x 3 squares with the nine columns corresponding to individual animals and the three rows corresponding to the three experimental periods. Following initial construction of the Latin Squares, rows and columns were randomized and animals were randomly allocated to columns. Details of the allocation of animals to treatments are given in Appendix 4.b.

Procedures

Animals were confined to metabolism cages during infusion periods to prevent damage to infusion lines. The infusates were prepared by mixing appropriate volumes of ACN (2.013 g/l) or AITC (2.975 g/l) in propylene glycol. Propylene glycol was chosen as an inert vehicle because of its capacity to dissolve both ACN and AITC and its lack of toxicity to ruminants (Ruddick, 1972). Infusates were placed in sealed polythene infusion packs and delivered to lambs along polythene infusion lines (0.0625 mm internal diameter) from a central multichannel peristaltic pump (Ismatec, Zurich, Switzerland) at the rate of 80 ml/head/d. The infusion packs were refilled with fresh infusate every three days. CONTROL animals received the propylene glycol vehicle only.

At the end of each 21 day infusion period, the pump was stopped, the lines removed and the animals transferred from the metabolism cages to individual pens for a 7 day period. *Ad libitum* feeding of forage rape was continued during these periods.

Measurements and analyses

Dry matter intake was determined daily throughout the experiment, feed refusals being collected before each morning feed. Dry matter determinations were conducted on both feed and refusal samples daily.

Two sets of blood samples were collected from the jugular vein of each animal at 1100 h on days 7, 14 and 21 of each infusion period. The first set of blood samples was collected in evacuated heparinised tubes (Vacutainer, Becton Dickinson, Oxford,

UK) and used for whole blood analyses. The second set of blood samples was collected in non-heparinised tubes. After leaving to stand for one to two hours, serum was removed and stored at -20°C for subsequent analysis.

Packed cell volume (PCV) was determined using the micro-haematocrit method; well mixed whole blood was drawn into capillary tubes which were flame-sealed and spun at 12,000 rpm for five minutes in a haematocrit centrifuge. PCV's were then read on a Hawksley haematocrit reader. Total blood haemoglobin was determined using a commercial kit (Sigma Procedure no. 525; Sigma, Dorset, UK) based on the reaction of haemoglobin with Drabkin's reagent to form cyanohaemoglobin (Stadie, 1920). Drabkin's reagent consists of an alkaline solution of potassium ferricyanide and potassium cyanide. The technique is based on the fact that in the presence of alkaline potassium ferricyanide, haemoglobin is oxidised to methaemoglobin. The latter then reacts with potassium cyanide to form cyanohaemoglobin which has a maximum absorption at 540 nm. The colour intensity at this wavelength, which is proportional to total haemoglobin concentration, is measured spectro-photometrically.

Serum samples were analysed in one batch for total tri-iodothyronine (T3) and total thyroxine (T4) using Amerlex RIA kits (Amersham International, Bucks., UK). Because the antibody was bound to magnetisable polymer beads, separation of the antibody-bound fraction was achieved using magnetic plates rather than centrifugation. Tubes were counted for ^{125}I on a Packard Gamma counter. Calibration curves were constructed using in-house computing software (GenAnalC; written by Dr J. Rogers). The sensitivity of the thyroid hormone assays, calculated as the concentration which is two standard deviations above the zero standard, was 0.15 nmol/l for the T3 assay and 4.0 nmol/l for the T4 assay. A pooled control serum sample was inserted every ten samples for quality control purposes. Intra-assay reproducibility cannot be quoted since the individual values were destroyed by fire before intra-assay reproducibility could be estimated; values quoted by Amersham International indicate coefficients of variation of less than 5% for these assays.

Statistical Analysis

Data were analysed by analysis of variance using the "GENSTAT 5" statistical package (Lawes Agricultural Trust; Release 1.3). A sample analysis of variance table is given in Appendix 4q. Only the results of the last week of each period were subject to analysis of variance to allow time for the development of effects.

Experiment 2.b - Effects of 21-day continuous infusion of allyl cyanide or allyl isothiocyanate on thyroid function, haematocrit and the voluntary food intake of dried grass pellets by lambs.

Animals

Nine Greyface x Suffolk wether lambs were prepared with rumen cannulae as described in Experiment 2a four weeks prior to the start of infusions. Animals were introduced to the experimental diet of dried grass pellets two weeks before the start of the experiment.

Feed

Dried grass pellets (160 g CP/kg DM)[2] were fed *ad libitum* in two approximately equal feeds at 0930 h and 1600 h daily. Refusals were collected before the morning feed and the current day's allowance was calculated as 1.10 that of the previous day's intake.

Treatments and experimental design

Treatments consisted of continuous intra-ruminal infusion for 21 days of ACN (4.8 mmol/d; treatment ACN) or AITC (4.8 mmol/d; treatment AITC) with CONTROL animals receiving the propylene glycol vehicle only. Infusion levels were chosen to be broadly similar to natural ingestion of glucosinolates by animals consuming one kilogram of dry matter of cabbage per day, assuming 0.50 hydrolysis to ACN and 0.50 hydrolysis to AITC.

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[2] Details of the chemical composition of the diet are unavailable as samples were destroyed by fire before they could be analysed.

Experimental design was the same as in Experiment 2a. Details of the layout of the design are give in Appendix 4c.

Procedures, measurements and analyses

Infusates were prepared by diluting appropriate amounts of ACN (4.026 g/l) and AITC (5.950 g/l) in propylene glycol. All other procedures, measurements and analyses were identical to those described in Experiment 2a.

Results

Experiment 2a

There was a tendency for a reduction in VFI by lambs given the ACN treatment compared to that of lambs on the CONTROL treatment, although the difference was not statistically significant at the 0.05 level of significance (Table 4.1; $p = 0.06$). AITC infusion resulted in a significant depression in T3 concentrations relative to CONTROL animals, while ACN infusion caused a slight elevation in plasma T3 concentrations. Plasma T4 concentrations showed similar trends with depressed concentration on the AITC treatment and elevated concentrations among ACN-treated animals relative to CONTROL animals but the differences were not statistically significant.

Haemoglobin concentrations were significantly higher on the ACN treatment than in CONTROL animals. Packed cell volume (PCV), as a second indicator of the anaemic status of the animals, was unaffected by treatment.

Experiment 2b

Although VFI of grass pellets was not significantly affected by treatment, similar trends in VFI to those seen in Experiment 2a were found in this experiment (Table 4.2). ACN treatment animals had lower VFI than either CONTROL or AITC-treatment animals. Voluntary food intake was about 0.50 higher in this experiment than in Exp-

Table 4.1. The effects of allyl cyanide (ACN) and allyl isothiocyanate (AITC) infusion on voluntary food intake (VFI), thyroid hormone concentrations and anaemic status of lambs offered forage rape ad libitum.

	Treatment				
	Control	AITC	ACN	SED	Level of significance
VFI (g DM/d)	982	968	721	111.0	p=0.06
Plasma tri-iodothyronine (T3) concentration (µmol/l)	1.76	1.42	1.94	0.172	P<0.05
Plasma thyroxine (T4) concentration (µmol/l)	81.4	72.9	84.4	8.53	NS
Blood haemoglobin concentration (g/dl)	13.6	13.2	14.6	0.39	p<0.01
Blood PCV (%)	35.2	34.3	36.4	1.16	NS

Table 4.2. The effects of allyl cyanide (ACN) and allyl isothiocyanate (AITC) infusion on voluntary food intake (VFI), thyroid hormone concentrations and anaemic status of lambs offered dried grass pellets ad libitum.

	TREATMENT				
	CONTROL	AITC	ACN	SED	Level of significance
VFI (g DM/d)	1567	1529	1478	80.6	NS
Plasma tri-iodothyronine (T3) concentration (µmol/l)	1.76	1.76	1.85	0.144	NS
Plasma thyroxine (T4) concentration (µmol/l)	78.7	71.4	80.7	10.47	NS
Blood haemoglobin concentration (g/dl)	14.71	14.58	14.56	0.380	NS
Blood PCV (%)	37.4	37.1	36.4	0.95	NS

eriment 2a in which lambs were offered forage rape.

Plasma thyroid hormone concentrations were not significantly affected by treatment in this experiment. Furthermore, the anaemic status of the experimental animals was not significantly altered by ACN or AITC infusion in this experiment and neither haemoglobin concentrations nor PCV values were significantly affected by treatment.

Discussion

Experiment 2a

The results of this experiment indicate that ACN is a stronger VFI depressant than AITC at the levels infused. Although the degree of VFI depression elicited by ACN was only moderate (27%), and just failed to reach statistical significance ($p = 0.060$), the fact that infusion levels in this experiment were relatively low (2.4 mmol/head/d) implicates ACN as a potential VFI depressant. Voluntary food intakes were variable in this experiment (coefficient of variation = 35 %) and this may have been partly due to the fact that the animals were cannulated and confined to allow infusion. The level of variability seen here would have required a larger experiment to provide more conclusive statistically significant evidence of VFI depression. Since this was a preliminary screening experiment, only a limited range of measurements were taken aimed at determining toxic mechanisms. The elevation in peripheral T3 concentrations elicited by ACN infusion was minor and probably not the main cause of its effects on toxicity. *In vivo* cyanide production from aliphatic nitriles (Willhite and Smith, 1981), combined with a subsequent elevation of plasma thiocyanate ion concentrations through the action of hepatic rhodanese on free cyanide, might have been expected to impair rather than enhance thyroid hormone production, due to the anti-thyroid properties of the thiocyanate ion. Increased plasma T3 concentrations were, therefore, probably associated with alterations in the peripheral metabolism of T4 to T3 or the cellular uptake of T3 and this issue will be addressed in more detail in later experiments.

Allyl isothiocyanate infusion had little effect on the VFI of forage rape at the level infused in this experiment. The observed depression in peripheral concentrations of T3 and the indication of a similar effect with respect to peripheral T4 concentrations reflect the known anti-thyroid effects of this compound (Langer and Stolc, 1965; Langer and Greer, 1968). Thyroid hormones have multiple activities at the cellular level but are thought to be primarily involved in the control of metabolic rate and so on growth rate and VFI. The reduction in concentrations of circulating thyroid hormones did not appear to be of sufficient magnitude to have marked effects on the appetite of the experimental animals, since reduced VFI was not observed; longer term administration of AITC might have had more serious toxic effects.

The haematological status of the experimental animals was measured to assess the contribution, if any, of AITC or ACN to the well-known haemolytic anaemia found among brassica-fed animals. The main cause of this anaemia has been established as the presence of S-methyl cysteine sulphoxide (SMCO) (Smith, 1974) in brassica herbage. This compound is broken down by rumen micro-organisms to the actual lysin, dimethyl disulphide (DMDS); lysis apparently follows perturbation of protective mechanisms within the erythrocyte with the role of reduced glutathione in protecting against free-radical damage being particularly affected. Conjugation with glutathione is also known to occur with isothiocyanates (Brusewitz et al., 1977) and nitriles (Szabo et al., 1977) and these compounds could potentially compound the effects of DMDS in causing haemolytic anaemia. Examination of the anaemic response to treatment with ACN or AITC in this experiment indicated that neither compound had significant haemolytic activity. Indeed, ACN caused significant elevation in blood haemoglobin concentrations after 3 weeks of infusion ; the reason for this is unclear, although it could conceivably have been a compensatory response to allow increased oxygen transport to the tissues to counteract inhibition to cellular respiratory pathways occurring as a result of free cyanide release in the tissues (Willhite and Smith, 1981).

In comparison with previous studies in which the haematological status of brassica-fed ruminants has been studied, the degree of anaemia in the current experiment was slight despite the fact that animals were offered forage rape *ad libitum* for a period of 13 weeks. Presumably the rape diet contained relatively low

concentrations of SMCO, although its content was not measured. In addition sheep are known to be less susceptible to haemolytic anaemia than cattle or goats (Smith, 1974). Had the anaemic response to brassica feeding been more typically acute, the potential additional contribution of glucosinolates might have been more effectively assessed.

Experiment 2b

Overall VFI was higher in this experiment in which animals were fed on dried grass than in Experiment 2a in which the diet was forage rape. Although some of this effect may have been associated with the physical form in which the diet was presented (dried grass pellets vs. fresh, chopped forage rape), the observed shortfall in VFI of the forage brassica crop does agree with previous comparative studies (see Literature Review).

The most remarkable result of this experiment, in which grass-fed animals were infused with physiological concentrations of glucosinolate breakdown products, was the apparent lack of detrimental effects of either compound. Although trends in a depression in VFI by ACN and a reduction in concentrations of plasma thyroid hormones on the AITC treatment were similar to the effects observed in Experiment 2a, none of the effects approached statistical significance. This observed lack of effect of either compound was surprising, bearing in mind that infusion levels in this experiment were double those in Experiment 2a. Because these animals received no dietary glucosinolates, however, actual absorption of glucosinolate breakdown products may have been less than in Experiment 2a, in which animals received both dietary and artificially administered glucosinolates, and may have been below a threshold at which detoxification mechanisms were overwhelmed and toxicity occurred.

Alternatively, as previously discussed, the toxic effects of glucosinolates may be dependent on their interaction with other components of the brassica diet, either due to direct interactions between the physiological effects of various compounds within the brassica diet or through effects on the metabolic fate of the compounds. For example, the effects of systemic free cyanide release from nitriles on respiration could be more pronounced in animals made anaemic by SMCO ingestion. This is a question

which will be addressed in Experiment 5. For example, damage to liver tissue caused by nitriles may reduce efficiency of excretion of other xenobiotics, such as DMDS and AITC, and so increase their potency.

Finally, the diet may have affected the potency of the compounds by leading to a basic difference between the nutritional status of the experimental animals. The effects of plane of nutrition on the efficiency of xenobiotic detoxification have been demonstrated (Kappas, Anderson, Conney and Alvares, 1976).

Conclusions

Two main conclusions emerge from these experiments; firstly ACN is a more potent VFI depressant than AITC when administered to sheep at equimolar rates and a more intensive long-term assessment of its toxicity to sheep is warranted. Secondly, the effects of ACN in reducing VFI were stronger on the brassica than on the non-brassica diet indicating the importance of other components of the brassica diet in eliciting the potential toxicity of ACN.

Chapter 5 - LONG TERM EFFECTS OF INTRA-RUMINALLY ADMINISTERED ALLYL CYANIDE ON LAMBS

Experiment 3 - The effect of long-term (9 week) intra-ruminal infusion of allyl cyanide on the voluntary food intake and metabolism of lambs.

Introduction

A preliminary assessment of the relative physiological effects of nitriles and isothiocyanates in Experiment 2 indicated that ACN had greater effects on the VFI of forage rape than AITC. On the basis of these results a more long-term examination of the effects of ACN administration on sheep with greater emphasis on the mechanisms of toxicity was conducted.

A number of potential biochemical lesions have been found to occur in monogastrics as a result of nitrile administration. Nitriles have been found to cause lesions in the liver and kidney tissues of rats (Van Etten et al., 1969; Nishie and Daxenbichler, 1980) though the mechanisms underlying the cellular injury are not clear. Nitriles may also have more general effects on cellular metabolism following release of free cyanide in liver and brain tissues (Willhite and Smith, 1981). The resulting inhibition of cytochrome oxidase activity seems to be important in the acute toxicity of aliphatic nitriles (Ahmed and Farooqui, 1982). The chronic repercussions of low rates of cyanide production in the tissues that may occur in ruminants fed on forage brassica crops are unknown.

As well as these direct effects, nitriles may compound the effects of other components of the brassica diet. For example, free cyanide, released in the tissues from nitriles (Willhite and Smith, 1981), is presumably detoxified by hepatic rhodanese to thiocyanate ions (Westley, 1988). Increased concentrations of circulating thiocyanate ions may then affect thyroid function (Paxman and Hill, 1974), adding to the direct goitrogenic effects of glucosinolate-derived isothiocyanates. Furthermore, the haemolytic anaemia caused by SMCO present in brassica herbage may be exacerbated by nitriles because of their activity as glutathione depletors. Maintenance

of adequate levels of reduced glutathione is important in maintaining the integrity of the erythrocyte membrane, and glutathione depletion by nitriles may therefore contribute to the haemolytic effects of SMCO.

In order to assess the importance of these various potential effects of nitriles, an experiment was conducted in which ACN was infused into the rumen of sheep offered dried grass pellets *ad libitum* over a period of nine weeks. Voluntary food intake was measured and renal sufficiency and hepatic damage were assessed using clinical function tests; effects on tissue cytochrome oxidase activity were examined at the end of the treatment period. Finally, the effects on erythrocyte glutathione concentrations were recorded to determine the potential contribution of ACN to haemolytic anaemia.

Sheep were again continuously infused, intra-ruminally with ACN, although in this experiment, two infusion rates were tested. The low level of ACN infusion (Treatment L) was similar to the rate applied in Experiment 2b and was based on approximate levels of ACN release to be expected, assuming ingested sinigrin to be broken down into equal proportions of ACN and AITC. The higher infusion rate (Treatment H) corresponded to maximum potential release of ACN assuming complete hydrolysis of sinigrin to ACN in the rumen. The higher infusion rate (Treatment H) may thus have resulted in higher than natural levels of ACN administration, given the results of Experiment 1 which indicated that a significant proportion of ingested sinigrin is hydrolysed to AITC. However, sinigrin is only one of a number of glucosinolates found in forage brassica crops and nitrile release from the full complement of glucosinolates may be considerably higher than the level given here. The higher ACN infusion rate was probably intermediate between levels likely to arise from total and individual glucosinolate hydrolysis.

A longer treatment period (9 weeks) was employed in this experiment to give sufficient time for potential chronic, cumulative effects to develop. Most previous work on nitrile toxicity has concentrated on the acute toxic effects associated with free cyanide release (Willhite and Smith, 1981; Ahmed and Farooqui, 1982). Forage brassica ingestion, however, does not lead to acute toxic symptoms and any detrimental effects are more likely to be as a result of chronic, long-term exposure which is more effectively investigated in a long-term experiment.

Materials and Methods

Animals

Twenty-one Scottish Blackface wether lambs (7-8 months; 30-40 kg W) were selected from the MLURI's, Hartwood Research Station in January, 1988 and surgically prepared with "Trocar" type rumen cannulae (1 cm diameter, Arnolds Veterinary Products, Essex, UK) three weeks before the start of the experiment (as described in Experiment 2a).

Feeds

All animals were offered a diet of dried grass pellets to appetite throughout both the pre-treatment and the treatment periods. The feed (160 g CP/kg DM)[3] was offered at 0900 h once daily; refusals were collected before feeding each morning and the amount offered was calculated as 1.10 that of the previous day's food intake.

Treatments and experimental design

The experiment took the form of a simple randomised block design with three treatments and seven animals per treatment. Animals were offered the experimental diet of dried grass pellets *ad libitum* for 21 days prior to the start of treatment imposition. Mean VFIs recorded between days 8 and 21 of this period were used to rank animals on the basis of their VFI. The rank order was then divided into seven blocks of three animals each, so that animals with similar VFI's were in the same block. Treatments were randomly allocated to animals within blocks. Details of treatment allocation are given in Appendix 5b.

[3] Details of the chemical composition of the diet are unavailable since samples were destroyed by fire before they could be analysed.

An infusion period of 63 days followed this pre-treatment period during which the following treatments were applied to each animal:

Treatment H - 9.6 mmol/d of ACN continually infused intra-ruminally.

Treatment L - 4.8 mmol/d of ACN continually infused intra-ruminally.

Treatment C - Continuously intra-ruminally infused with propylene glycol infusion vehicle.

Procedures

The infusates were prepared by diluting appropriate volumes of ACN in propylene glycol as in Experiment 2a (H, 8.052 g/l; L, 4.026 g/l; C, 0.000 g/l). Infusates were delivered to sheep (80 ml/d) along polythene infusion lines (0.0625 mm i.d.) from individual peristaltic infusion pumps strapped to each animal's back by means of canvas harnesses. Each infusion point consisted of a 10 cm length of stainless steel tubing (2 mm i.d.) which was attached to the end of the infusion line and which had been drilled with a number of holes at the end situated in the rumen to prevent blockage with digesta. Sealed polythene infusion packs (0.5 l) acted as reservoirs which sat behind each pump and were secured by plastic clips. The pump apparatus is illustrated in Figure 5.1. Pump batteries were replaced at weekly intervals to ensure constant infusion rates; sealed infusion packs were replenished at three to four day intervals and were filled using a multi-channel peristaltic pump (Ismatec SA, Zurich, Switzerland; model mp13r). Pumps and lines were checked for leakage two or three times a day.

Two blood samples were collected from the jugular vein of each animal on days 13 and 20 of the pre-treatment period and weekly during the treatment period starting on day 7, using evacuated, heparinised tubes (Vacutainers, Becton Dickinson, Oxford, UK). One set of blood samples was centrifuged at 3000 rpm and the plasma removed and stored at -20°C. Plasma samples were later analysed to determine the plasma concentrations of clinical indicators of kidney (creatinine, urea) and liver (gamma glutamyl transpeptidase (GGTP)) function. The second set of blood samples were analysed on the day of collection for PCV, blood haemoglobin concentration and blood reduced glutathione concentration. Animals were slaughtered using intravenous pentobarbitone

Figure 5.1 Infusion apparatus used in Experiment 3 consisting of portable peristaltic pump, harness and infusion cannula.

at the end of the experiment in three batches of seven animals each over three days. Infusions were continued until within one hour of slaughter. Following slaughter liver and kidney tissues were quickly excised and weighed fresh. Samples (approximately 1 g) of liver and kidney tissues were removed and placed in ice-cold phosphate buffer (0.033 M; pH 7.4) prior to analysis for cytochrome oxidase activity and tissue glutathione concentrations.

Measurements and analyses

Voluntary food intake was measured daily throughout the infusion period by recording the amounts of dry matter offered and refused. Plasma concentrations of creatinine, urea and GGTP were determined in samples which had been stored at -20°C. Samples were analysed by continuous flow analysis as follows. Creatinine was analysed using the Jaffe reaction in which a red complex is formed with alkaline picrate (Butler, 1975), which is measured colorimetrically. Urea was determined using a urease method to produce ammonia, which was subsequently measured using the Berthelot reaction (Wilcox, Caroll, Sterling, Davis and Ware, 1966). GGTP was determined using the method of Fuke, Yagi, Takegoshi and Kondo, (1976) in which γ-L-glutamyl-p-nitroanilide, added as a substrate, was hydrolysed to p-nitroaniline, which was subsequently dye-linked, and the absorption of the derivative measured at 550 nm.

Haemoglobin and PCV analyses were conducted on the day of collection according to the methods described in Experiment 2a. Blood reduced glutathione was analysed by the method of Beutler, Duron and Kelly (1963). In this method, erythrocytes are lysed and de-proteinised before being filtered and the concentration of sulphydryl groups in the filtrate is then determined by measuring the absorbance of a coloured complex, formed following addition of 5,5′-dithiobis-(-2-nitrobenzoic) acid (DTNB reagent).

Samples of liver and kidney tissue were homogenised in ice cold phosphate buffer (0.033 M; pH 7.4) using a PTFE barrel and plunger homogeniser (Philip Harris Scientific, London, UK). Reduced glutathione concentrations in the homogenates were determined employing the reaction of sulphydryl groups with DTNB reagent to form

a coloured complex (Sedlak and Lindsay, 1968). Cytochrome oxidase activity was determined by following the change in absorbance of a reduced cytochrome c preparation (Sigma, Poole, Dorset) at 550 nm, which resulted from enzymatic oxidation following addition of an aliquot of tissue homogenate (Cooperstein and Lazarow, 1951). All tissue analyses were complete within six hours of slaughter.

Statistical Analysis

Data were analysed statistically using "GENSTAT 5" (Lawes Agricultural Trust; Release 1.3). The main effects of treatment and time together with their interaction were analysed using analysis of variance, having accounted for variation due to blocks and animals within blocks, using the BLOCKSTRUCTURE statement. A sample ANOVA table is presented in Appendix 5.a.

Results

The overall effects of the treatments on VFI, blood glutathione concentrations and plasma concentrations of creatinine, urea and GGTP are presented in Table 5.1. Voluntary food intake was depressed ($p < 0.05$) by the infusion of ACN (Treatments H and L) compared with control values (Treatment C) and the effect was greater on Treatment L than on Treatment H. Blood glutathione concentrations were similar on all treatments.

Plasma creatinine concentrations were significantly affected by treatment ($p < 0.05$); animals on Treatment L had significantly elevated concentrations of plasma creatinine while those on Treatment H had depressed plasma creatinine concentrations relative to Treatment C animals. Mean plasma urea concentrations were unaffected by treatment.

Plasma GGTP activity was significantly higher on Treatment H than on Treatment C. Control animals and those on the Treatment L had similar plasma GGTP concentrations.

In addition to examining the overall effects of treatment on the above parameters, treatment by time interactions were examined in order to determine the consistency

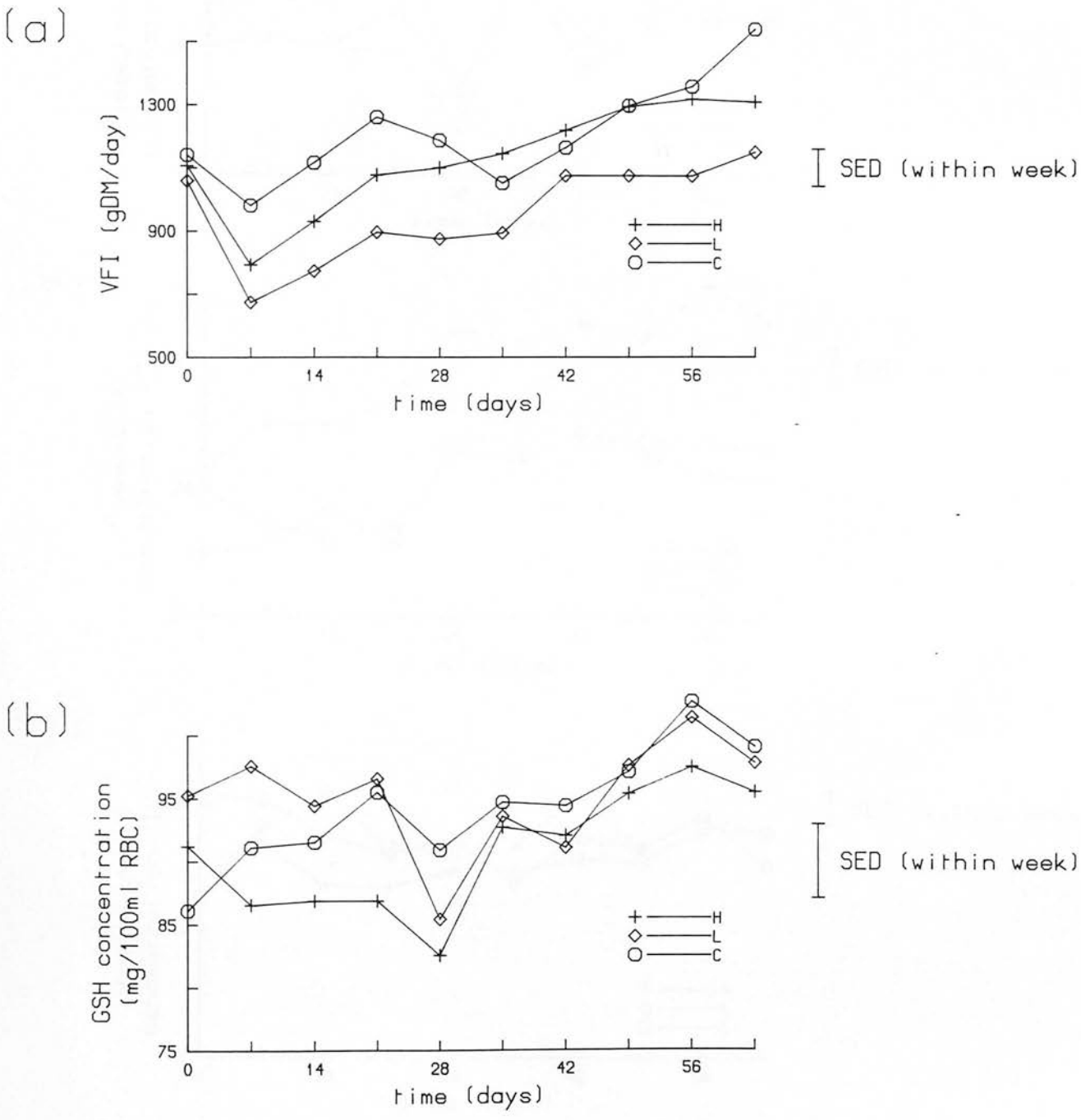


Figure 5.2 Effects of long-term intra-ruminal infusion of allyl cyanide on voluntary food intake (VFI) (a) and blood glutathione concentrations (GSH) (b) in sheep.

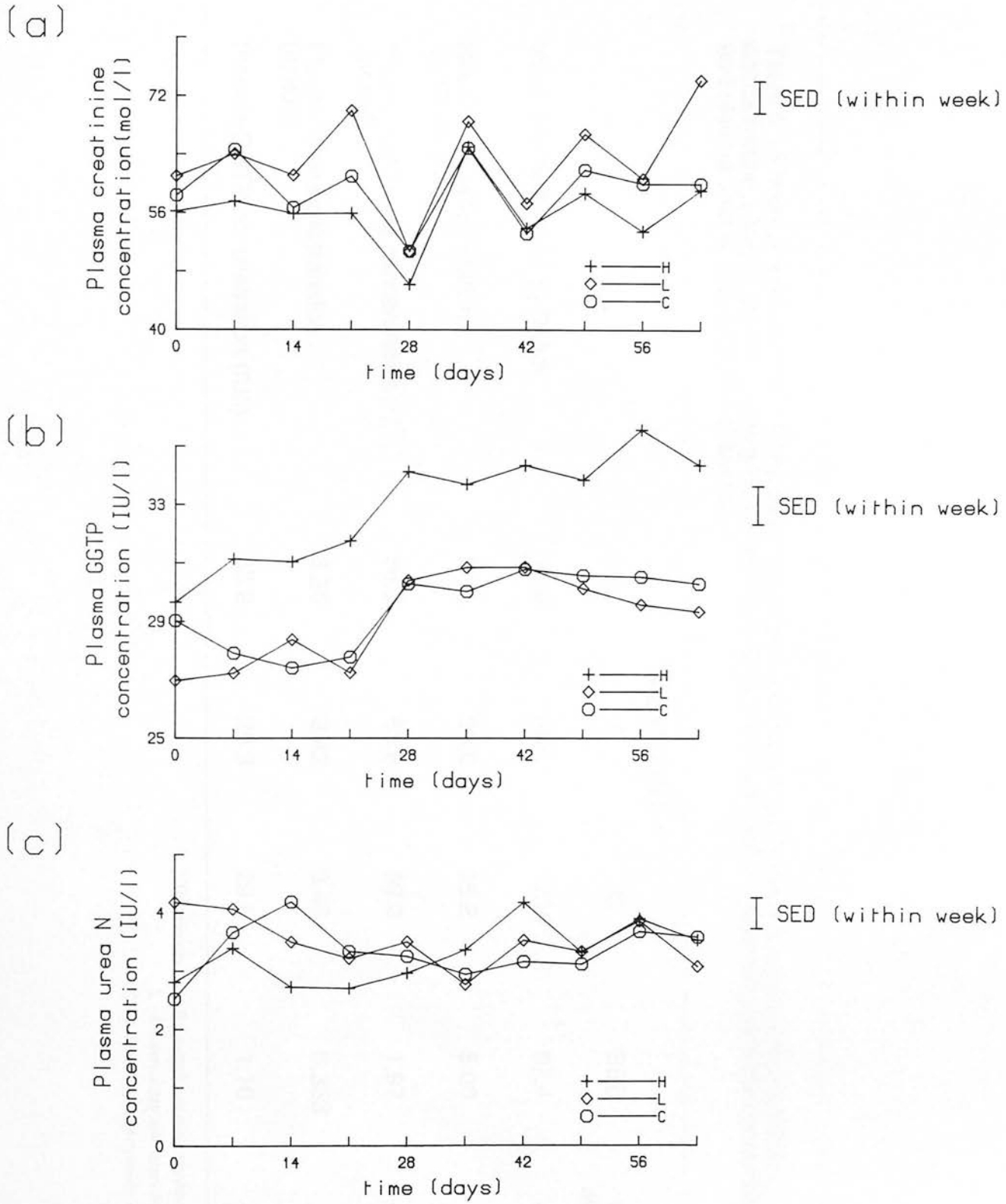


Figure 5.3 Effects of long-term intra-ruminal infusion of allyl cyanide on plasma creatinine (a), gamma glutamyl transpeptidase (GGTP) (b) and urea concentrations (c) in sheep.

Table 5.1 Effects of intra-ruminal allyl cyanide infusion on voluntary food intake, blood glutathione (GSH) concentration, plasma gamma glutamyl transpeptidase (GGTP) concentration, plasma creatinine concentration and plasma urea N concentration of lambs.

	TREATMENT *				
	H	L	C	SED	Level of significance
Voluntary food intake (g DM/d)	1098	958	1225	87.4	<0.05
Blood GSH concentration (g/100ml RBC)	90.8	95.0	95.2	5.00	NS
Plasma creatinine concentration (µmol/l)	56.3	63.8	59.0	1.97	<0.05
Plasma urea concentration (mmol/l)	3.35	3.42	3.46	0.353	NS
Plasma GGTP concentration (IU/l)	33.2	29.3	29.5	1.10	<0.05

* Treatments H - 9.6 mmol/day allyl cyanide for 63 days
L - 4.8 mmol/day allyl cyanide for 63 days
C - 0 mmol/day allyl cyanide for 63 days

TABLE 5.2 Effects of intra-ruminal allyl cyanide infusion on tissue weight, tissue glutathione (GSH) concentration and tissue cytochrome oxidase activity in lambs.

	TREATMENT **				
	H	L	C	SED	Level of significance
Kidney weight (g/kg LW)	3.73	3.63	3.75	0.095	NS
Liver weight (g/kg LW)	19.91	18.74	19.32	0.689	NS
Kidney GSH (mg/100g tissue)	331	326	327	37.5	NS
Liver GSH (mg/100g tissue)	818	730	680	138.0	NS
Kidney cytochrome oxidase activity *	18.3	21.9	18.2	3.17	NS
Liver cytochrome oxidase activity *	8.9	12.4	12.6	1.26	$p<0.05$

* units = delta log[ferrocytochrome]/min for 1:100 tissue dilution

** Treatments H - 9.6 mmol/day allyl cyanide for 63 days
L - 4.8 mmol/day allyl cyanide for 63 days
C - 0 mmol/day allyl cyanide for 63 days

of the response over time and specifically to investigate whether the treatment effects developed over time or were subject to adaptation. The effects of treatment over time on VFI, blood glutathione concentrations and plasma creatinine, urea and GGTP concentrations are shown in Figures 5.2 and 5.3. The treatment by time term of the analysis of variance was not significant for VFI, blood glutathione, plasma creatinine and plasma GGTP concentrations, indicating that the effects of treatment were consistent over time (Figure 5.2 and 5.3).

Plasma urea concentrations showed a significant treatment by time interaction. Examination of the corresponding graph (Figure 5.3 c) indicates that some of this effect could be attributed to a developing increase in plasma urea concentrations over time among Treatment H animals when compared to Treatment C and Treatment L animals.

Following slaughter of the experimental animals at the end of the infusion period, liver and kidney tissues were removed for determination of tissue glutathione concentrations and tissue cytochrome oxidase activity and results of tissue analyses are presented in Table 5.2. Kidney and liver fresh weights expressed as a proportion of liveweight were not significantly affected by treatment. Liver and kidney glutathione concentrations were not significantly affected by treatment. Liver cytochrome oxidase activity was significantly affected by treatment ($p < 0.05$) with animals on Treatment H having depressed activity compared with Treatment C and Treatment L animals, which had similar values. Kidney cytochrome oxidase activity on the other hand was unaffected by treatment. Cytochrome oxidase activities were higher in the kidney than in the liver tissues in agreement with previous studies (Cooperstein and Lazarow, 1951).

Discussion

This experiment was conducted as a more long-term, intensive investigation of the effects of the sinigrin-derived nitrile, ACN, on growing sheep. Although VFI was depressed in animals infused with ACN compared to that of control animals, a classical "dose-response effect" was not observed with the higher level of ACN

(Treatment H) reducing VFI to a lesser degree than the low level of ACN (Treatment L). Whether the higher VFI on Treatment H than on Treatment L was a genuine biological phenomenon or an artefact of treatment imposition is unclear. Examination of the individual animal data (Appendix 5.c) shows that of the twenty-one original animals, four had to be removed from the experiment because of exceptionally low VFI's. The low VFI's appeared unrelated to treatment and appeared to be linked to the presence of the pumps and harnesses, since removal of these resulted in higher VFIs within a few days. This extreme behavioural effect may have occurred to a lesser degree in other animals obscuring treatment effects on appetite. This is borne out by the variability in VFI data among the seventeen remaining animals; the coefficient of variation for VFI data was 31.8% for the experiment and this is high in relation to other studies.

A number of associated measurements were made in this experiment aimed at determining the physiological mechanisms underlying chronic nitrile toxicity. Plasma urea and creatinine concentrations are used clinically as indicators of kidney function and were measured in the current experiment because of the previously described toxicity of nitriles to kidney tissues (Gould et al., 1985). Although plasma creatinine concentrations were elevated on the low treatment in this experiment, the effect was small and concentrations were not higher than previously quoted normal values for sheep (100-170 µmol/l; Kaneko, 1980). (The fact that creatinine concentrations were below the quoted normal range probably reflects the juvenile status of the animals since the normal range in humans increases with maturity (Gowenlock, McMurray and McLauchlan, 1988)). Much higher levels would need to be recorded before diagnosing renal insufficiency.

Plasma urea concentrations may be less useful as an indicator of kidney function in ruminants than in monogastrics, since recycling of urea *via* the saliva into the rumen provides an additional route for urea loss in ruminants. Quantitatively, however, renal excretion is a more important route than rumen recycling and renal insufficiency is likely to lead to elevated plasma concentrations of urea. Plasma urea concentrations in this experiment, however, gave no indication of renal insufficiency.

Plasma gamma glutamyl transpeptidase concentrations were determined to assess potential liver toxicity. Concentrations were elevated by the higher level of ACN infusion but again the increase was slight and not sufficient to serve as firm evidence for liver damage. Elevated GGTP concentrations on the H treatment provided some evidence for liver damage. However, to place these results in context, in an assessment of the value of GGTP for clinical diagnosis of hepato-toxicity in sheep, serum GGTP concentrations reached twenty times normal values in cases of severe liver damage (Towers and Stratton, 1978).

As well as their potential pathological effects on specific tissues such as the liver and kidney, nitriles may have more generalised effects on cellular metabolism *via* release of free cyanide in the tissues (Willhite and Smith, 1981). Cyanide is known to inhibit activity of cytochrome oxidase, the terminal enzyme in the electron transport chain. This is thought to be the mechanism underlying the acute toxic effects of cyanide, though its importance in chronic toxicity is not clear. Liver cytochrome oxidase activity was depressed when measured at the end of the experiment, indicating a possible long-term inhibition of oxidative phosphorylation by the chronic release of free cyanide in the liver. Kidney cytochrome oxidase activity was unaffected by treatment and the more severe effects in the liver may have been because higher concentrations of absorbed toxins were present in the portal circulation than in the peripheral circulation.

Liver and kidney glutathione concentrations were also unaffected by ACN administration. Glutathione is involved in the excretion of foreign compounds including nitriles (Orrenius and Moldeus, 1984; Pilon, Roberts and Rickert, 1988) from the body *via* the mercapturic acid pathway (Boyland and Chasseaud, 1969). Nitriles are known to act as glutathione depletors, although this tends to be an immediate, short-term effect. Tissue glutathione concentrations were not reduced on the ACN treatments, which suggests that adaptation to chronic administration of ACN may have occurred. Thus, a new equilibrium between glutathione synthesis and excretion *via* the mercapturic acid pathway may have been reached which was sufficient to maintain tissue glutathione levels in ACN-treated animals by the end of the experiment when samples were taken. Sequential measurements of tissue glutathione

concentrations might have revealed the time scale at which this equilibrium was reached but the intrusive nature of tissue sampling procedures precluded this data being gathered. Maintenance of hepatic glutathione levels indicated that the capacity of the mercapturic acid excretion pathway had not been exhausted and that little absorbed ACN may have entered the peripheral circulation. This was reflected in the fact that blood glutathione concentrations were also unaffected by ACN infusion. The interactions between nitriles and glutathione will be considered in more detail in Experiment 5.

In summary, intra-ruminal ACN administration reduced VFI although not in a predictable, dose-related manner. In addition, there was limited evidence for pathological effects on liver and kidney tissues. More severe effects were seen at the cellular level with significant depression in cytochrome oxidase activity, an enzyme of primary importance in cellular respiration.

Previous studies have demonstrated the toxic nature of ACN and related nitriles in other species (Ahmed and Farooqui, 1982; Gould et al., 1985) although information on ruminants is lacking. The animals in this experiment showed considerable tolerance to this compound. Presumably they were able to detoxify the compound in some way and this could have occurred either before absorption in the digestive tract or following absorption in the liver. The capacity of rumen micro-organisms to degrade plant toxins prior to their absorption has been demonstrated with other classes of plant secondary compound (Carlson and Breeze, 1984; Dickinson, Smith, Randel and Pemberton, 1988) and this could have been occurring in the current experiment. This possibility will be investigated in a further experiment (Experiment 4).

Chapter 6 -RUMINAL FATE OF SINIGRIN BREAKDOWN PRODUCTS.

Introduction

Results of infusion experiments, in which the sinigrin breakdown products AITC (Experiment 2a and 2b) and ACN (Experiment 2a,2b and 3) were infused continuously into the rumen of sheep, showed the compounds to have only moderate effects on VFI with evidence of limited tissue toxicity. Previous tests of isothiocyanate toxicity (Becker and Plaa, 1965) and nitrile toxicity (Van Etten et al., 1969; Nishie and Daxenbichler, 1980) in rodents have shown both groups of compounds to be potentially toxic at the levels infused here. The less severe effects of these compounds in ruminants than in monogastrics may be the result of different species susceptibility to the systemic effects of the compounds. Alternatively, ruminants may be more efficient in detoxification of the compounds either by hepatic pathways, or more probably in the rumen itself.

Studies with other groups of secondary compounds have demonstrated the capacity of rumen micro-organisms to degrade potentially toxic compounds in the diet and so protect the host from detrimental effects. For example, *in vitro* experiments have demonstrated the capacity of rumen micro-organisms to degrade β-nitropropionic acid found in *Coronilla varia* and so reduce the toxicity of this plant (Gustine, Moyer, Wangsness and Shenk, 1977) and similar phenomena have been reported with mimosine (Jones, 1981), pyrrolizidine alkaloids (Lanigan, 1971) and other compounds.

The apparent tolerance of sheep to ruminal infusion of ACN and AITC, the toxicity of which has been demonstrated in other species (Becker and Plaa, 1965; Van Etten et al., 1969; Nishie and Daxenbichler, 1980), prompted an investigation of the fate of the compounds in rumen fluid. Initial experiments were aimed at quantifying the stability of ACN in rumen fluid from sheep fed on either brassica or non-brassica diets and assessing the role of the microbial fraction of rumen fluid in ACN degradation (Experiment 4a). Further work sought to determine the time course of adaptation to a glucosinolate-containing diet and the development of the ability to degrade ACN and AITC (Experiment 4b).

Of particular interest was the possibility that the time taken for rumen micro-organisms to adapt to the presence of secondary compounds arising from brassica herbage could be responsible for the "check" in growth following introduction of sheep to brassica crops which is often observed. Feeding studies with forage brassicas have regularly shown growth rates to be lower during the initial 2 - 3 weeks of brassica feeding than later in the grazing period (Nicol and Barry, 1980).

Materials and Methods

Experiment 4a - *In vitro* stability of allyl cyanide in rumen fluid from sheep fed fresh cabbage or dried grass pellets.

Animals and diets

Two Greyface ewes (age 4-5 years; 70-80 kg) were used. Both animals were equipped with permanent rumen cannulae (4cm diameter; Kay and Mackenzie, 1968) which had been in place for more than a year and prior to the experiment the animals had been habitually sampled for rumen fluid *via* the rumen cannulae. One animal was introduced to a diet of fresh chopped cabbage, while the other was offered dried grass pellets (160 g CP/kg DM)[4]. Cabbage was stored at 4°C and was chopped by hand immediately prior to feeding. Both animals were fed 1.0 kg DM/d of their respective feeds in two equal meals at 0800 h and 1600 h daily. The diets were offered in this manner throughout the experiment and there were no refusals. There was a 14-day period between the introduction of the experimental diets and the start of experimental measurements.

Experimental procedures and measurements

1. Assessment of stability of allyl cyanide in rumen fluid

After the initial 14-day period on the diets, the first rumen samples were collected for the assessment of the stability of ACN in rumen fluid. Approximately 500 ml of

[4] Precise details of the chemical composition of the feed are unavailable since feed samples were destroyed by fire before they could be processed.

rumen fluid was collected *via* the rumen cannula from each animal at 0900 h using a vacuum pump . Samples were aspirated directly into pre-warmed vacuum flasks which had been purged with CO_2 prior to collection. Rumen samples were immediately transferred to the laboratory (within 10 minutes) and strained through muslin. Two hundred millilitres of liquid from each source was decanted into separate 1 litre round-bottomed flasks. The whole procedure was conducted under CO_2 to preserve microbial activity and CO_2 was bubbled through the strained rumen fluid for 2-3 minutes before sealing the flasks. Flasks were sealed with rubber bungs fitted with bunsen valves and sampling ports and placed in a water bath at 37 ^{o}C.

Allyl cyanide was diluted as follows. Twenty-seven milligrams (400 μmol) of ACN were pipetted into 2 ml of distilled water in a glass scintillation vial. The mixture was sonicated for 2 minutes by suspending the vial in an ultrasonic bath and half the mixture (1 ml) was then added to each of the two incubation flasks. In this way 200 ul of ACN was added to each flask within one hour of collection from the animal.

Samples (20 ml) of rumen fluid were collected from each flask by syringe at 0, 5, 10, 20, 40 and 160 minutes after addition of ACN and immediately processed as follows. One millilitre of internal standard solution (1mg butanol/ml distilled water) and two to three drops of anti-foaming agent (Antifoam A Emulsion; Sigma, Poole, Dorset) were added to a long-necked round-bottomed flask (50 ml capacity). The rumen fluid sample was then added to the flask which was placed on a steam distillation apparatus and distilled to remove the volatile components. The first 10 ml of distillate was collected and extracted twice with 5 ml of dichloromethane and the pooled extracts were then dried over anhydrous sodium sulphate. The dried extract was concentrated under an air stream to approximately 500 μl before being transferred to stoppered glass vials ready for GLC analysis.

2. Role of rumen micro-organisms in degrading allyl cyanide.

The capacity of rumen micro-organisms to degrade ACN was assessed in a second incubation using rumen fluid from the cabbage-fed sheep after 21 days of cabbage feeding. To this end, a rumen fluid sample (600 ml) was collected from the cabbage-fed animal at 0900 h as before. The sample was transferred to the laboratory and

divided into two equal aliquots of 300 ml each. One aliquot was strained through muslin to remove coarse particulate matter and stored at 4°C for 30 minutes ("whole" rumen fluid), while the other aliquot was centrifuged at 20,000 g for 30 minutes at 4°C to remove micro-organisms (supernatant= "centrifuged" rumen fluid). The two rumen fluid samples (200 ml each) were then decanted into round- bottomed flasks which were placed in a water bath at 37°C. Allyl cyanide was diluted as before by adding 19 mg (280 µmol) of ACN to two millilitres of distilled water and sonicating the mixture. One millilitre of the mixture was then added to each of the two incubation vessels such that each flask received 140 µmol of ACN. Samples (20 ml) were removed as before at 0, 15, 45, 60 and 140 minutes after ACN addition and prepared for GLC analysis as previously described.

Analysis

GLC analysis was carried out on a Philips PU4400 gas chromatograph. The column was a BP20 fused silica capillary column (SGE) of length 25 m, an internal diameter of 0.33 mm and a film thickness of 1.0 um. The sample (0.5 ul) was introduced onto the column by "Grob splitless" injection using a purge delay of 45 seconds and an initial column temperature of 30°C. Column temperature was increased at a rate of 9°C/min to a final temperature of 120°C. Both carrier and make-up gases were helium and detection was by flame ionisation detection. Peak areas were measured on a Philips PU4810 computing integrator and the response factor of ACN relative to the butanol internal standard peak was determined using pure standards (response factor:ACN = 1.181). Allyl cyanide concentrations in test samples were subsequently determined relative to the internal standard.

Experiment 4b - Determination of the time taken for rumen micro-organisms to develop the ability to degrade sinigrin breakdown products.

The time scale involved in the development of the capacity of rumen micro-organisms to degrade ACN and AITC was investigated in a further experiment.

Animals

Six Scottish Blackface wethers of liveweight 45 - 55 kg were ranked on the basis of live-weight and assigned to two treatment groups balanced for live-weight in October 1988.

Feeds and treatments

The two treatment diets consisted of 800 g DM of either dried grass pellets (Treatment DG) or fresh chopped cabbage (Treatment CA). Dried grass pellets (160 g CP/kg DM)[5] were fed as purchased while the cabbage was stored at 4°C prior to use and hand chopped as required. In a pre-treatment period lasting seven days, all sheep were offered 800 g DM/d of dried grass pellets in two equal meals at 0800 h and 1600 h. During the ensuing 30-day treatment period, the three Treatment CA animals were offered fresh cabbage (800 g DM/d) while Treatment DG animals continued to receive dried grass pellets (800 g DM/d). In the final seven day post-treatment period which followed, all animals were again offered 800 g/d of dried grass pellets.

Measurements and procedures

Rumen fluid samples (approximately 150 ml per animal) were collected from each animal using a stomach tube and vacuum pump into a Buchner flask and pooled by treatment in pre-warmed vacuum flasks which had been purged with CO_2. Samples were taken at 0900 h on day 4 of the pre-treatment period, on days 1, 6, 8, 15, 22 and 29 of the treatment period and on day 7 of the post-treatment period.

The incubation procedure was broadly similar to that used in Experiment 4a except that individual incubation tubes were used in place of the sequentially sampled common vessel. Following collection, pooled rumen fluid samples (approximately 450 ml) were strained through muslin and diluted with an equal volume of artificial saliva (McDougall, 1948). Collection and filtration were conducted under CO_2 to maintain

[5]Precise details of the chemical composition of experimental diets are unavailable since samples were destroyed by fire before analyses were conducted.

microbial activity and CO_2 was then bubbled through the diluted sample for 2-3 minutes before decanting into incubation tubes.

Two sets of 21 thick-walled test tubes (100 ml capacity) were purged with CO_2, capped with bunsen valves and placed in racks in a circulating water bath maintained at 37 ^{o}C ready to receive the rumen fluid. An ACN/AITC solution was prepared by diluting 200 mg of ACN and 200 mg of AITC in 100 ml distilled water and sonicating the mixture as in Experiment 4a. One millilitre of the mixture was added to each tube. At time zero, 50 ml of diluted rumen fluid from the cabbage-fed sheep was rapidly added to each of 15 tubes using an automatic dispenser (Oxford Laboratories, County Kildare, Ireland) while an additional 6 control tubes each received 50 ml of phosphate buffer (0.05 M, pH 7). The headspace of all tubes was rapidly purged with CO_2 and the tubes were immediately stoppered. Three control tubes and three treatment tubes were immediately removed from the water bath (time 0 minutes) and their contents transferred to long-necked round bottomed flasks (100 ml capacity) to which had been added 2 ml of internal standard (1 mg butanol/ml distilled water) and 2-3 drops of anti-foam reagent. Flasks were then placed on a steam distillation apparatus to remove volatiles. Volatiles were extracted from the first 10 ml of distillate with dichloromethane as described in Experiment 2a. Incubation tubes were subsequently removed in triplicate at 15, 60, 120 and 300 minutes after addition of the AITC/ACN solution and immediately processed. The remaining 3 control tubes were removed and their contents steam distilled at 300 minutes. An identical procedure was conducted with rumen fluid from the grass-fed animals; addition of the ACN/AITC mixture and sampling of rumen fluid from cabbage and grass-fed animals was staggered by 5 minutes to allow time for the steam distillation step.

Analysis

Samples were analysed for ACN and AITC by GLC as described in Experiment 4a. Response factors relative to the butanol internal standard were determined and used in calculation of ACN and AITC concentrations (response factors: ACN = 1.181; AITC = 1.565).

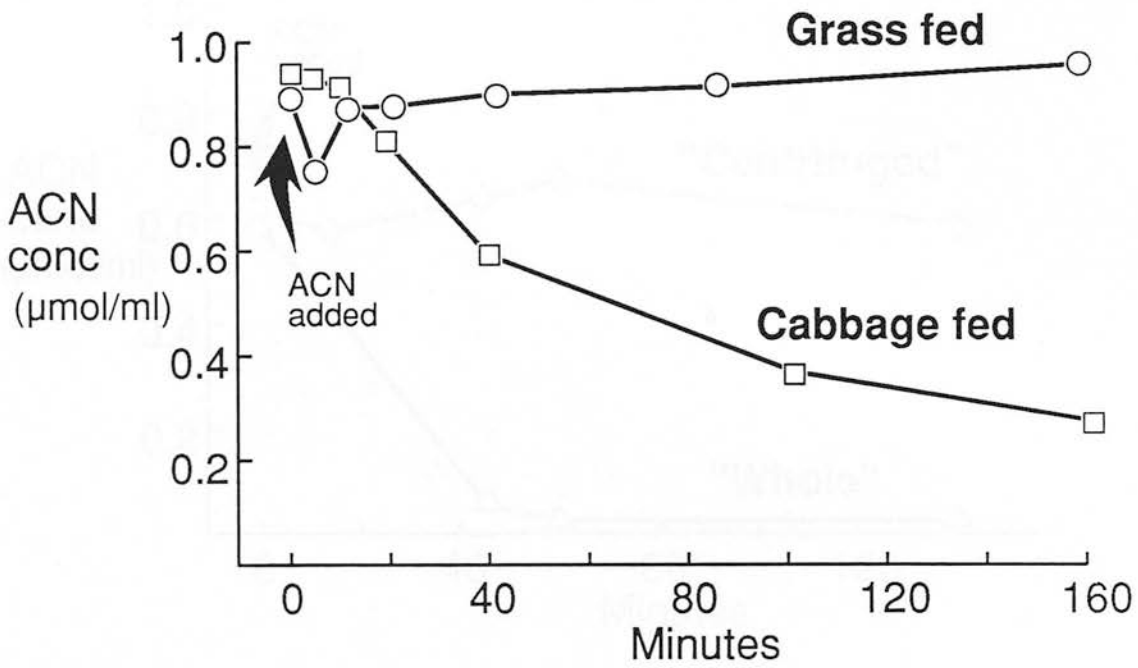


Figure 6.1 Allyl cyanide (ACN) degradation in rumen fluid from grass and cabbage-fed sheep.

Results

Experiment 4a -*In vitro* stability of allyl cyanide in rumen fluid from cabbage and grass-fed sheep

The results of this preliminary investigation of the stability of ACN in rumen fluid are shown in Figure 6.1 and Figure 6.2. ACN was stable in whole rumen fluid from the sheep fed dried grass over the 160-minute incubation period. In contrast, the concentration of the compound in rumen fluid from the cabbage-fed animal declined steadily such that its concentration after 160 minutes was approximately 0.30 of the original concentration.

Removal of the micro-organisms from rumen fluid by high-speed centrifugation removed the capacity of rumen fluid from the cabbage-fed sheep to degrade ACN; thus in "centrifuged" rumen fluid from the cabbage-fed animal, ACN was stable over the 140-minute measurement period. In "whole" rumen fluid tested at the same time,

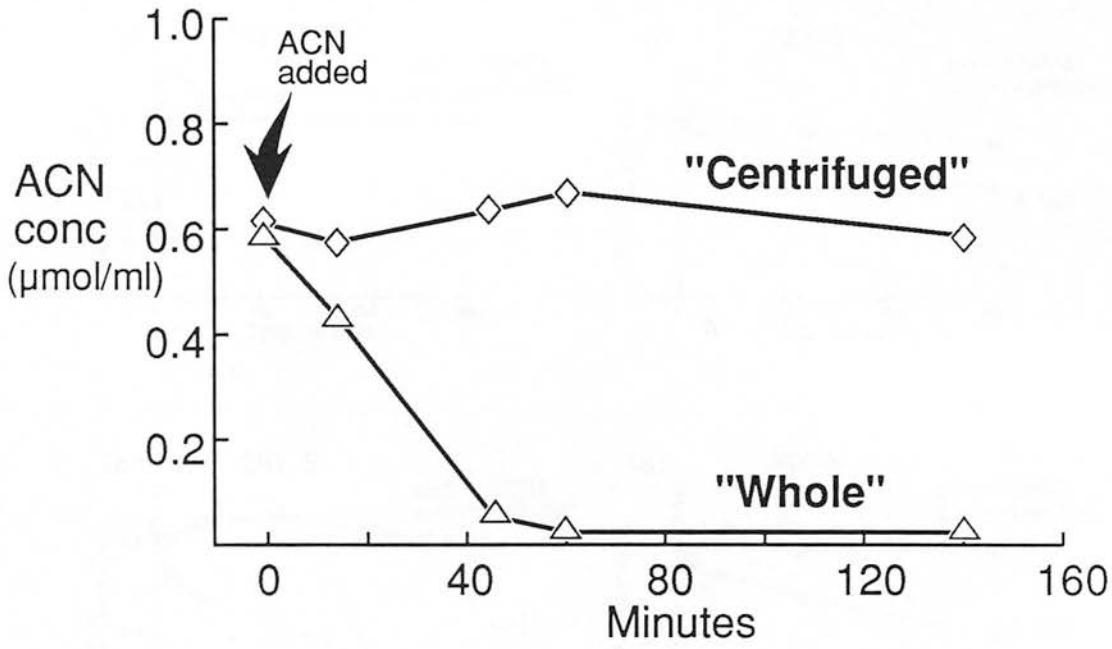


Figure 6.2 Allyl cyanide (ACN) stability in "centrifuged" and "whole" rumen fluid from cabbage-fed animal.

however, there was a rapid decline in ACN concentrations which approached complete disappearance 45 minutes after addition of ACN.

Experiment 4b- Assessment of the time taken for rumen micro-organisms to develop the ability to degrade allyl cyanide.

By abruptly changing the diet from a glucosinolate-free diet (dried grass pellets) in the pre-treatment period to one containing glucosinolates (fresh cabbage) in Treatment CA and comparing the stability of sinigrin breakdown products in rumen fluid at intervals over the following 30 days with that of Treatment DG, the time-course of adaptation by rumen-organisms to the presence of sinigrin in the diet was quantified; results are shown in Figure 6.3 and Figure 6.4. Individual data values are given in Appendices 6a and 6b.

In the pre-treatment period, ACN was stable in both sources of rumen fluid over 300 minutes (Figure 6.3 a). After one day of cabbage feeding there was some evidence for a decline in ACN concentration in rumen fluid from sheep on the CA treatment

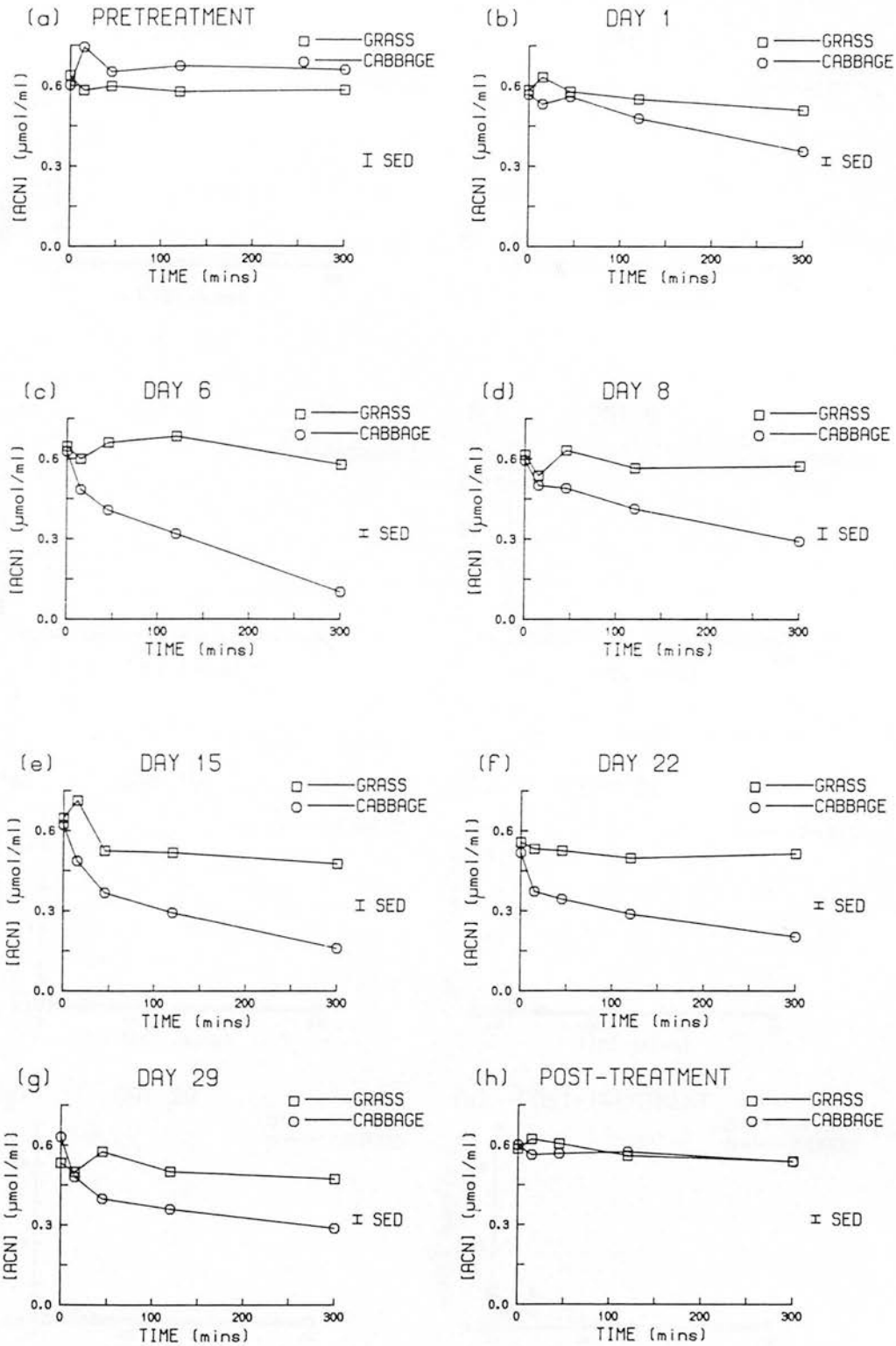


Figure 6.3 Stability of ACN in rumen fluid from sheep fed grass or cabbage and sampled at intervals over 4 weeks.

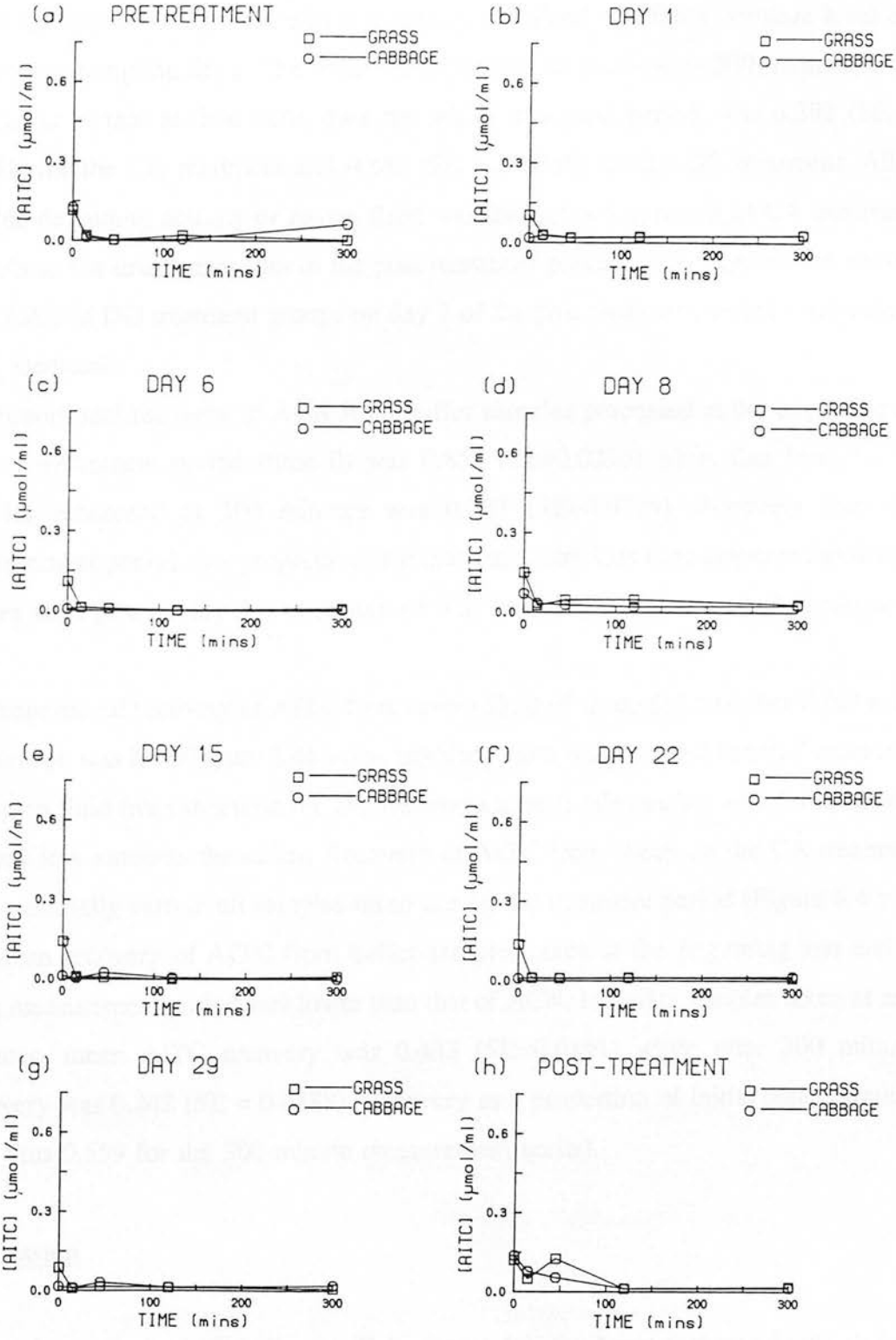


Figure 6.4 Stability of AITC in rumen fluid from sheep fed grass or cabbage and sampled at intervals over 4 weeks.

and this effect was pronounced by day 6 of the treatment period (Figure 6.3 c). On day 8 of the treatment period the ACN-degrading activity of rumen fluid from sheep on the CA treatment had declined and activity remained at a fairly constant level on subsequent sampling days. The mean concentration of ACN after 300 minutes, as a proportion of that at time zero, over the whole treatment period, was 0.392 (SE = 0.0691) for the CA treatment and 0.842 (SE = 0.0356) for the DG treatment. Allyl cyanide-degrading activity of rumen fluid was lost following return of CA treatment animals to the dried grass diet in the post-treatment period; ACN degradation curves from CA and DG treatment groups on day 7 of the post-treatment period were essentially identical.

Proportional recovery of ACN from buffer samples processed at the beginning of each measurement period (time 0) was 0.853 (SE=0.0296) while that from buffer samples processed at 300 minutes was 0.763 (SE=0.0218). Recovery over the measurement period as a proportion of initial concentrations thus amounted to 0.894. Losses were presumably due to factors such as evaporation and chemical decomposition.

Proportional recovery of AITC from rumen fluid of sheep fed on either dried grass or cabbage was low (Figure 6.4). Allyl isothiocyanate was detected in small quantities in rumen fluid from sheep on the DG treatment at zero minutes but was detected only in very low amounts thereafter. Recovery of AITC from sheep on the CA treatment was essentially zero in all samples taken during the treatment period (Figure 6.4 b-f).

Mean recovery of AITC from buffer samples taken at the beginning and end of each measurement period was lower than that of ACN. In buffer samples taken at zero minutes, mean AITC recovery was 0.433 (SE=0.0191) while after 300 minutes recovery was 0.242 (SE = 0.0288). Recovery as a proportion of initial concentrations was thus 0.559 for the 300-minute measurement period.

Discussion

In the preliminary experiment (Experiment 4a), the basal diet was found to have a marked influence on ACN stability. Thus, in rumen fluid from a cabbage-fed animal,

ACN was found to degrade rapidly over a period of 2-3 hours, while in rumen fluid from a grass-fed animal the compound was found to be stable. In order to determine whether this degradation of ACN was due to simple chemical breakdown or whether it occurred as a result of microbial activity, rumen fluid which had been collected from a cabbage-fed animals, was centrifuged to remove micro-organisms. Allyl cyanide was found to be stable in the resulting supernatant, implicating rumen micro-organisms as the cause of ACN degradation. Presumably, the microbial species responsible for ACN degradation are widespread in ruminants but exist in low numbers in the absence of a suitable substrate. Cabbage feeding would have provided a source of substrate as a result of glucosinolate hydrolysis to nitriles precipitating a rapid increase in numbers of nitrile-degrading micro-organisms. Microbial hydrolysis of nitriles to yield the corresponding carboxylic acid has been demonstrated in a number of non-ruminal bacteria (Ingvorsen, Godtfredsen and Tsuchiya, 1988) and similar pathways may occur among rumen species.

Having demonstrated the capacity of rumen micro-organisms to adapt to the presence of glucosinolates in the diet, a further experiment was conducted to quantify the time taken to develop the ability to hydrolyse nitrile breakdown products. To quantify the time taken for the rumen microbial population to adapt to brassica feeding, an experiment was conducted in which animals fed on dried grass were abruptly switched to a brassica diet; rumen samples collected over subsequent days were tested for their capacity to degrade glucosinolate breakdown products. In order to obtain a single measure of "ACN degrading potential" linear regression equations were determined for each of the decay curves shown in Figure 6.3 (x = time (mins); y = log[ACN conc.]). The slopes from these regression lines (as a measure of ACN degrading potential) plotted against the length of time on the experimental diet are shown in Fig. 6.5. Grass-fed animals showed no capacity to degrade ACN over the experimental period; cabbage-feeding on the other hand resulted in rapid breakdown of ACN in rumen fluid within 6 days. There was little evidence for a gradual adaptation period and subsequent rumen samples showed considerable variability in "ACN- degrading potential". Indeed, if the day 8 value is excluded as an out-lier, there is a suggestion of an early maximum (day 6) in ACN-degrading potential, followed

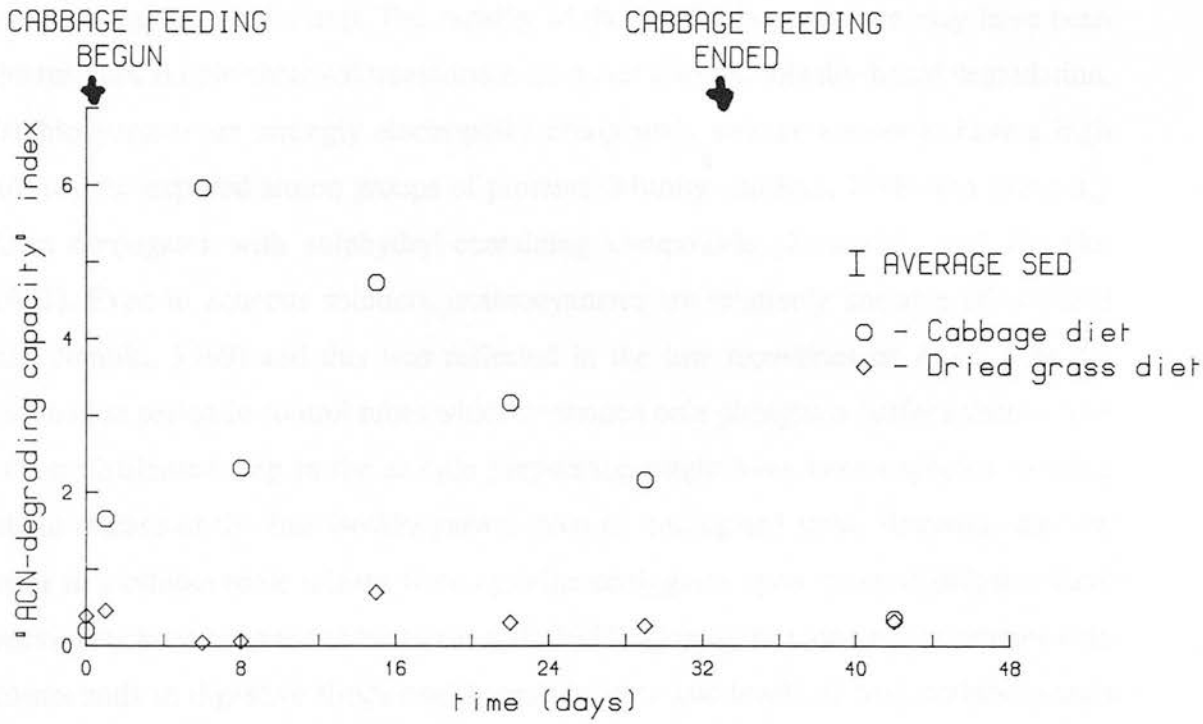


Figure 6.5 Effect of length of cabbage feeding period on "ACN-degrading capacity" index of rumen fluid (calculated as (slope of regressions of log [ACN] against incubation time) x 1000)

by a steady decline over the subsequent three weeks. Alternatively, the observed trend in ACN-degrading potential may have occurred by chance due to sampling variation. For example, differences in the microbial density of rumen fluid samples due to the position of the stomach tube in the rumen at sampling, could have had significant effects on the ACN-degrading potential of the resulting sample. If rumen samples from individual experimental animals had been tested separately rather than being pooled before testing, it would have been possible to determine how much of the observed variability in ACN-degrading potential was due to sampling differences and how much

was genuinely attributable to differences over time. Such an approach would have been logistically difficult but would have provided more reliable information on the time-course involved in adaptation to the presence of ACN in the rumen.

Allyl isothiocyanate showed very rapid disappearance in rumen fluid from either grass- or cabbage-fed sheep. The rapidity of the AITC disappearance may have been the result of simple chemical transformation rather than microbially-based degradation. Isothiocyanates are strongly electrophilic compounds and are known to have a high affinity for exposed amino groups of proteins (Murthy and Rao, 1986) and to readily form conjugates with sulphydryl-containing compounds (Kawakishi and Namiki, 1982). Even in aqueous solution, isothiocyanates are relatively unstable (Kawakishi and Namiki, 1969) and this was reflected in the low recoveries of AITC over the incubation period in control tubes which contained only phosphate buffer solution. The steam distillation step in the sample preparation might have been expected to bring about release of the free isothiocyanate from its conjugated state. However, the low rates of isothiocyanate release from cysteine conjugates upon steam distillation have previously been reported (Mennicke et al., 1983). Conjugation of isothiocyanates with compounds in digestive fluids could result in very low levels of free isothiocyanates in the digestive tract. The extent to which this might reduce their systemic effects is unclear, since it is possible that isothiocyanates could be absorbed in conjugated form with the free compounds then being released systemically. The fact that conjugation of isothiocyanates with sulphydryl compounds is reversible was demonstrated by Bruggemann et al., (1986), who showed glutathione conjugates to release free benzyl isothiocyanate in chemically favourable conditions. Further work on the digestive fate of isothiocyanates is clearly needed and this would most usefully involve radio-labelled compounds.

In conclusion, this work has demonstrated the capacity of rumen micro-organisms to degrade ACN and so potentially reduce the detrimental effects of ingested glucosinolates. The time taken for adaptation to the presence of glucosinolates could be partially responsible for the "check" in growth observed among ruminants fed on forage brassicas. The practical significance of this degradation process will depend on the relative rates of nitrile release, absorption and degradation *in vivo* and further work

is required. Allyl isothiocyanate was not detectable in free form following addition to rumen fluid and conjugation with macro-molecules in the digestive fluid may offer an explanation for this. The significance of such reactions in relation to subsequent physiological effects is not known.

Chapter 7- EFFECTS OF INTERACTIONS BETWEEN SECONDARY METABOLITES DERIVED FROM FORAGE BRASSICAS ON THE PHYSIOLOGY OF SHEEP.

Experiment 5 - Effects of interactions between allyl cyanide, allyl isothiocyanate and dimethyl disulphide on the voluntary food intake and metabolism of lambs.

Introduction

In investigations of the detrimental effects of plant secondary compounds to animals, recent studies have recognised the importance of considering all components of the diet when assessing the potential effects of individual compounds. Thus, the physiological effects of secondary plant compounds may be influenced by levels of macro-nutrients (Kappas et al., 1976) and the presence of other groups of secondary compounds (Freeland, Calcott and Anderson, 1985; Goldstein and Spencer, 1985; Scofield, Rossiter, Witham, Kite, Nash and Fellows, 1990) in the diet. This potential for interactions between separate groups of secondary plant compounds is particularly pertinent in *Brassica* species which commonly contain two separate groups of secondary compounds. The potential interaction between S-methyl cysteine sulphoxide (SMCO) and glucosinolates in producing detrimental effects in the animal has been hypothesised before (Smith, 1980) but never tested. Previous experiments have shown that artificial administration of either glucosinolates or SMCO results in more severe effects when the compounds are given to animals already consuming a brassica diet. Thus, when SMCO was added to basal diets of either fresh kale or dried lucerne at various concentrations, VFI was markedly depressed with the lowest SMCO intake in kale-fed lambs (0.3 g/kg W /d) while even at the highest SMCO intake in lucerne-fed lambs (0.7 g/kg W /d) effects were small and transient (Barry et al., 1982). In the current experiments, infusion of ACN into forage rape-fed sheep resulted in significant VFI depression, while infusion at double the rate into dried-grass fed animals had no effect (Experiments 2a and 2b). The more severe effects of brassica secondary compounds when given to animals fed on brassica herbage may have been associated

with the different levels of nutrition associated with the basal diets. Alternatively, interactions between brassica secondary metabolites may have been important in exacerbating their individual effects. Interactions between glucosinolates and SMCO could occur at various levels. For example, the anaemia caused by DMDS could reduce oxygen transport to the tissues and so exacerbate the effects of inhibition of oxidative phosphorylation at the cellular level caused by cyanide release from glucosinolate-derived nitriles. On the other hand, at the metabolic fate level, induction of cellular detoxification processes, such as the mixed function oxidase (MFO) system and glutathione transferase activity by isothiocyanates (Sparnins et al., 1982; Stoewsand et al.,1986), could increase the efficiency of excretion of unrelated compounds, such as DMDS and nitriles, and so reduce their systemic effects.

In order to investigate this considerable potential for interactions between brassica secondary compounds, a further experiment was conducted in which animals were given mixtures of brassica secondary compounds. A range of blood and tissue measurements were taken aimed at assessing the effects of combinations of compounds on overall metabolism. In addition, excretion rates of selected catabolites of the administered compounds were determined to quantify alterations in the metabolic fate of compounds resulting from their dual administration.

A treatment period of 5 weeks was chosen to be of sufficient duration to allow development of anaemia as a result of DMDS administration (Smith, 1974), the rationale being that physiological interactions between glucosinolate and SMCO-derived compounds might not occur until anaemic symptoms had developed.

Materials and Methods

Animals and feed

Forty-four Scottish Blackface wether lambs (15 months old; mean liveweight 37.2 (range 32-44) kg from the MLURI's Sourhope Research Station were housed at the beginning of June 1989. Animals were immediately introduced to a sole diet of dried grass pellets (N, 23.9 g/kg DM; NDF, 549 g/kg DM; ADF, 310 g/kg DM; Ash, 81 g/kg DM).

Treatments and experimental design

Animals were offered dried grass pellets *ad libitum* and daily dry matter intakes were recorded for a 21-day pre-treatment period. At the end of this period, animals were ranked on the basis of VFI and the first forty in the rank order selected for the experiment. The rank order was divided into five blocks of eight animals each and treatments were randomly allocated to animals within blocks.

The experiment had a 2 x 2 x 2 factorial design with levels consisting of presence or absence of each of three brassica secondary compounds. Treatments factors consisted of daily oral administration to each sheep for 35 days of ACN (0 or 10 mmol/d), AITC (0 or 10 mmol/d) and DMDS (0 or 25 mmol/d). All combinations of factors and levels yielded eight possible treatments and details of the treatments assigned to each animal are given in Appendix 7b.

The higher levels of the compounds given, were chosen on the basis of typical concentrations of brassica secondary metabolites in cabbage, together with likely forage intakes. For example, sinigrin concentrations in cabbage are in the region of 10 mmol/kg DM (Bradshaw et al., 1984) and 40 kg wethers might be expected to consume cabbage at the rate of 1 kg DM/d. A dose rate of 10 mmol/d of ACN is thus roughly equivalent to the potential *in vivo* release assuming complete hydrolysis of sinigrin to ACN. Similar reasoning was applied in selecting dose rates of AITC and DMDS. The dose rate of the higher level of ACN was thus similar to the higher level of ACN given in Experiment 3 and the rationale behind the choice of dose levels is further discussed in the Introduction to Chapter 5.

Procedures

The means of administering compounds in this experiment was different to that used in Experiments 2a, 2b and 3. In these experiments, variation in VFI among surgically prepared animals encumbered with infusion apparatus was high in relation to normal indoor experiments. In the present experiment, intact animals were used and thus continuous intra-ruminal infusion was not possible. Instead, animals were given discrete doses of mixtures of the compounds under investigation twice daily. Animals were dosed with gelatin capsules containing the brassica secondary compounds twice

daily at 0800h and 1600h throughout the treatment period. Care was taken to avoid breakage of the capsules during administration with a dosing gun.

Capsules were prepared in batches every two to three days as follows. Gelatin capsules (3 ml vol.; Davcaps, Monmouth, UK) were arranged in rubber test tube racks (BDH, Dorset, UK) in a fume cupboard. Pre-punched cardboard templates were then placed over the racks before each compound was pipetted to ensure correct dispensing of compounds. Allyl cyanide (Aldrich, Dorset, UK), allyl isothiocyanate (Aldrich, Dorset, UK) and dimethyl disulphide (Sigma, Dorset, UK) were dispensed using automatic pipettes. Templates were then removed and gelatin caps were replaced on each capsule. Capsules were then sealed with a strip of cellulose tape (3 cm x 1 cm) ready for dosing.

The stability of mixtures of ACN, AITC and DMDS was verified by gas chromatography prior to the experiment. An equimolar mixture of the three compounds was prepared from which aliquots were removed at intervals over 144 hours and analysed on a Philips PU4400 gas chromatograph; at each sampling 15 mg of the mixture was added to 5 ml dichloromethane containing butanol as an internal standard (1 mg/ml) and 0.5 ul of this solution was injected onto the GLC. The column was a BP20 fused silica capillary column (SGE (UK) Ltd, Milton Keynes, UK; length 25 m, i.d. 0.33 mm, film thickness 1.0 um); injection was in "split mode" with an initial column temperature of 50^{o}C which was maintained for 5 minutes before being increased at 5^{o}C/min to a final temperature of 120^{o}C. Peak areas were measured on a Philips PU4810 computing integrator and expressed relative to the internal standard. The results of the GLC analyses which are summarised in Appendix 7c indicate no reaction between compounds or degradation of compounds over six days.

Voluntary food intake was measured as the difference between dry matter offered and refused with refusals being collected before each morning feed. Blood samples were collected using evacuated heparinised tubes (Vacutainer, Becton Dickinson, Oxford, UK) from the jugular vein. Urine samples were collected by placing a large clean polythene sheet under each pen for two hours and collecting the run-off. Polythene bags were stapled into funnels at the lower end to allow urine collection

and the resulting samples, which were relatively free of faecal contamination, were immediately frozen pending analysis.

Muscle biopsies (0.5 g) were taken from the left hind limb of each animal under general anaesthetic (Thiopentone injection followed by halothane/oxygen inhalation) between 0800h and 1200 h (The 0800h oral administration of compounds was withheld on the day of muscle sampling.) The procedure was conducted under aseptic conditions and the animals were administered with a long-acting antibiotic after the procedure. Following excision, samples were immersed in ice-cold phosphate buffer (0.05 M, pH 7.2) pending analysis for cytochrome oxidase activity.

Measurements and analyses

Samples of feed offered and feed refused were taken daily, pooled by week and analysed for ash, total nitrogen, ADF and NDF. Ash content was determined by weighing residues after ashing in a furnace for 16 hours at 450°C. NDF was measured using the method of Van Soest and Wine (1967) and ADF was determined by the method of Van Soest (1963). Total N was estimated by a semi-micro kjeldahl block digestion method followed by a continuous flow method based on the Berthelot reaction (Berthelot, 1859) (Alpkem Corp. RFA, method no. A303-S072).

Concentrations of plasma tri-iodothyronine (T3), plasma thyroxine (T4), blood haemoglobin, blood reduced glutathione, plasma albumin, plasma creatinine, plasma urea, activities of plasma gamma glutamyl transpeptidase and plasma alkaline phosphatase together with Heinz body counts and blood PCV's were measured on days 8 and 15 of the pre-treatment period in order to establish baseline levels, and on days 2, 9, 16, 23 and 30 of the treatment period. Muscle cytochrome oxidase activity was measured in samples taken on day 25 of the treatment period and urinary thiocyanate ion concentration and urinary N-acetyl-L- (allyl thiocarbamoyl)- L-cysteine (AATCC) concentration were measured in samples collected between 1300 h and 1500 h on day 17 of the treatment period.

Plasma T3 and T4 concentrations were measured using Amerlex RIA kits (Amersham International, Bucks., UK), as described in Experiment 2a. Free thyroxine index (FTI) was estimated from "T3 uptake" values which were determined using a

commercial kit (T3 uptake (MAA) kit, Amersham International, Bucks., UK). The sensitivity of the T3 assay, calculated as the concentration which is two standard deviations above the zero standard, was 0.15 nmol/l, while the within-assay coefficient of variation was 4 % (n = 50). The sensitivity of the T4 assay was 4.0 nmol/l and the within-assay coefficient of variation was 7 % (n = 56). The within-assay coefficient of variation for the T3 uptake test was 6 % (n = 19).

Plasma albumin concentration was measured by the method of Spencer and Price (1977) in which albumin is bound by bromocresol green dye in succinate buffer at pH 4.1. Plasma creatinine concentration was analysed using the Jaffe reaction, as described in Experiment 3 (Butler, 1975). Plasma urea concentration was determined using the Berthelot reaction, as described in Experiment 3 (Wilcox et al., 1966). Plasma alkaline phosphatase activity was measured using a commercial diagnostic kit (Sigma procedure no. DG 1245, Sigma, Poole, Dorset), based on the enzymatic hydrolysis of p-nitrophenyl phosphate to p-nitrophenol by alkaline phophatase. p-Nitrophenol shows an absorbance maximum at 405 nm and the rate of increase in absorbance at 405 nm, following addition of alkaline phosphatase, is used to measure its activity. Plasma GGTP was measured by the method of Fuke et al., (1976), as described in Experiment 3.

Blood PCV and blood haemoglobin were measured using the methods described in Experiment 2a. Blood reduced glutathione was measured by the method of Beutler et al. (1963), as described in Experiment 3. The percentage of red blood cells containing Heinz bodies was determined on stained erythrocytes. For this purpose 4 drops of whole blood and 4 drops of Brilliant Cresyl Blue (1% in 0.9% physiological saline) were incubated in a test tube for 15 minutes at 37°C. Blood smears were prepared using a drop of the above mixture and the resulting smears were examined using an oil immersion microscope. Heinz bodies appeared as darkly staining granules at the periphery of the erythrocytes. The number of cells containing Heinz bodies was expressed as a percentage of the total number of cells counted (> 300).

Muscle cytochrome oxidase activity was measured within six hours of tissue excision by the methods described in Experiment 3 (Cooperstein and Lazarow, 1951); activity was expressed relative to the protein content of the homogenates measured by

the Lowry method (Sigma procedure no. P5656, Sigma, Poole, Dorset). Urinary thiocyanate ion was measured by the method of Bowler (1944). The problem of non-specific absorbance due to urine pigments was avoided using the modification of Johnston and Jones (1966). In brief, the absorbance of a coloured complex formed by the reaction of thiocyanate ion with ferric nitrate was measured at 450 nm. Duplicate tubes were prepared to which 10 ul of 5% mercuric chloride (Sigma, Dorset, UK) was added to destroy the coloured complex. The difference in absorbance between the two tubes was taken as absorbance due to thiocyanate ion. Concentrations were calculated following construction of a calibration curve using potassium thiocyanate standards.

Statistical Analysis

Data were analysed by analysis of variance using "GENSTAT 5" (Lawes Agricultural Trust; Release 1.3, 1988). Block, animal and week effects were taken into account in the analysis. The main effects of DMDS, AITC, ACN and time together with their respective interactions were analysed by analysis of variance and a sample ANOVA table is given in Appendix 7a.

When analysing data relating to urinary metabolites of ACN (thiocyanate ion outputs) and AITC (AATCC outputs), only those animals receiving the precursor produced significant quantities of the urinary metabolites. In each case, because half the animals produced essentially zero concentrations of the urinary metabolites (see Results), the data did not fit a normal distribution and the assumptions relating to the analysis of variance were not satisfied. For these two data-sets, therefore, only animals receiving the administered precursor were included in statistical analyses of the urinary metabolite data.

Results

Main Effects

The main effects of dosing with DMDS, ACN and AITC on VFI, blood glutathione concentrations, anaemic status, thyroid hormone concentrations and a range of clinical diagnostic parameters are shown in Table 7.1. These results refer to mean values over

the whole treatment period. The way in which the above parameters changed with time is discussed later under Effects of time.

Voluntary food intake was significantly depressed by the presence of ACN ($p < 0.01$) and AITC ($p < 0.001$) but not by the presence of DMDS. The presence of AITC caused a greater reduction in VFI over the experiment (VFI = 0.863 of control values) than did the presence of ACN (VFI = 0.924 of control values).

The higher level of all three factors had highly significant effects on blood glutathione concentrations. The presence of ACN had the greatest effect in reducing mean blood reduced glutathione concentrations which were 0.635 of those in the absence of ACN ($p < 0.001$). A similar but less pronounced depression was caused by the presence of AITC with animals in this group having glutathione concentrations which were 0.825 of those in the absence of the compound ($p < 0.001$). Conversely, the presence of DMDS produced a rise in glutathione concentrations (1.22 of control values, $p < 0.001$) when concentrations were averaged for the whole experiment.

The extent of the anaemic response to treatments was relatively slight and neither haemoglobin concentrations nor PCV values were significantly altered by any of the treatments. Heinz body counts were elevated in the presence of DMDS ($p < 0.001$).

The presence of each factor tended to increase plasma concentrations of T3 and T4, although the effects of the presence of AITC and DMDS were non-significant. The increase in T4 concentrations attained significance in the presence of ACN ($p < 0.01$); the presence of ACN also caused a significant increase in free thyroxine index (FTI) values ($p < 0.05$).

Clinical indicators of liver and kidney function showed a number of significant treatment effects. Plasma albumin concentration was slightly depressed in the presence of AITC. Similarly, plasma urea concentrations were depressed when AITC was present. Plasma creatinine concentrations and plasma alkaline phosphatase activity were unaffected by treatment. Plasma GGTP activity was significantly elevated in the presence of ACN; a similar, but less pronounced effect, which approached significance ($p = 0.07$), was observed when AITC was present.

Muscle cytochrome oxidase activity measured on day 25 of the treatment period was not significantly altered by the presence or absence of any of the three

Table 7.1 Effects of oral administration of allyl cyanide (ACN), allyl isothiocyanate (AITC) and dimethyl disulphide (DMDS) on the voluntary food intake, blood glutathione concentration, haematology, thyroid status and plasma albumin, urea and creatinine concentrations of sheep.

VARIABLE		ACN	Level of significance		AITC	Level of significance		DMDS	Level of significance	SED
Voluntary food intake (g DM/d)	0*	2106	P<0.01	0	2175	P<0.001	0	2056	NS	58.3
	1	1946		1	1878		1	1996		
Blood glutathione concentration (g/100ml RBC)	0	91.8	P<0.001	0	82.3	P<0.001	0	67.6	P<0.001	4.05
	1	58.3		1	67.9		1	82.6		
Blood haemoglobin concentration (g/dl)	0	11.79	NS	0	11.66	NS	0	11.78	NS	0.222
	1	11.77		1	11.90		1	11.78		
PCV (%)	0	32.7	NS	0	32.0	NS	0	32.6	NS	0.55
	1	31.9		1	32.6		1	32.1		
Heinz bodies (log(% of red blood cells))	0	0.637	NS	0	0.647	NS	0	0.426	P<0.001	0.0586
	1	0.687		1	0.677		1	0.898		
Plasma tri-iodothyronine concentration (µmol/l)	0	3.15	NS	0	3.45	NS	0	3.37	NS	0.370
	1	3.82		1	3.50		1	3.59		
Plasma thyroxine concentration (µmol/l)	0	72.2	P<0.01	0	77.9	NS	0	75.1	NS	3.87
	1	83.9		1	78.3		1	81.0		
Free thyroxine index (%)	0	20.6	P<0.05	0	22.2	NS	0	21.4	NS	0.98
	1	22.9		1	21.3		1	22.1		
Plasma albumin concentration (g/litre)	0	26.3	NS	0	26.8	P<0.05	0	26.2	NS	0.32
	1	26.5		1	26.0		1	26.5		
Plasma urea concentration (µmol/l)	0	4.97	NS	0	5.22	P<0.05	0	5.12	NS	0.196
	1	5.01		1	4.76		1	4.86		
Plasma creatinine concentration (µmol/l)	0	43.3	NS	0	44.1	NS	0	45.1	NS	1.29
	1	45.8		1	45.0		1	44.0		
Plasma gamma glut-amyl transpeptidase (GGTP) activity (IU/l)	0	74.2	P<0.001	0	77.8	P=0.067	0	79.4	NS	4.29
	1	89.5		1	85.9		1	84.3		
Plasma alkaline phosphatase activity (IU/l)	0	68.8	NS	0	75.8	NS	0	69.5	NS	7.42
	1	71.7		1	64.7		1	71.0		
Muscle cytochrome oxidase activity **	0	0.455	NS	0	0.443	NS	0	0.445	NS	0.4210
	1	0.406		1	0.408		1	0.406		

* 0 = Absence of factor
1 = Presence of factor

** units = log[ferrocytochrome c]/min for 1:100 tissue dilution

compounds administered.

Both AITC and ACN had large effects on the concentrations of their respective metabolites in the urine. Thus, the presence of AITC resulted in mean urinary concentrations of AATCC of 29.5 (SE = 4.94) μg/ml, while in its absence the mean concentration of AATCC was 0.9 (SE = 0.30) μg/ml. Similarly, the mean urinary concentration of thiocyanate ions was 8.73 (SE = 1.250) μg/ml in the presence of ACN but only 0.32 (SE = 0.225) μg/ml in its absence.

Interaction effects

Interaction effects are presented in Table 7.2 and in Appendices 7f - 7i. There was a significant interaction between the effects of ACN and DMDS on VFI ($p < 0.05$), whereby the presence of each compound singly caused a reduction in VFI but the extent of the reduction caused by the presence of the two compounds in combination was not significantly different from the other treatments.

There was a significant interaction between the effects of ACN and AITC on PCV values (Table 7.2). The effect of ACN in the absence of AITC was to marginally depress PCV values, while in the presence of AITC, values were slightly elevated by the presence of ACN (PCV, $p < 0.05$). The presence of DMDS caused a reduction in PCV values in the presence of ACN or AITC but had an opposite effect in their absence, although the effects just failed to attain statistical significance ($p = 0.07$). There were no significant interaction effects in relation to blood reduced glutathione concentrations, Heinz Body counts, T3 and T4 concentrations, FTI values, plasma albumin concentrations, plasma urea concentrations, plasma GGTP activity, plasma alkaline phosphatase activity or muscle cytochrome oxidase activity (Appendices 7.f - 7.i).

Catabolism of ACN to urinary thiocyanate ions was affected by the additional presence of AITC or DMDS. The presence of AITC significantly increased the urinary concentration of thiocyanate ion when compared with concentrations in the absence of AITC (presence, 11.43 μg/ml; absence, 5.55 μg/ml; SED 1.433; $p < 0.005$). Conversely, the urinary thiocyanate ion concentration in the presence of DMDS was lower than in its absence (presence, 6.59 μg/ml; absence, 10.39 μg/ml; SED 1.433; p

Table 7.2 Two-way interactions between allyl cyanide (ACN), allyl isothiocyanate (AITC) and dimethyl disulphide (DMDS); effects on voluntary food intake and blood packed cell volume

VOLUNTARY FOOD INTAKE (g DM/d)

	AITC		DMDS		DMDS	
	0	1*	0	1	0	1
ACN 0	2201	2012	2206	2007	2186	2163
ACN 1	2149	1743	1906	1986	1926	1830
level of significance	NS		p<0.05		NS	
SED	82.4		82.4		82.4	

PACKED CELL VOLUME (%)

	AITC		DMDS		DMDS	
	0	1	0	1	0	1
ACN 0	33.0	32.4	32.4	33.0	31. 8	32.3
ACN 1	31.0	32.8	32.7	31.1	33.3	31.9
Level of significance	<0.05		NS		NS	
SED	0.77		0.77		0.77	

* 0 = Absence of compound
1 = Presence of compound

< 0.05). Catabolism of AITC to urinary AATCC on the other hand was not significantly affected by the additional presence of ACN or DMDS.

Effects of time

Weekly measurements made over the five-week treatment period enabled investigation of the extent to which responses to the treatments developed over time. The time-course of changes in the main effects of the parameters investigated are shown in Figures 7.1 - 7.13. Significant treatment by week interactions indicate a changing response over time to application of the treatment; a summary of the significance of treatment by time interaction terms is presented in Table 7.3.

Voluntary food intake changed significantly with time in response to the presence of AITC ($p < 0.001$) and ACN ($p < 0.01$). In both cases, VFI reduction in the presence of the factors occurred during the first two weeks of the treatment period and remained at the same level subsequently.

Blood glutathione concentrations changed with time in response to the presence of DMDS and ACN. Thus, in the presence of DMDS, blood reduced glutathione concentrations increased steadily with time over the 5-week treatment period. The presence of ACN, however, caused a reduction in blood reduced glutathione concentrations with most of the change occurring during weeks 1 to 3 of the treatment period.

Anaemic variables showed some evidence of a changing response to treatment over time. The presence of AITC caused a small but significant depression in haemoglobin concentrations ($p < 0.01$) and PCV values ($p < 0.05$), particularly during weeks 3-5 of the treatment period, while PCV values of ACN-treated animals decreased relative to controls over the first four weeks of the treatment period ($p < 0.001$). The increase in Heinz body count in the presence of DMDS occurred chiefly during the first two weeks of the experiment ($p < 0.001$) and differences in Heinz body numbers due to the presence of the compound remained fairly constant thereafter (Figure 7.5).

The only significant treatment by time interaction associated with thyroid status measurements was with plasma T3 concentrations in ACN-treated animals. Most of the increase in plasma T3 concentrations caused by the ACN treatment occurred

Table 7.3 - Summary of level of significance of time x treatment interaction terms in the analysis of variance.

Variable	Treatment	Time x treatment interaction Level of significance
Voluntary food intake	DMDS	NS
	AITC	<0.001
	ACN	<0.01
Blood glutathione	DMDS	<0.001
	AITC	NS
	ACN	<0.001
Blood haemoglobin	DMDS	NS
	AITC	<0.05
	ACN	NS
PCV	DMDS	NS
	AITC	<0.05
	ACN	<0.001
Heinz body count	DMDS	<0.001
	AITC	NS
	ACN	NS
Plasma tri-iodothyronine	DMDS	NS
	AITC	NS
	ACN	<0.001
Plasma thyroxine	DMDS	NS
	AITC	NS
	ACN	NS
Free thyroxine index	DMDS	NS
	AITC	NS
	ACN	NS
Plasma albumin	DMDS	NS
	AITC	p<0.001
	ACN	NS
Plasma urea	DMDS	NS
	AITC	NS
	ACN	NS
Plasma creatinine	DMDS	NS
	AITC	NS
	ACN	NS
Plasma gamma glut-amyl transpeptidase	DMDS	NS
	AITC	p<0.001
	ACN	p<0.001
Plasma alkaline phosphatase	DMDS	NS
	AITC	p<0.05
	ACN	p<0.05

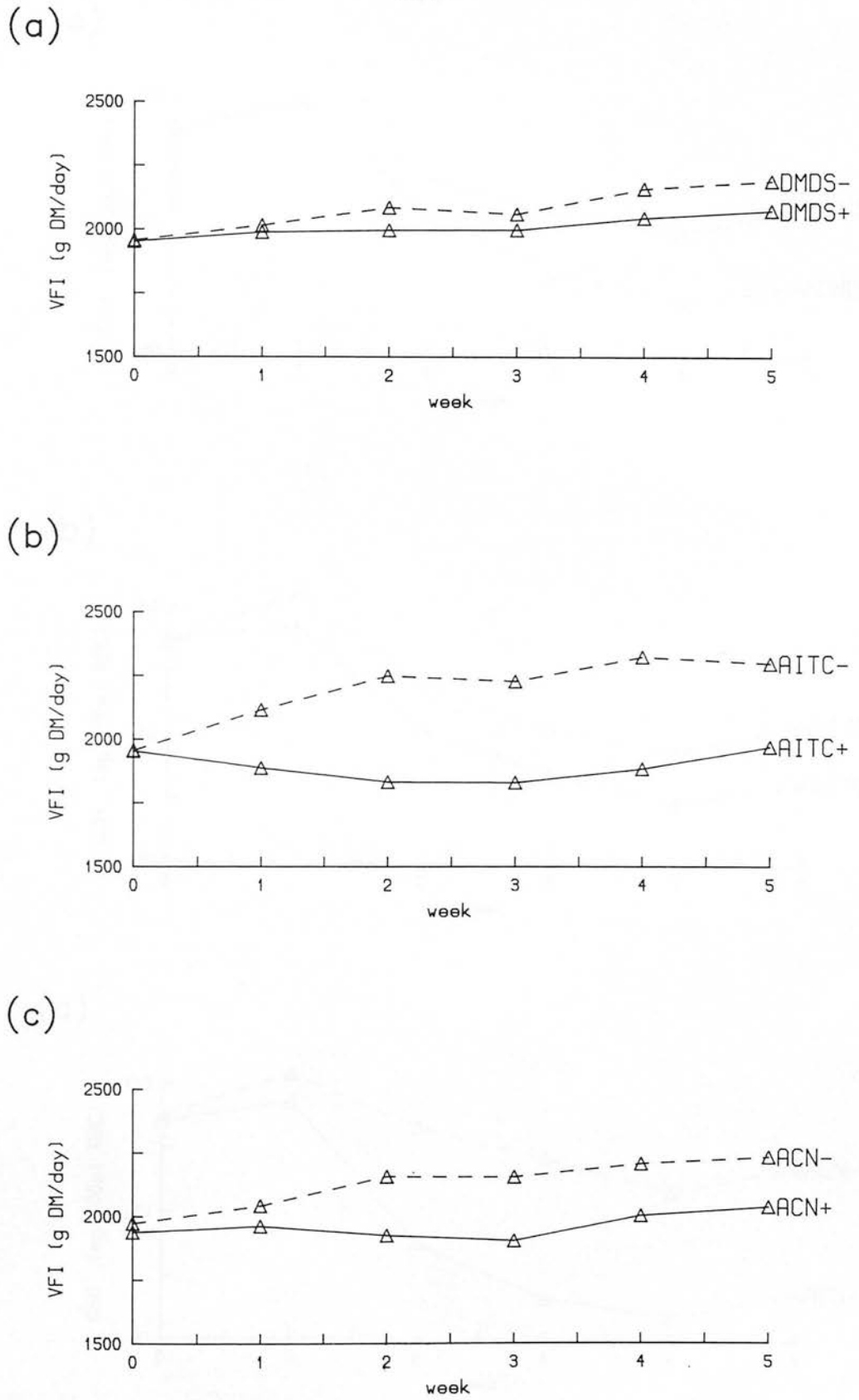


Figure 7.1 Main effects of DMDS (a), AITC (b) and ACN (c) on voluntary food intake (VFI) over the five week treatment period.

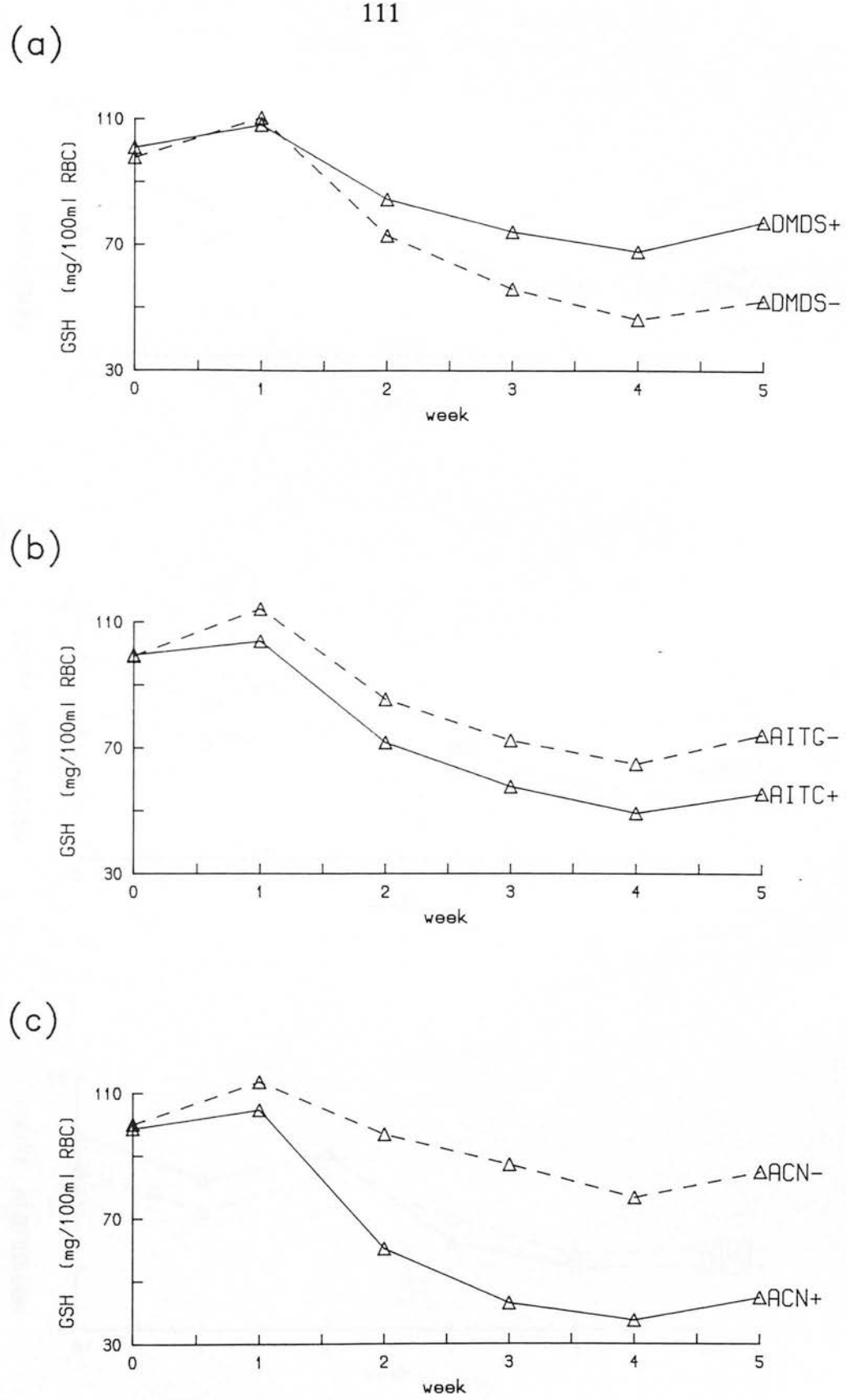
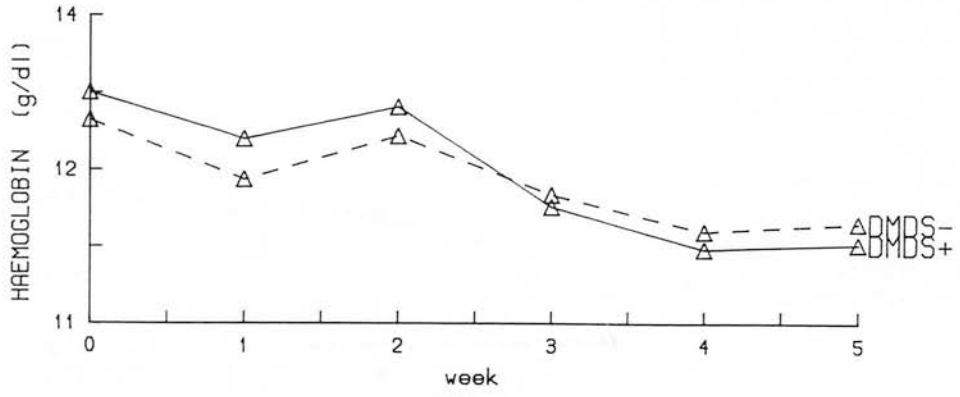
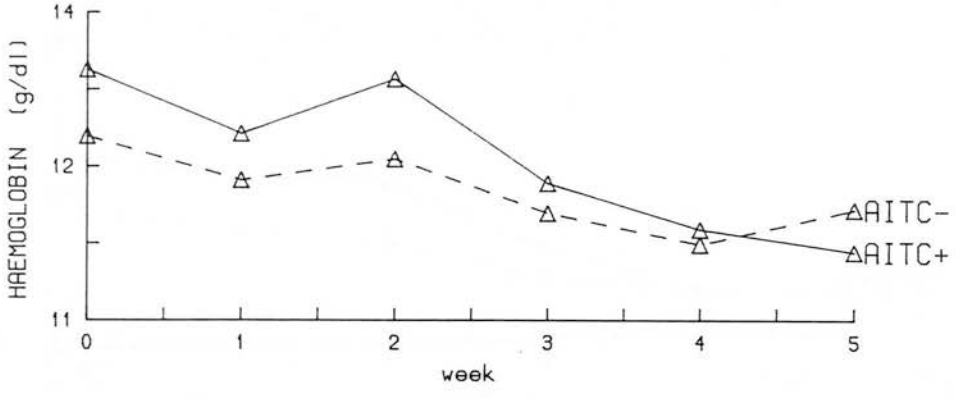


Figure 7.2 Main effects of DMDS (a), AITC (b) and ACN (c) on blood reduced glutathione (GSH) concentrations over the five week treatment period.

(a)



(b)



(c)

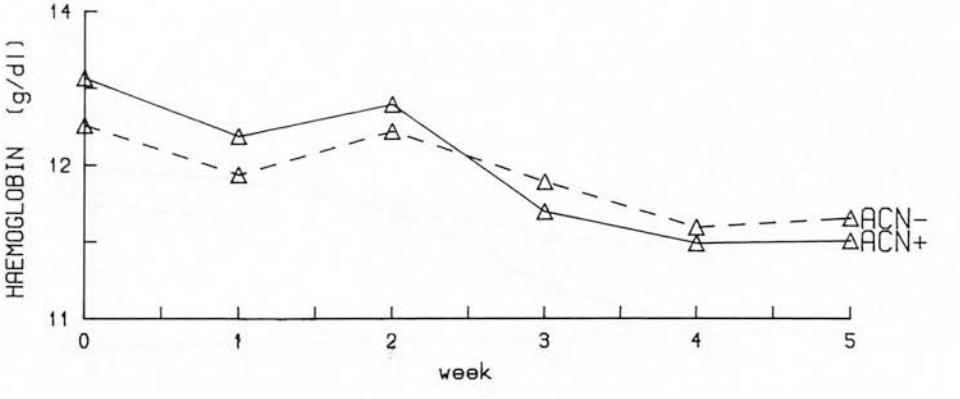


Figure 7.3 Main effects of DMDS (a), AITC (b) and ACN (c) on blood haemoglobin concentration over the five week treatment period.

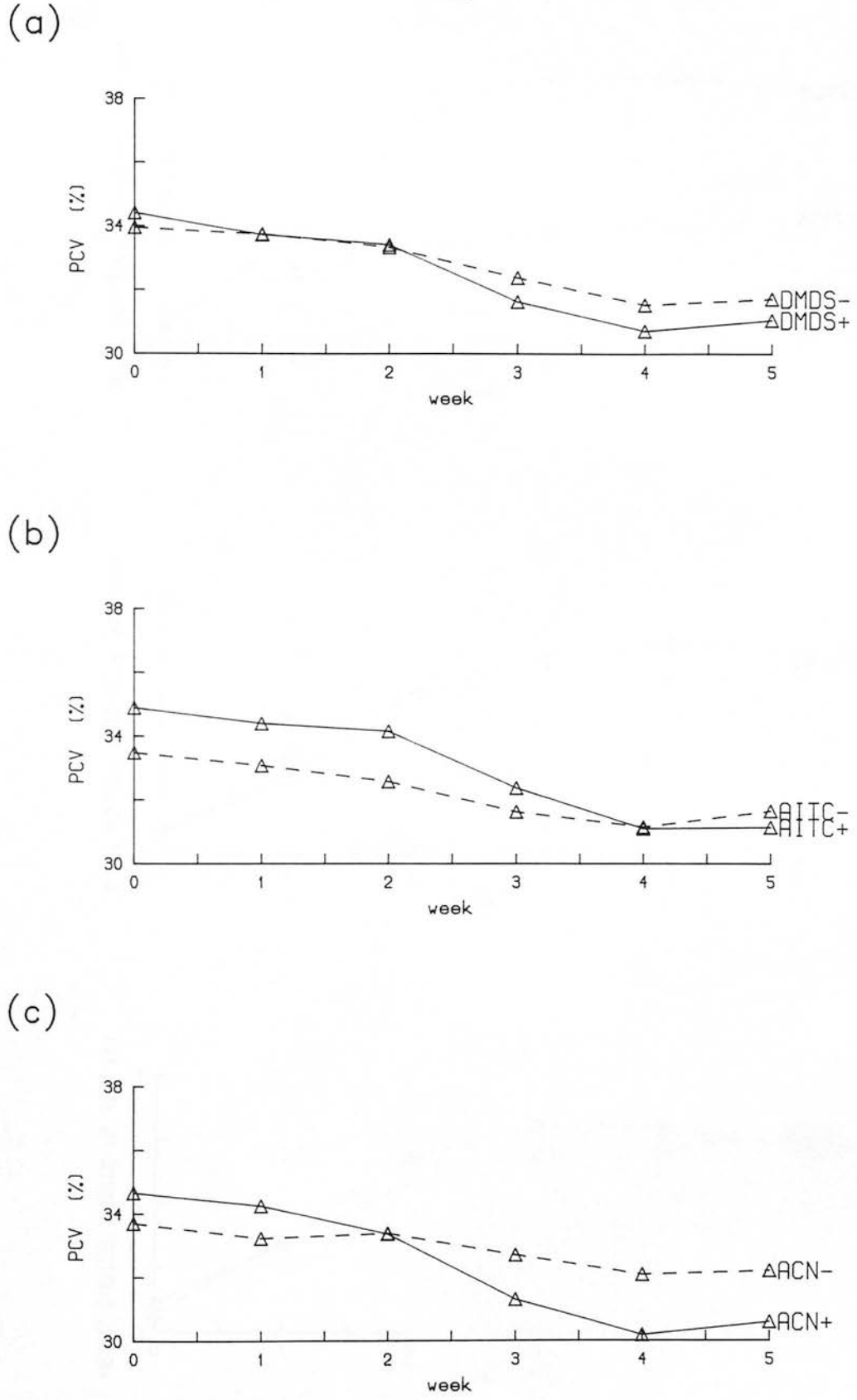


Figure 7.4 Main effects of DMDS (a), AITC (b) and ACN (c) on packed cell volume over the five week treatment period.

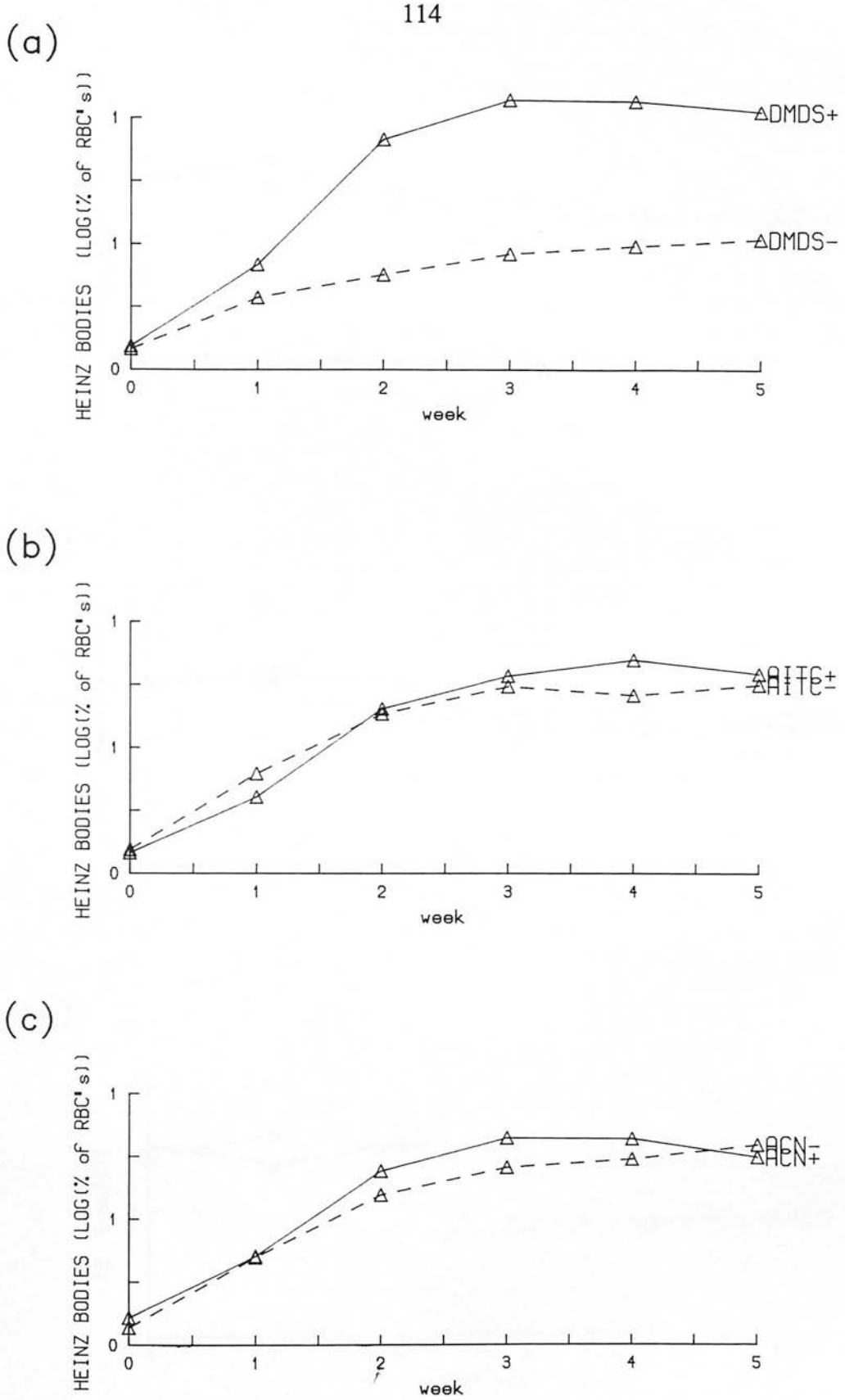


Figure 7.5 Main effects of DMDS (a), AITC (b) and ACN (c) on Heinz body count over the five week treatment period.

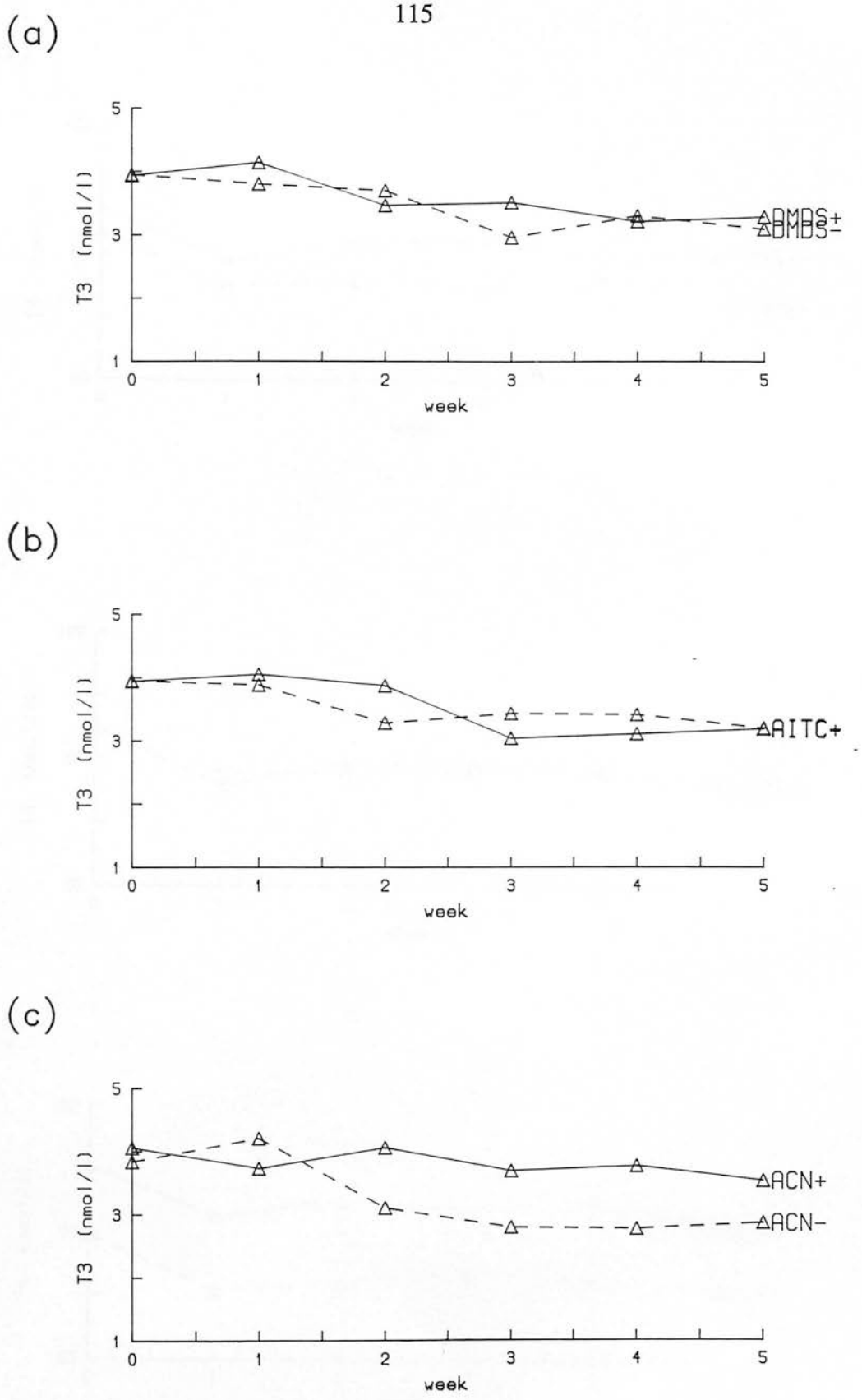


Figure 7.6 Main effects of DMDS (a), AITC (b) and ACN (c) on plasma tri-iodothyronine (T3) concentrations over the five week treatment period.

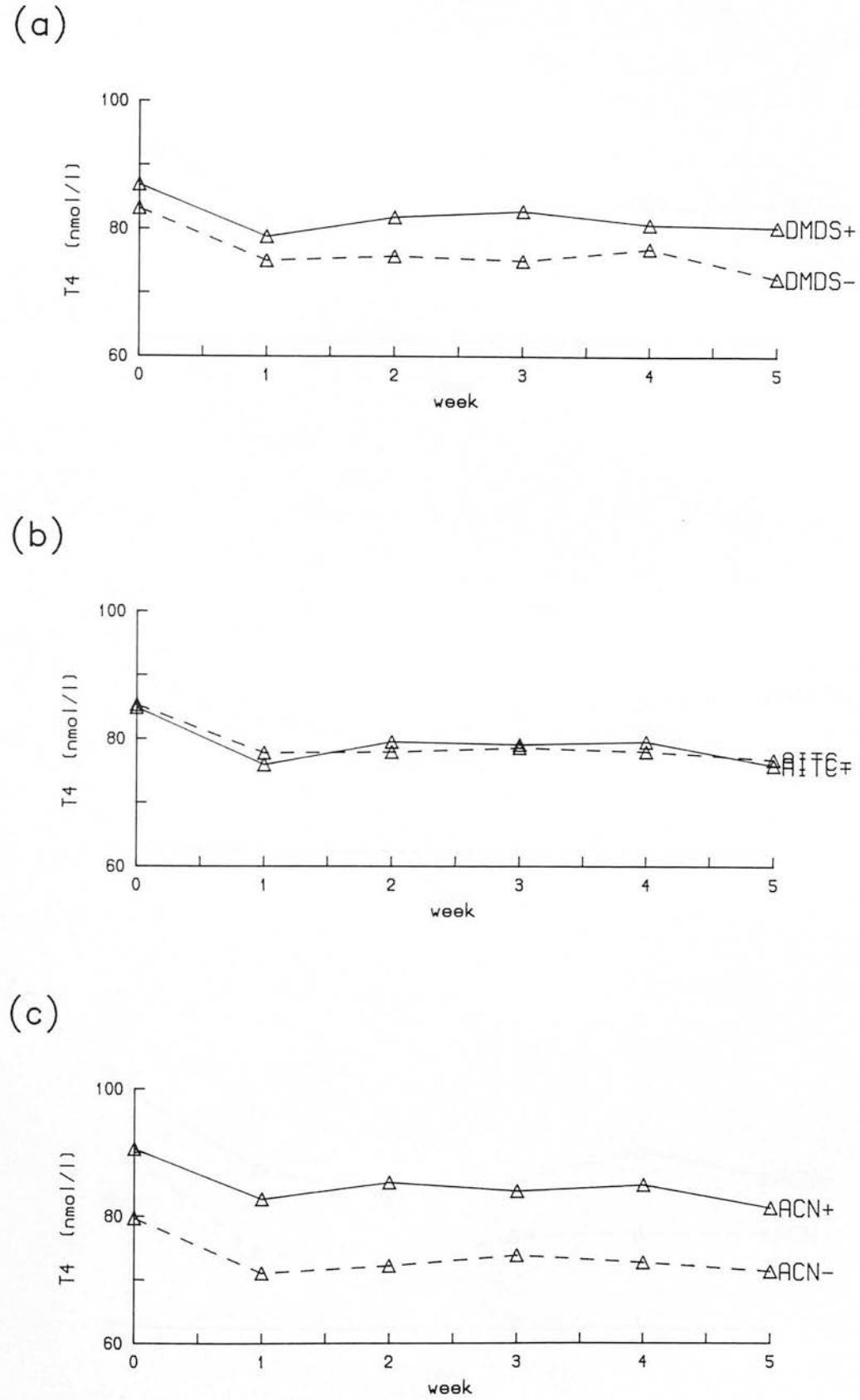
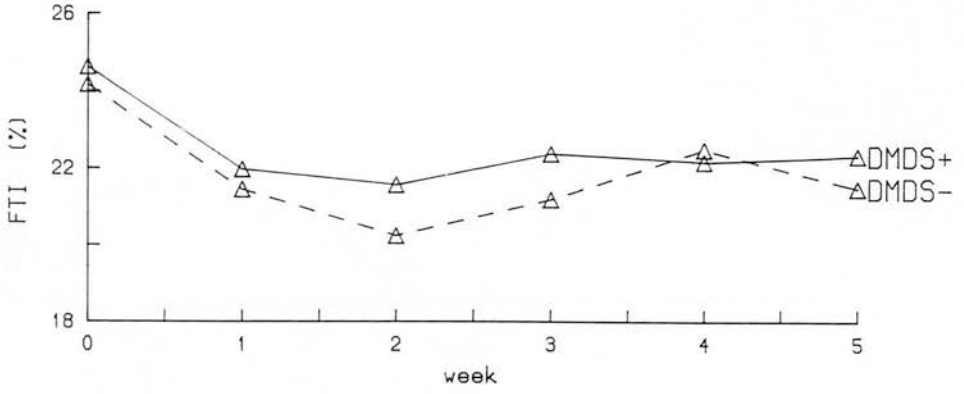
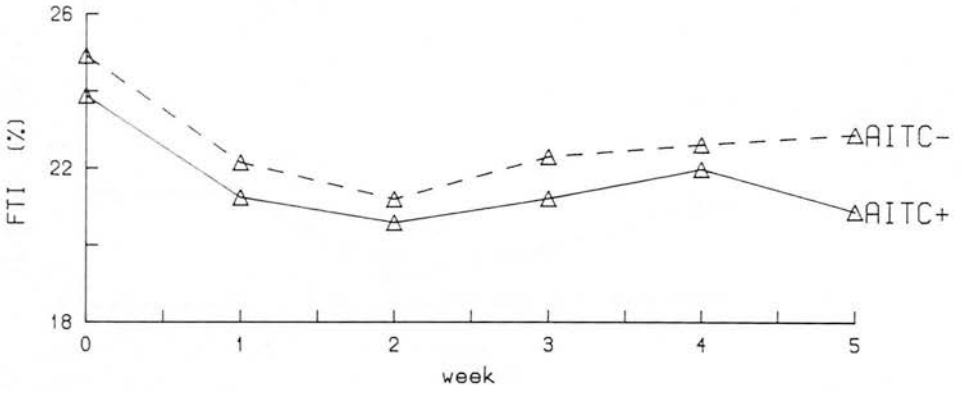


Figure 7.7 Main effects of DMDS (a), AITC (b) and ACN (c) on plasma thyroxine (T4) concentrations over the five week treatment period.

(a)



(b)



(c)

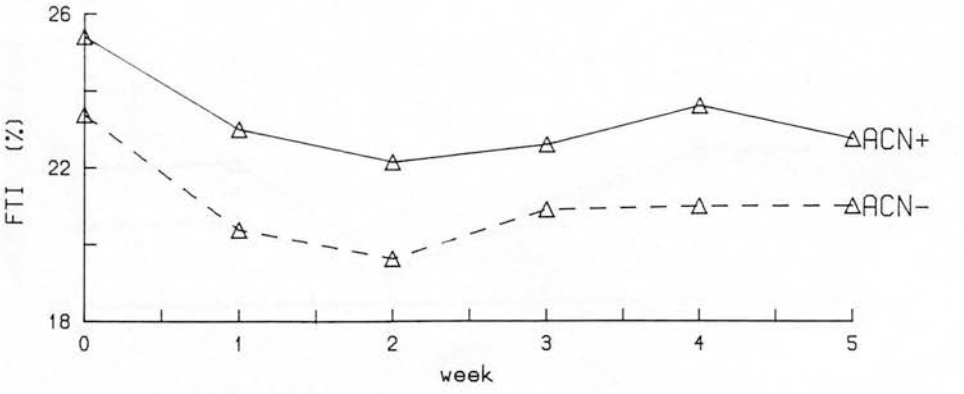
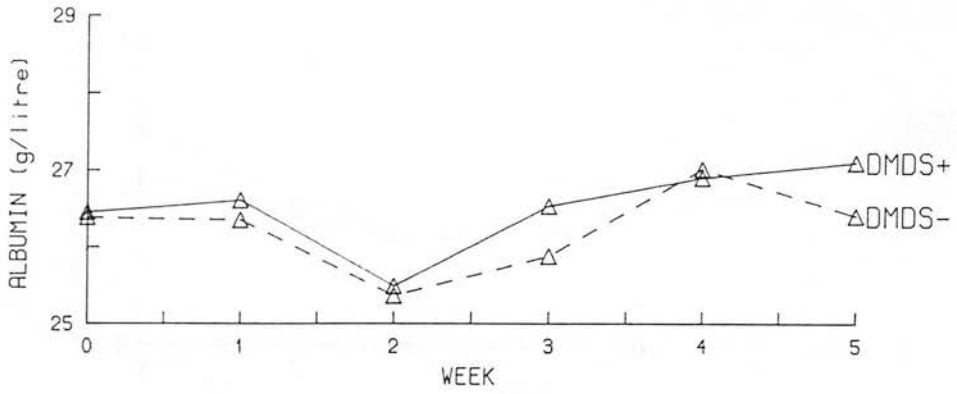
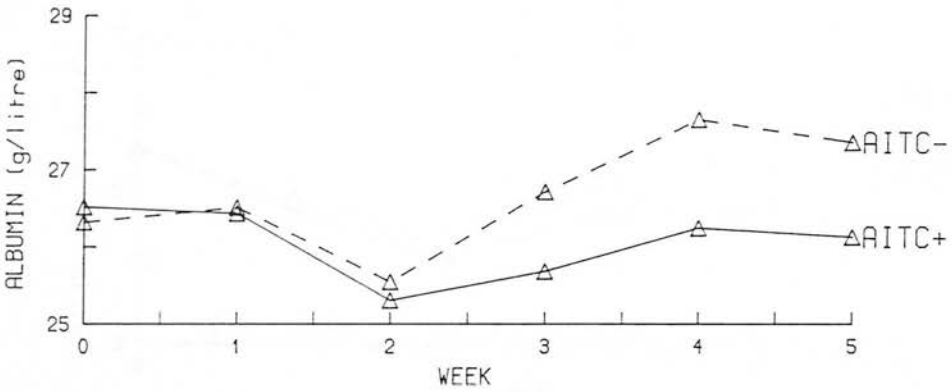


Figure 7.8 Main effects of DMDS (a), AITC (b) and ACN (c) on free thyroxine index (FTI) over the five week treatment period.

(a)



(b)



(c)

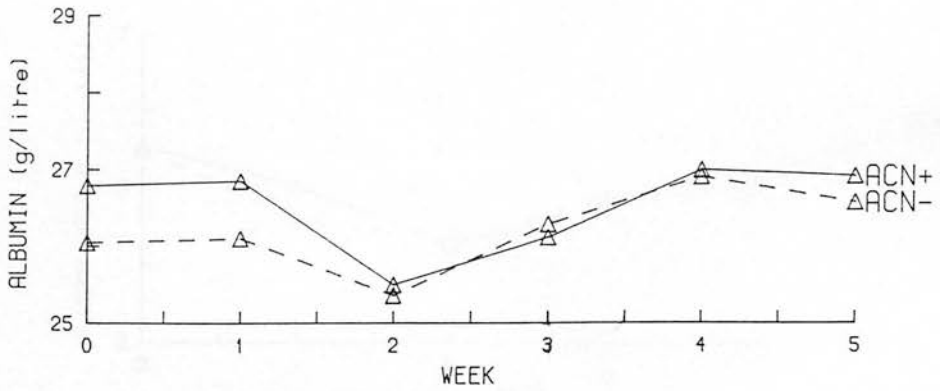
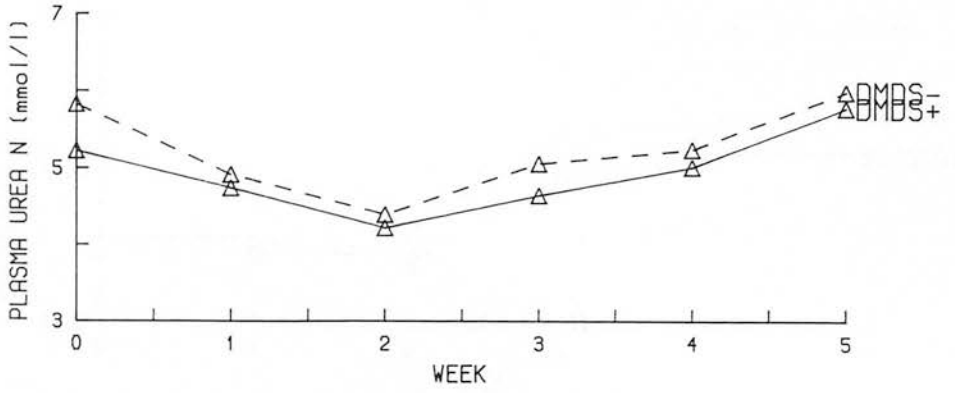
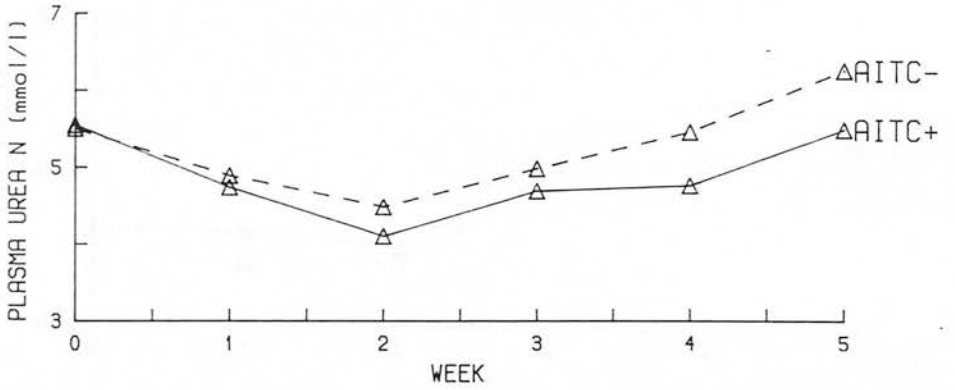


Figure 7.9 Main effects of DMDS (a), AITC (b) and ACN (c) on plasma albumin concentrations over the five week treatment period.

(a)



(b)



(c)

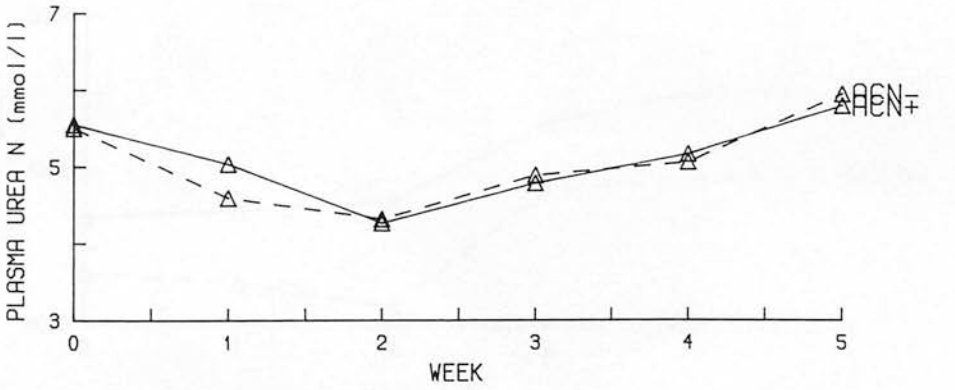
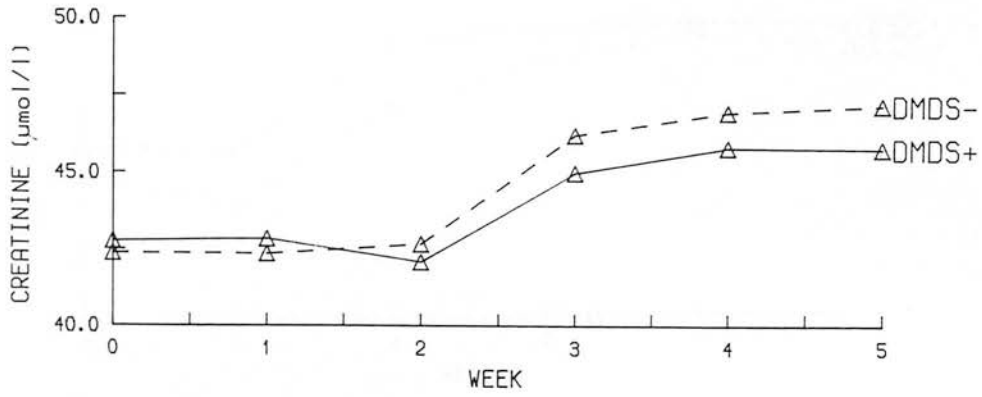
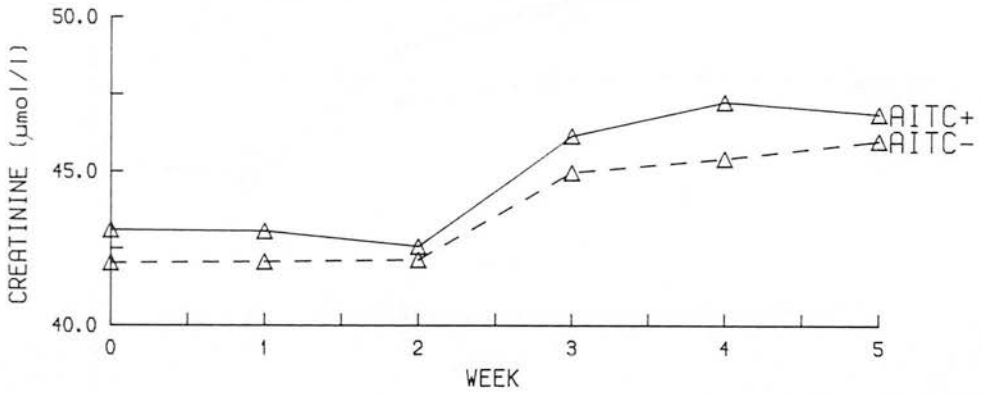


Figure 7.10 Main effects of DMDS (a), AITC (b) and ACN (c) on plasma urea concentrations over the five week treatment period.

(a)



(b)



(c)

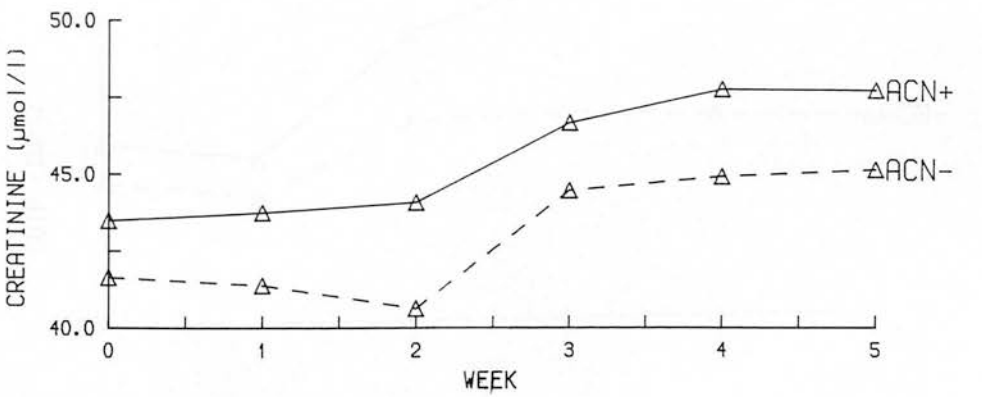


Figure 7.11 Main effects of DMDS (a), AITC (b) and ACN (c) on plasma creatinine concentrations over the five week treatment period.

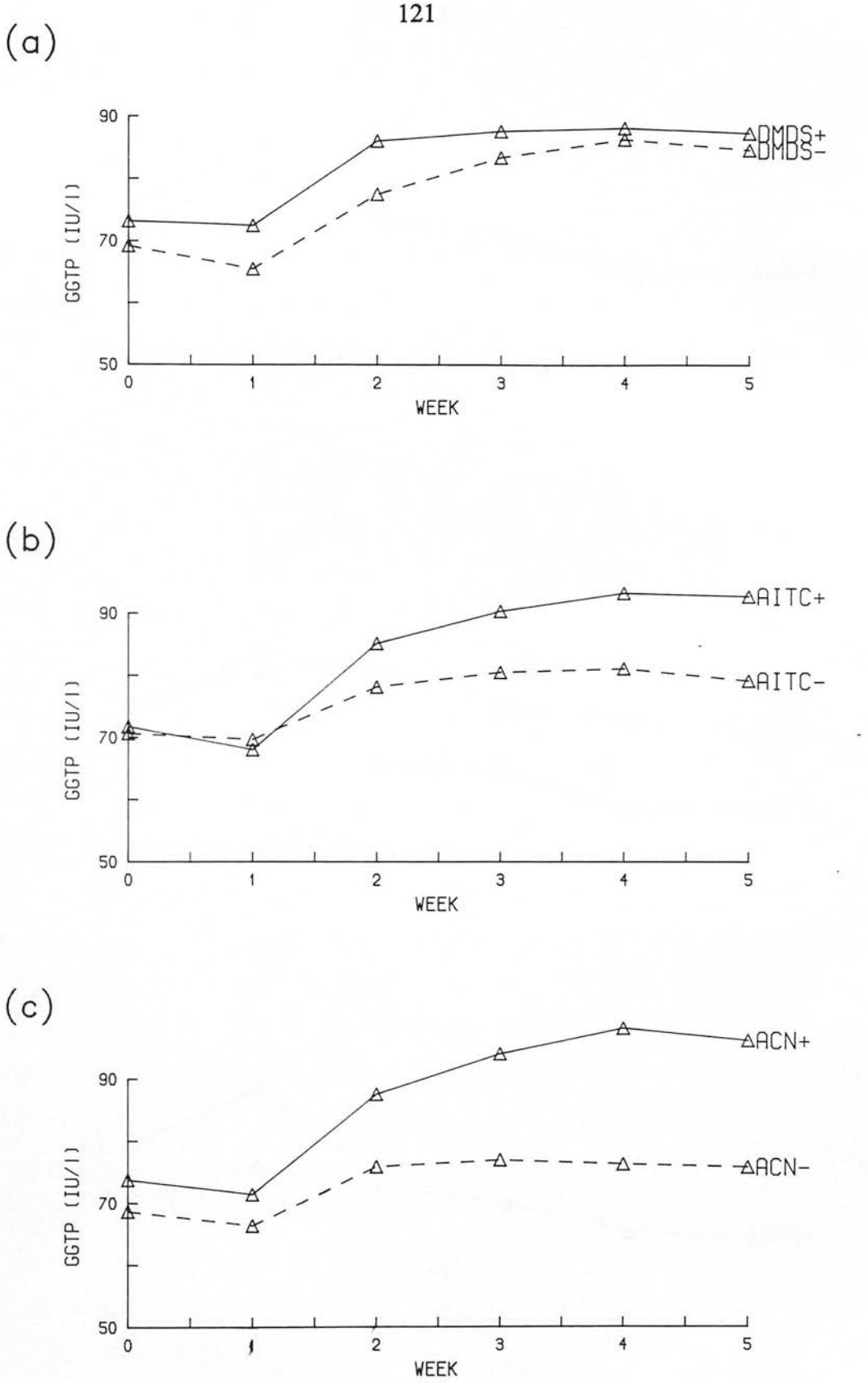
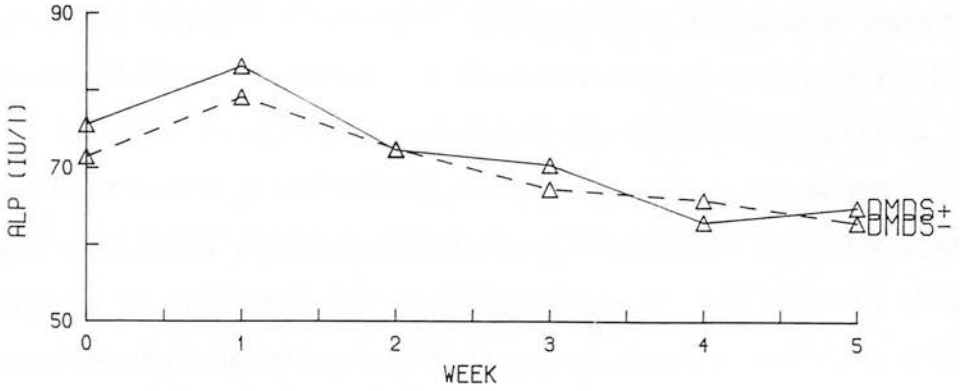
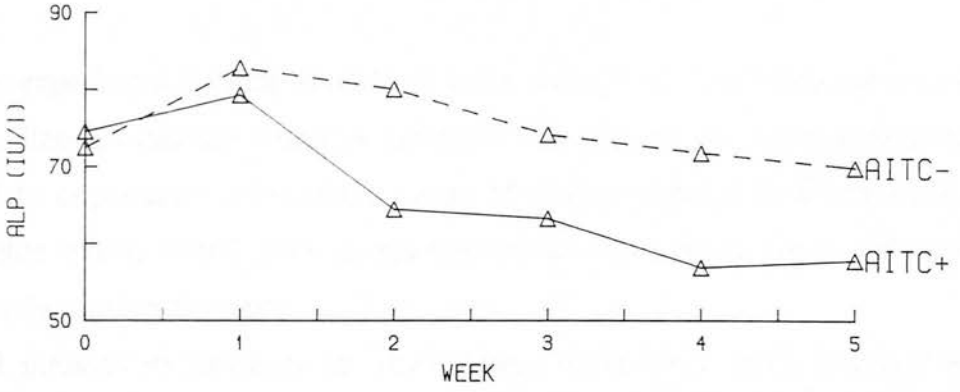


Figure 7.12 Main effects of DMDS (a), AITC (b) and ACN (c) on plasma gamma glutamyl transpeptidase (GGTP) activity over the five week treatment period.

(a)



(b)



(c)

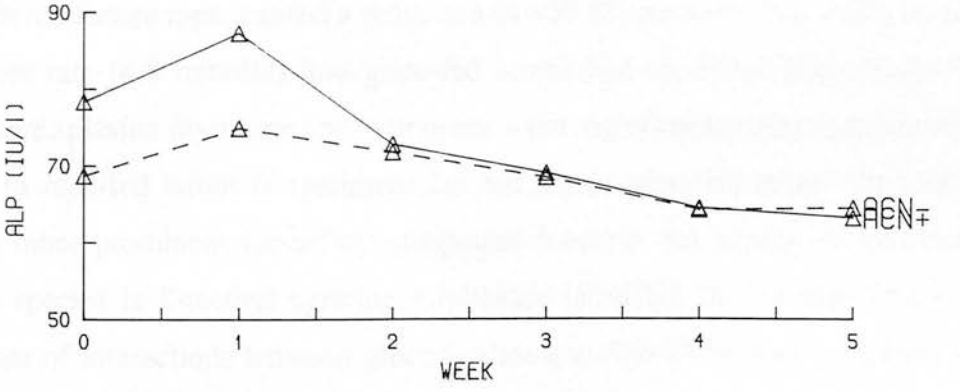


Figure 7.13 Main effects of DMDS (a), AITC (b) and ACN (c) on plasma alkaline phosphatase (ALP) activity over the five week treatment period.

during week two of the treatment period ($p < 0.001$) (Figure 7.6c).

Among clinical indicators of liver and kidney function, plasma albumin concentration and plasma GGTP activity showed significant treatment x time interactions; in the presence of AITC plasma albumin concentration was significantly depressed after week 2 of the treatment period relative to concentrations in the absence of the compound. Plasma GGTP activity increased steadily throughout the treatment period in the presence of ACN and also in the presence of AITC. Plasma alkaline phosphatase concentrations decreased with time in the presence of AITC ($p < 0.05$), and also in the presence of ACN ($p < 0.05$), with the largest change in concentrations occurring during week 2 of the treatment period in both cases.

Discussion

In this experiment animals were dosed twice daily rather than being continuously infused. Although this less intrusive approach was adopted for practical reasons, it provided the opportunity to examine a means of applying treatments which was more akin to that which would arise in grazing animals consuming meals rather than continuously grazing the crop.

Initial infusion experiments in which glucosinolate breakdown products were infused into lambs fed on forage rape or dried grass pellets indicated that interactions between glucosinolates and other components of the brassica diet might be important in dictating their effects on the animal. Thus, infusion of ACN (2.4 mmol/d) into lambs offered forage rape, caused a reduction in VFI (Experiment 2a), while infusion at a higher rate (4.8 mmol/d) into grass-fed lambs had no effect (Experiment 2b). Furthermore, plasma thyroxine concentrations were significantly depressed by AITC infusion in rape-fed lambs (Experiment 2a) but not in grass-fed lambs (Experiment 2b). The other prominent secondary compound found in the vegetative material of *Brassica* species is S-methyl cysteine sulphoxide (SMCO). In this experiment, the importance of interactions between glucosinolate and SMCO-derived compounds, in influencing their effects on the animal, was investigated.

Effects of ACN - In agreement with experiments on the effects of chronic administration of ACN (Experiment 2a and Experiment 3), ACN caused a significant depression of VFI in this experiment. The possibility that ACN was acting directly on rumen micro-organisms by reducing their activity and so reducing VFI *via* reduced digestibility and rate of passage cannot be excluded. Such effects of plant secondary compounds on rumen microflora have been demonstrated previously, for example, with phenolic acids (Hartley and Akin, 1989; Martin, 1988) and essential oils (Oh, Sakai, Jones and Longhurst, 1967). However, the most likely mechanism of toxicity of nitriles, involving inhibition of aerobic respiration following release of free cyanide, suggests that the toxicity of ACN is more likely to be manifested systemically than in the anaerobic rumen environment.

Systemic effects were certainly apparent. Among clinical indicators of liver and kidney function, plasma GGTP activity was elevated by ACN, indicating liver damage to have been caused by the compound, while plasma albumin concentrations and plasma alkaline phosphatase activity were unaffected by treatment. Gamma glutamyl transpeptidase is a more sensitive indicator of liver damage than alkaline phosphatase and is also specific to liver tissues, while alkaline phosphatase may be also be elevated in cases of bone lesion (Gowenlock et al., 1988). Similarly, plasma albumin concentrations are only depressed in cases of advanced liver necrosis. This evidence of liver damage caused by glucosinolate-derived nitriles is in agreement with studies with monogastric animals (Pearson, Butler and Fenwick, 1979; Campbell, 1987) and indicates that impaired liver function may be an important factor in the reduced performance of animals consuming glucosinolate-containing diets.

Allyl cyanide administration caused a dramatic reduction in erythrocyte glutathione concentrations. The effects of nitriles and other electrophilic compounds in reducing glutathione concentrations of other tissues, and in particular liver tissues, are well known (Orrenius and Moldeus, 1984) but the parallel depletion in erythrocytes has not previously been demonstrated. Indeed, the sustained nature of the depletion over the 5-week experimental period suggests that glutathione synthesis may have been affected.

Metabolism of ACN to thiocyanate ions in the liver by rhodanese would be expected to increase concentrations of circulating thiocyanate ions whose goitrogenic properties have previously been demonstrated (Paxman and Hill, 1974). Thyroid function, as measured by plasma concentrations of thyroid hormones, however, did not appear to be depressed by ACN. Peripheral T4 levels were elevated in the presence of ACN and this may have been the result of effects on peripheral thyroid hormone metabolism rather than thyroid function *per se*. Recent work has demonstrated the role of reduced glutathione in the de-iodination of thyroxine to tri-iodothyronine in rats with glutathione depletion leading to a build up in circulating T4 concentrations (Suberville, Higueret, Taruoura, Garcin and Higueret, 1988). ACN may thus have had an indirect effect on T4 concentrations through its action as a glutathione depletor.

Effects of AITC - In contrast to Experiments 2a and 2b, in which the short term effects of AITC infusion were found to be minimal, in this experiment AITC had pronounced effects on intake. The higher level of administration probably contributed to the more severe effects of AITC on VFI in this experiment. In addition, administration of AITC as discrete doses is likely to have resulted in higher transient concentrations of AITC, both in the digestive tract and in the circulation, than when infused continuously, and potential detoxification pathways may have been overwhelmed, leading to more severe physiological effects. The mechanism underlying VFI depression did not appear to be associated with the previously described goitrogenicity of the isothiocyanates, since peripheral thyroid hormone concentrations were unaffected by AITC administration.

In common with ACN, AITC caused severe depletion of erythrocyte glutathione concentrations, although the significance of this effect in relation to reduced VFI is unclear. Reduced erythrocyte glutathione concentrations presumably reflect the fact that the capacity of hepatic detoxification pathways (including hepatic glutathione conjugation) had been exceeded. However, erythrocyte glutathione reserves appeared to be sufficient to maintain protection in the red blood cells against peroxide and singlet oxygen toxicity since there was no evidence of haemolysis and anaemia in the presence of AITC. Kidney function did not appear to be affected by AITC since neither circulating creatinine nor urea concentrations were elevated in the presence of

the compound. Reduced plasma concentrations of albumin and increased plasma GGTP activity provided some indication of effects on liver metabolism, although the effects were less marked than with ACN.

The possibility that AITC exerted its effects in the digestive tract itself was not investigated but cannot be excluded. A number of potential modes of action can be conceived including effects on rumen motility, inhibition of microbial activity, or direct inhibition of digestive enzymes. *In vitro* studies have demonstrated the anti-bacterial action of isothiocyanates (Drobnica, 1967) and their inhibitory effects on proteins including digestive enzymes (Tang, 1974). The importance of these processes *in vivo* requires clarification.

Effects of DMDS - Dimethyl disulphide did not affect VFI at the level tested in this experiment. Dimethyl disulphide has been shown to cause haemolytic anaemia in previous studies (Smith, 1974). The only measurement associated with haemolytic anaemia to be affected by the treatment was the proportion of erythrocytes containing Heinz bodies. Heinz body numbers in the presence of DMDS (10 - 15% of red blood cells) indicated a mild anaemic effect; by comparison, typical levels produced as a result of the haemolytic anaemia caused by brassica ingestion would be 30 - 50 % (Greenhalgh, Sharman and Aitken, 1970). Since PCV levels and blood haemoglobin concentrations were unaffected by DMDS, the production of Heinz bodies in the erythrocytes was apparently not sufficient to bring about haemolysis and anaemia. The fact that severe haemolytic anaemia was not produced by the presence of DMDS treatment indicates that the basal diet may be an important factor in DMDS toxicity. In a previous experiment on the effects of SMCO supplementation on VFI, more severe effects were found when kale was offered than when the basal diet was dried lucerne (Barry et al., 1982). This was attributed to the higher concentration of water soluble carbohydrates in the kale diet, which, it was argued, would result in higher microbial activity and more rapid hydrolysis of SMCO to DMDS. However, the lack of effects when DMDS was administered to dried grass-fed sheep in the present experiment indicates that interactions between DMDS and components of the brassica diet may be important in determining ultimate toxicity, either through effects on the digestive fate of the compounds or, following absorption, in the tissues.

The elevation in blood glutathione concentrations as a result of DMDS administration was unexpected given conventional explanations of the mechanism underlying haemolytic anaemia. These state that haemolysis follows perturbation of erythrocyte protective mechanisms, following binding of reduced glutathione by DMDS. Depletion of erythrocyte glutathione levels by DMDS thus reduces the cell's protection against free radicals, which arise as a result of normal cellular oxidative metabolism (Halliwell and Gutteridge, 1985). However, in the present experiment erythrocyte GSH concentrations were increased in the presence of DMDS. This unexpected result may have been associated with the low rates of DMDS administration which may have resulted in increased GSH biosynthesis as a compensatory response. Alternatively, conventional explanations of the events leading to haemolysis following DMDS administration may need to be re-examined.

Effects of Time - Consideration of the time course of development of physiological effects is potentially useful in correlating VFI depression with underlying biochemical events. The effects of ACN in reducing VFI were most pronounced in weeks 1 and 2 of the treatment period. Similarly, changes in other biochemical variables were most marked during this period and it is difficult to correlate effects on VFI with any single variable. Changes in VFI due to the AITC treatment were also greatest in the first two weeks of the treatment period. Conversely, effects on the haematological variables occurred later in the treatment period, casting doubt on their importance in relation to VFI depression. Changes in plasma GGTP activity, indicating liver damage, were apparent early on in the treatment period, although reduced plasma albumin concentrations appeared later in the treatment period. This may reflect the fact that GGTP is an indicator of cellular damage in the liver, while albumin is a measure of liver function. The later development of changes in plasma albumin concentrations suggest a progressive effect of AITC on the liver.

Interaction effects - Although this experiment provided the opportunity to examine further, the main physiological effects of glucosinolate breakdown products in sheep, the primary aim of the experiment was to determine the importance of interactions between different brassica secondary compounds in reducing VFI, by sheep. Such interactions would most obviously occur as a result of the anaemia caused by DMDS.

Such anaemia might increase susceptibility to the effects of glucosinolates. Unfortunately, such an interaction was not adequately tested in the present experiment, since the extent of the anaemia resulting from DMDS administration was slight in comparison to that seen in animals grazing forage brassica crops, and did not affect the animals sufficiently to allow an assessment of the importance of potential interactions with the effects of glucosinolates. However, other interactions between glucosinolate breakdown products and DMDS were found, particularly in relation to their metabolic fate. For example, thiocyanate ion concentrations in the urine of animals given ACN were decreased by the additional presence of DMDS, indicating cyanide release to be decreased by the presence of DMDS. Conversely, the presence of AITC increased urinary SCN^- excretion by animals given ACN, indicating increased cyanide production in the tissues. Interestingly, parallel interactions were apparent with respect to VFI. Thus, reduced free cyanide release in the presence of DMDS was accompanied by less marked effects of the presence of ACN on VFI when DMDS was also present. Similarly, in an interaction which approached significance ($p = 0.07$) the presence of AITC increased free cyanide release from ACN and also increased the effects of ACN on VFI. These parallel interactions suggest that free cyanide release (measured as urinary SCN^- excretion) may be an important factor in the effects of ACN on VFI. This conclusion is not borne out by the clinical indicators of liver and kidney damage; the effects of ACN on plasma GGTP activity, for example, were not lessened by the additional presence of DMDS. It may be, therefore, that although ACN causes cellular damage in the liver, other tissue sites such as the brain are more important in mediating the effects of ACN on VFI.

Examination of interactions among the other physiological parameters measured provided little evidence for more pronounced effects as a result of the combined administration of compounds. Such effects might have been more apparent had the haemolytic anaemia caused by DMDS been more severe. Further investigation in this area would be valuable.

In conclusion, this experiment did not show interactions between different brassica secondary compounds to be of major significance in exacerbating their harmful effects. However, because the infusion levels of DMDS did not produce anaemia of the

magnitude observed in animals consuming forage brassica herbage in the field, the potential importance of such interactions cannot be ruled out. Furthermore, changes in the magnitude of effects which resulted from the combined administration of brassica secondary compounds and which could be correlated with altered metabolic fate, highlighted the importance of considering all components of a diet in combination before judging the harmful potential of an isolated constituent.

CHAPTER 8 - GENERAL DISCUSSION

Plant secondary compounds can be defined as those compounds that have no known function in the fundamental life processes of the plant in which they are present (Bell, 1981). The question of whether secondary plant compounds function as metabolic regulators, storage sinks, excretion products or whether they have adaptive significance in the defence of the plant against herbivores or pathogens is still in debate (Rhoades, 1979). The role of the secondary compounds found in *Brassica* species, the glucosinolates and SMCO, is not clear; the effects of glucosinolate concentrations on predation by insect herbivores has been demonstrated (Finch, 1978; Hardman and Ellis, 1978) with a protective role for glucosinolates being suggested. Similarly, susceptibility to fungal pathogens has been shown to be related to glucosinolate concentrations in the vegetative material (Mithen, Lewis and Fenwick, 1986). The effects of glucosinolate concentrations in herbage on predation by mammalian herbivores is not clearly understood (Tapper, 1989). There is some evidence that the VFI of ruminant herbivores is affected by the SMCO content of the consumed herbage (Barry et al., 1984) but the effects of SMCO concentration on insect predation or pathogen susceptibility are not known. Since the adaptive significance of brassica secondary metabolites is not clearly defined and given that *Brassica* species contain moderate concentrations of compounds with considerable toxic potential (Chapter 2), the current series of experiments was conducted to determine the extent to which such effects were actually realised or evaded through detoxification pathways within the animal.

The General Discussion begins by considering the experimental approach adopted with emphasis on its relative advantages and disadvantages in determining the effects of the compounds and the animals' ability to evade such effects. Potential toxic mechanisms of brassica secondary compounds will then be re-examined before discussing, in the light of the experimental results, the extent to which the various routes operate in practice or are limited by the animal's detoxification mechanisms. The mechanisms by which the observed physiological effects might influence VFI will then be dis-

cussed. Finally the practical importance of brassica secondary compounds and potential methods of alleviating their effects will be considered.

Experimental Approach

In examining the manner by which brassica secondary compounds affect the physiology of sheep, a ruminal infusion approach was chosen, since this facilitates examination of the physiological responses to infused compounds without the complications of sensory effects on VFI, which are inherent when compounds are presented as part of the feed. Infusion of a compound directly into the rumen removes the opportunity of animals to limit their ingestion of the compound and thus is more likely to reveal toxic effects. Sensory discrimination may be important in limiting the ingestion of such compounds but is likely to be associated with underlying toxic effects since sensory discrimination has presumably evolved, at least in part, to facilitate avoidance of toxic compounds (Provenza and Balph, 1987). Thus a harmful effect of a particular compound is likely to lead, in evolutionary time, to the development of sensory aversion to that compound (Chapman and Blaney, 1979).

With ruminants, rumination may result in sensory discrimination following entry of rumen contents into the mouth, since ruminants presumably associate the taste of the regurgitated bolus with the current food source (Provenza and Balph, 1987). The importance of sensory aversion as a result of regurgitation of infused compounds was not assessed in the current experiments. However, absorption of the infused compounds was probably rapid as a result of their small molecular weight. In addition, although rumination frequency was not quantified, the milled nature of the dried grass pellet diet (Experiment 2b, 3 and 5) probably reduced the extent of rumination and both these factors diminish the significance of rumination as a means of sensory discrimination.

Although infusion of compounds is an informative experimental approach, a number of potential theoretical disadvantages exist. The infusion of individual compounds takes no account of potential interactions between compounds and their chemical milieu within the plant in producing effects. The way in which the chemical composition of the diet may affect the rumen fate of brassica secondary compounds is illustrated in Figure 8.1. Many secondary compounds, including the glucosinolates,

Abbreviations

SMCO	S-methyl cysteine sulphoxide
AITC	Allyl isothiocyanate
ACN	Allyl cyanide
DMDS	Dimethyl disulphide

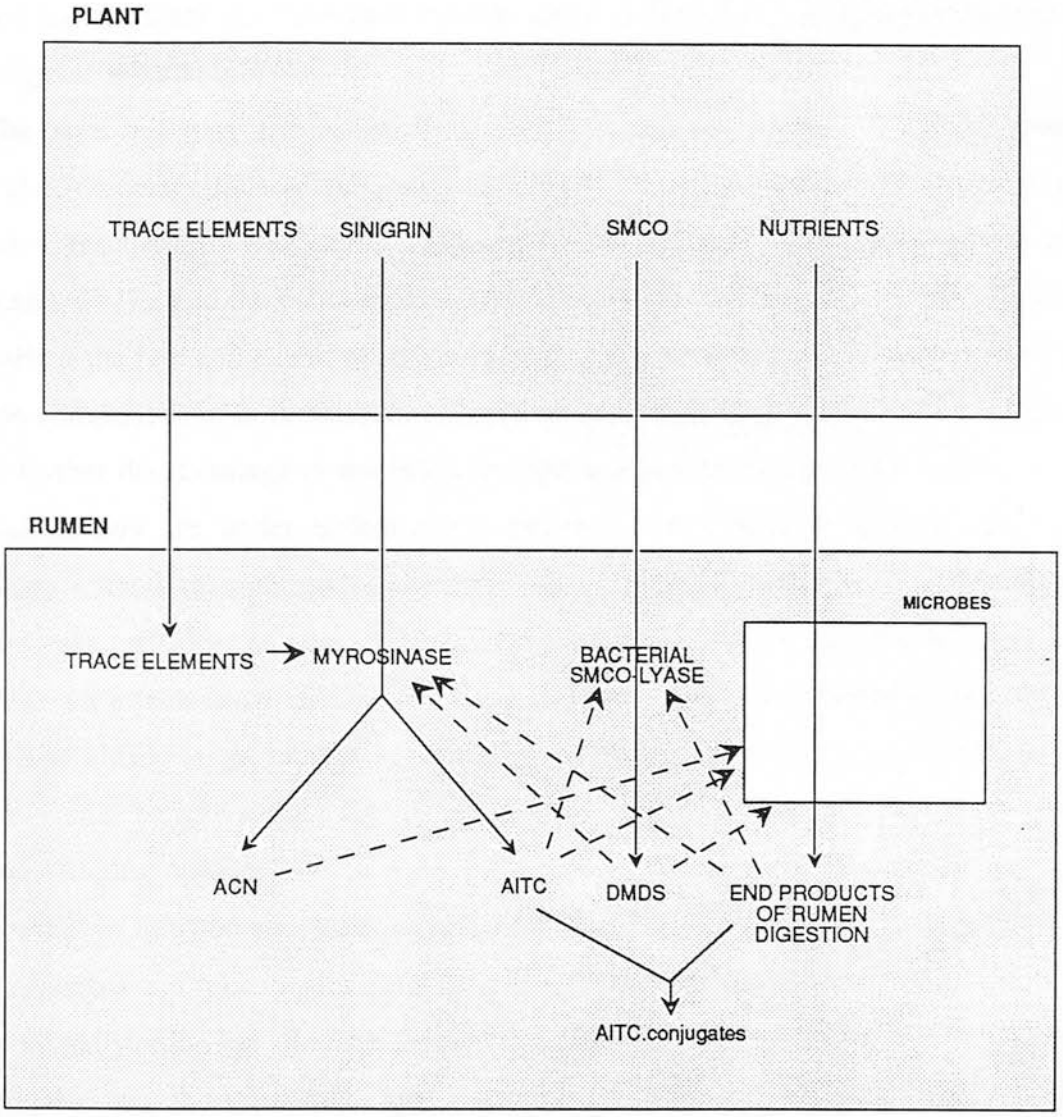


Figure 8.1 Potential interactions between components of brassica diet on production of toxic breakdown products.

exist in benign form in the plant, presumably as an adaptive mechanism to avoid auto-toxicity, and only release toxic products when chemically united with a degradative enzyme, in this case myrosinase. It would thus be futile to test the toxicity of a parent glucosinolate by gastric infusion in the absence of plant myrosinase. Even if infused

together, the resulting hydrolysis would not necessarily produce the same range of toxic products as occurs naturally, since the hydrolysis of glucosinolates is dependent on the chemistry of the hydrolysis environment. It was for this reason that the hydrolysis of parent glucosinolates and the toxic effects of resulting products were investigated separately.

The basal diet may also influence the metabolic fate of infused compounds, and thus alter the magnitude of physiological effects. For example, isothiocyanates are known to react readily with exposed amino groups of proteins (Drobnica, Kristian and Augustin, 1977), and the concentration and conformation of plant protein may therefore affect the rate and extent of isothiocyanate absorption. This was the justification for the comparison of both brassica and non-brassica diets in Experiments 2a and 2b.

A further disadvantage of the infusion approach is that animals kept indoors and fed *ad libitum* are under different environmental constraints to animals grazing outdoors. Effects may not occur as readily under these conditions as if experiments were conducted under harsher, outdoor conditions and assessment of the importance of observed effects must take account of this. For example, the haemolytic anaemia caused by SMCO might ultimately lead to an oxygen deficit in the tissues. The effects of this would be less serious for an animal presented with food indoors than for an animal foraging outdoors.

Finally, in infusion experiments, it is difficult to mimic the natural *in vivo* release rates, patterns and release sites pertaining to animals ingesting the crop *in situ*. Realistic administration of the compounds under investigation requires supporting information on the metabolic fate and, in particular, the digestive fate of the compounds. It was for this reason that the digestive fate of parent glucosinolates and of subsequent toxic breakdown products was investigated in Experiments 1 and 4.

Two methods of administering compounds were employed. Continuous infusion (Experiments 2a, 2b and 3) probably resulted in digestive tract concentrations of brassica secondary compounds similar to those among sheep grazing forage brassica crops continuously. Release of the compounds in the digestive tract, absorption into the blood stream and clearance by excretion would presumably reach equilibrium and this would afford animals the maximum opportunity to detoxify the compounds, either

in the digestive tract or systemically. Twice-daily dosing, on the other hand, would be equivalent to two daily meals of brassica forage provided release of compounds from the herbage was rapid. This would result in much higher instantaneous concentrations of compounds, both in the digestive tract and in the blood and tissues, and the potential for digestive tract or systemic detoxification would be much reduced. Information on ingestive behaviour during forage brassica grazing is limited but the meal pattern lies somewhere between the two extremes represented by continuous infusion and discrete dosing (R.H. Armstrong, personal communication). Both approaches are, therefore, informative.

Post-ingestive fate and physiological effects of brassica secondary metabolites.

Allyl cyanide

Allyl cyanide was selected for investigation as a representative of the aliphatic nitriles, which are potential breakdown products of all aliphatic glucosinolates. Nitriles result from the myrosinase-catalysed hydrolysis of parent glucosinolates and tend to predominate when such hydrolysis occurs in conditions of low pH and in the presence of sulphydryl compounds and certain metallic ions. A previous study of the fate of glucosinolates in ovine rumen fluid showed nitriles to form a significant proportion of glucosinolate-derived compounds (Forss and Barry, 1983); in these *in vitro* experiments in which swede or kale vegetative tissue was macerated in rumen fluid, nitrile production from glucosinolates present in the herbage was estimated to represent 0.40 of the total hydrolysis of glucosinolates. Although the results of Experiment 1 gave no direct evidence of nitrile production *in vivo*, they indicated that 0.53 of the sinigrin hydrolysed in the digestive tract yielded AITC. The proportion of sinigrin hydrolysed to ACN may be estimated by difference to be in the region of 0.50, although further work is required to clarify this.

Following hydrolysis of sinigrin to ACN in the rumen, further catabolism may occur (Figure 8.2). Rumen microbial degradation of secondary plant compounds has been demonstrated with a variety of compounds and may represent an evolutionary adaptation to permit ingestion of otherwise toxic food material (Carlson and Breeze,

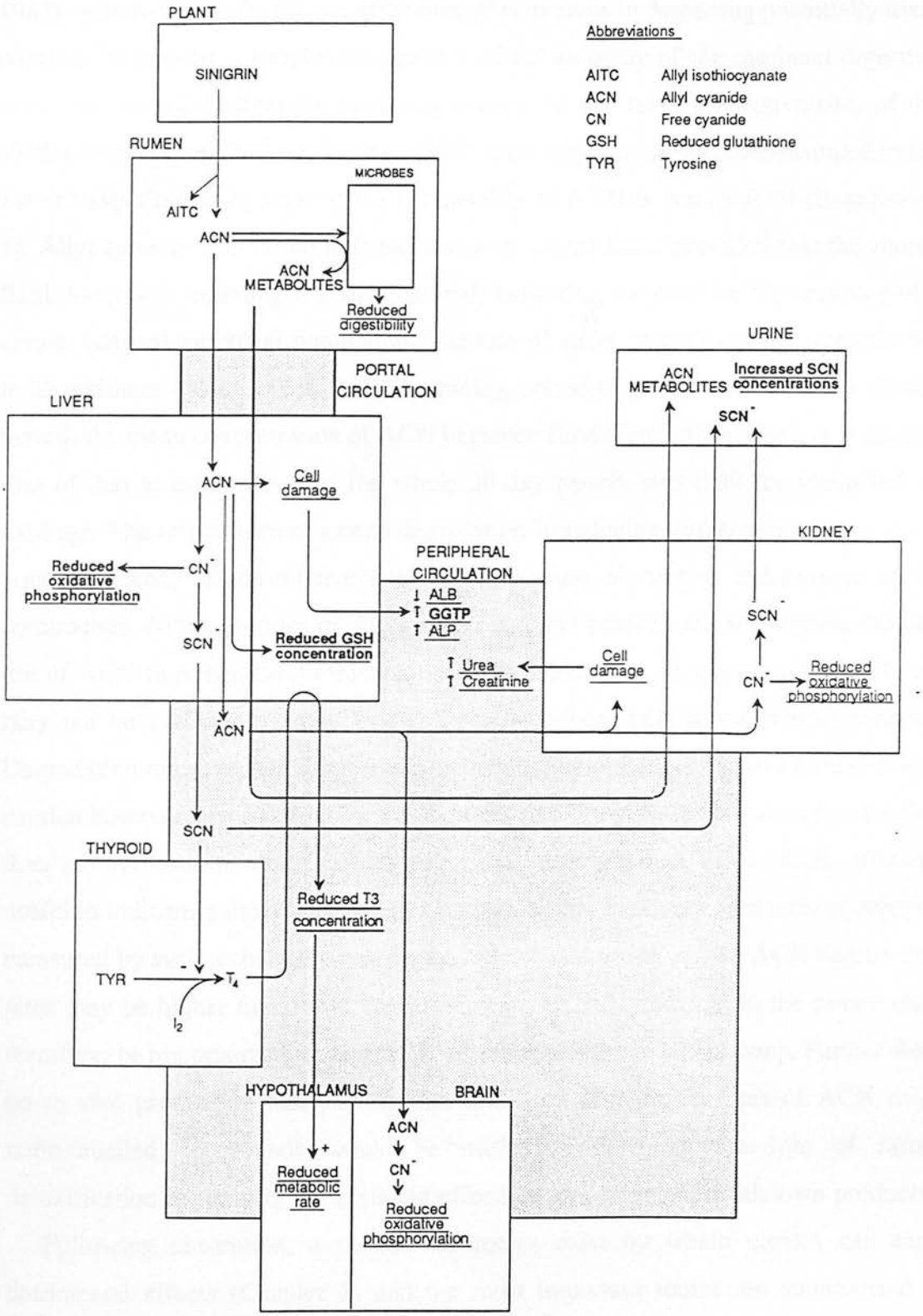


Figure 8.2 Potential metabolic fate and sites of action of allyl cyanide in sheep. Observed effects in bold text.

1984). Alternatively, the greater efficiency of ruminants in degrading potentially toxic compounds may be a simple consequence of the anatomy of the ruminant digestive tract with microbial digestion occurring anterior to the major absorptive sites of the small intestine (Freeland and Janzen, 1974). Such degradation was demonstrated in the current experiments by monitoring the stability of ACN in rumen fluid (Experiment 4). Allyl cyanide was found to break down in rumen fluid, provided that the rumen fluid donor was ingesting brassica material, indicating the need for the presence of a viable, adapted microbial population. The rate of ACN breakdown was variable but in Experiment 4b, in which ACN degrading potential was assessed over a 30-day period, the mean concentration of ACN in rumen fluid after 300 minutes, as a proportion of that at time zero over the whole 30-day period, was 0.39 for sheep fed on cabbage. The importance of rumen degradation in reducing absorption of toxic compounds depends on the relative rates of degradation, absorption and passage of the compounds. Although rates of ACN absorption and passage are not known, the half life of ACN in rumen fluid measured in Experiment 4b as approximately 2 - 3 hours may not be sufficiently rapid to significantly reduce ACN absorption in practice. Degradation rates measured by *in vitro* batch culture techniques must be treated with caution however, since factors such as reduced viability of micro-organisms and higher than natural concentrations of test substrates may introduce error. Thus, although useful in indicating the occurrence of reactions within the rumen, the rates of reaction measured by such techniques may not be reliable and actual *in vivo* ACN degradation rates may be higher than those measured here. ACN degradation in the rumen may, therefore, be important in limiting ACN absorption in brassica-fed sheep. Further work on *in vivo* production rates, absorption rates and degradation rates of ACN using radio-labelled compounds would be useful in clarifying the role of rumen detoxification in limiting the systemic effects of glucosinolate breakdown products.

Following absorption, a number of routes exist by which nitriles can exert detrimental effects (Chapter 2) and the most important routes are summarised in Figure 8.2. Previous tests of the physiological effects of glucosinolate-derived nitriles in mono-gastrics have emphasised their nephrotoxic and hepatotoxic properties, although the range of compounds and animal species investigated has been limited

(Nishie and Daxenbichler, 1980; Gould et al., 1985). In the current experiments, there was some evidence for hepato-cellular damage, as indicated by elevated plasma GGTP concentrations in ACN-infused sheep (Figure 8.2). In Experiment 3, lambs infused with 9.6 mmol ACN /d over 8 weeks had mean plasma GGTP concentrations which were 1.11 those of control animals which received no ACN. Similarly, in Experiment 5, animals dosed with 10 mmol/d ACN over a 5 week period, had mean GGTP concentrations which were 1.21 those of animals receiving no ACN. Other indicators of liver function, including plasma albumin concentrations and plasma alkaline phosphatase activity, however, did not indicate any gross malfunction. Thus, although liver damage was suggested by elevated plasma GGTP concentrations, liver function did not appear to be unduly affected and the toxicological importance, in sheep, of hepato-cellular damage resulting from glucosinolate-derived nitriles is questionable. Effects on kidney tissue were even less marked with little or no elevation of plasma creatinine or urea concentrations when ACN was infused (Experiment 3 and Experiment 5).

Another potential route of nitrile toxicity, which is rarely considered in the context of glucosinolate-derived nitrile toxicity, is the tissue biotransformation of nitriles to free cyanide with resulting chronic effects on cellular respiration (Figure 8.2). The acute toxicity of cyanide is thought to be associated with its properties as an inhibitor of cytochrome oxidase activity, with resulting inhibition of oxidative phosphorylation leading to respiratory shock, particularly in brain and cardiac tissues (Way, Leung, Cannon, Morgan, Tamulinas, Leong-Way, Baxter, Nagi and Chui, 1988). The importance of cytochrome oxidase inhibition in the chronic toxicity of cyanide, released from organic nitriles *in vivo,* is not so clearly defined. Biotransformation of nitriles to free cyanide has been demonstrated in rats with a number of aliphatic nitriles including ACN through detection of cyanide in brain, liver and kidney tissues with concomitant inhibition of cytochrome oxidase activity (Willhite and Smith, 1981; Ahmed and Farooqui, 1982). The importance of this route of toxicity was determined in the current experiments by measurement of cytochrome oxidase activity in liver and kidney tissues (Experiment 3) and in muscle tissues (Experiment 5), as well as the indirect approach of quantifying urinary thiocyanate ion concentrations, as a measure

of free cyanide release (Experiment 5) (Figure 8.2). Liver cytochrome oxidase activity was inhibited by ACN infusion (cytochrome oxidase activity in liver of animals infused with 9.6 mmol ACN /d was 0.72 of that of control animals; Experiment 3, Table 5.2) but the activity of the enzyme in kidney and muscle tissue was unaffected (Experiments 3 and 5), perhaps reflecting their distal position with respect to the digestive tract. Urinary thiocyanate ion concentration was elevated by ACN infusion (ACN present, 8.73 ug/ml, absent 0.32 ug/ml; Experiment 5), indicating that free cyanide release was indeed occurring.

Detoxification of free cyanide in the liver by hepatic rhodanese could influence thyroid function as a result of increased concentrations of circulating thiocyanate ions (Figure 8.2). This did not seem to be important in these experiments, since peripheral thyroid hormone concentrations were not depressed by ACN infusion.

In summary, the systemic effects of ACN may be reduced by microbial degradation of the compound in the rumen. Following absorption, ACN may exert its effects directly, through cellular damage in liver and kidney tissues, or indirectly via free cyanide release and inhibition of cellular respiration. The results of current experiments indicate the latter route to be of primary importance.

Allyl isothiocyanate

Isothiocyanates may also appear as products of glucosinolate hydrolysis in the ruminant digestive tract. When sinigrin was given orally, AITC formed a significant proportion of the resulting hydrolysis products (Experiment 1). Investigation of the further digestive fate of AITC yielded equivocal results because of analytical difficulties, although from knowledge of isothiocyanate chemistry conjugation with sulphydryl compounds and proteins may occur in the digestive tract and also following absorption (Drobnica et al., 1977).

The potential metabolic fate and toxic routes of AITC in sheep are indicated in Figure 8.3. The toxic effects of isothiocyanates have previously been attributed to their goitrogenic nature (Figure 8.3), when tested in rats (Langer and Stolc, 1965). There was little evidence for such an effect in the present experiments. Plasma tri-iodothyronine concentrations were marginally depressed when AITC was infused intra-

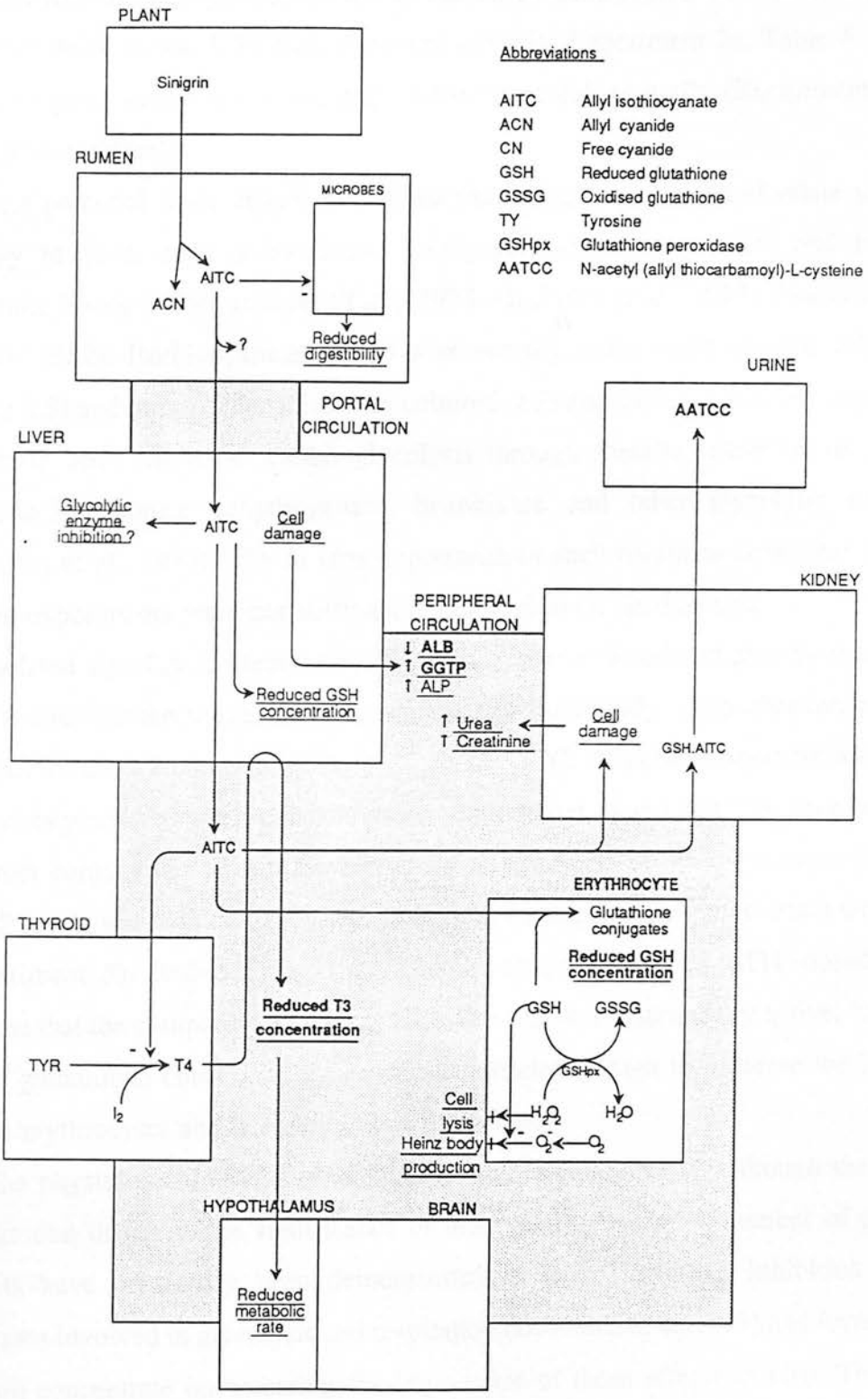


Figure 8.3 Potential metabolic fate and sites of action of allyl isothiocyanate in sheep. Observed effects in bold text.

ruminally into forage rape-fed lambs (plasma T3 concentration in lambs infused with 2.4 mmol ACN /d was 0.81 that of control animals; Experiment 2a, Table 4.1), but not when given either intra-ruminally (Experiment 2b) or orally (Experiment 5) to dried grass-fed lambs.

Other potential toxic effects of isothiocyanates are manifold and relate to their capacity to bind, and so inactivate, sulphydryl groups of proteins and to split disulphide bonds within proteins (Tang, 1974; Drobnica et al., 1977; Kawakishi and Namiki, 1982). Isothiocyanates are thus potentially multi-target enzyme inhibitors (Figure 8.3) and in *in vitro* studies with cultured cells and eukaryotic micro-organisms they have been shown to inhibit glycolysis through specific inhibition of glyceraldehyde-3-phosphate dehydrogenase, hexokinase and other glycolytic enzymes (Drobnica et al., 1977). The *in vivo* importance of such reactions is unclear and the present experiments were not sufficiently detailed to tackle this area.

Isolated significant effects of AITC on a number of blood and plasma parameters were found, but the range of measures was not sufficiently comprehensive to draw conclusions as to the important toxic effects of AITC. Allyl isothiocyanate was found to depress plasma albumin concentrations (Experiment 5) and this may have been due to direct conjugation with plasma proteins or to effects on liver function. Elevated GGTP levels were indicative of hepato-cellular damage, although the effect was small (Experiment 5). Reduced blood glutathione concentrations in AITC-dosed lambs implied that the compound was being absorbed and was systemically active; however, blood glutathione concentrations remained sufficiently high to preserve the integrity of the erythrocytes and haemolysis did not occur.

The physiological effects of isothiocyanates remain unclear, although the current results cast doubt on the significance of their goitrogenicity. A number of potential effects have previously been demonstrated *in vitro*, including inhibition of key enzymes involved in glycolysis and respiration (Drobnica et al, 1977) and further work should concentrate on assessing the importance of these effects *in vivo*. This could begin with an assessment of effects on glycolytic reactions *in vivo*. For example, hepatic activities of enzymes important in the control of glycolysis, such as phospho-

fructokinase and pyruvate kinase, could be determined following administration of isothiocyanates.

Dimethyl disulphide

The effects of SMCO in ruminants grazing forage brassicas have received more attention than those of glucosinolates. The link between the SMCO content of forage and the severity of the resultant haemolytic anaemia has been demonstrated (Barry et al., 1984a). In addition, supplementation of both brassica and non-brassica diets with artificial SMCO, albeit at higher than physiological levels, has shown VFI to be affected by SMCO (Barry et al., 1982). The biochemical events leading to cell lysis and potential interactions with the effects of glucosinolates are unknown.

The key sites of toxicity of DMDS are indicated in Figure 8.4. The haemolytic properties of SMCO have been attributed to the rumen product, DMDS, which is thought to interfere with protective mechanisms within the erythrocytes, following absorption, leading to membrane peroxidation (Smith, 1974). Simple conjugation of glutathione by DMDS did not appear to be an important factor leading to haemolysis in the current experiments, since blood glutathione concentrations were not reduced by DMDS administration (Experiment 5) (Figure 8.4). The protective reactions occurring in erythrocytes are complex (Suttle et al., 1987), and DMDS may exert its effects at a number of other biochemical sites. For example, inhibition of glutathione peroxidase would also have resulted in a build-up of harmful peroxides within the cell (Figure 8.4). Since glutathione is a substrate for glutathione peroxidase, such inhibition could have led to an intracellular build-up of reduced glutathione, as observed here. Previous investigations of the effects of SMCO on red cell metabolism have always assumed the glutathione-lowering effects of brassica-feeding to be due to SMCO ingestion. By separating the effects of SMCO and glucosinolates, the current experiments provide new evidence that blood glutathione concentrations are lowered by glucosinolate breakdown products and that DMDS increases membrane fragility by a different mechanism.

Future studies in this area should tackle the problem of determining the site of action of DMDS within the erythrocyte. This would most easily be achieved using *in*

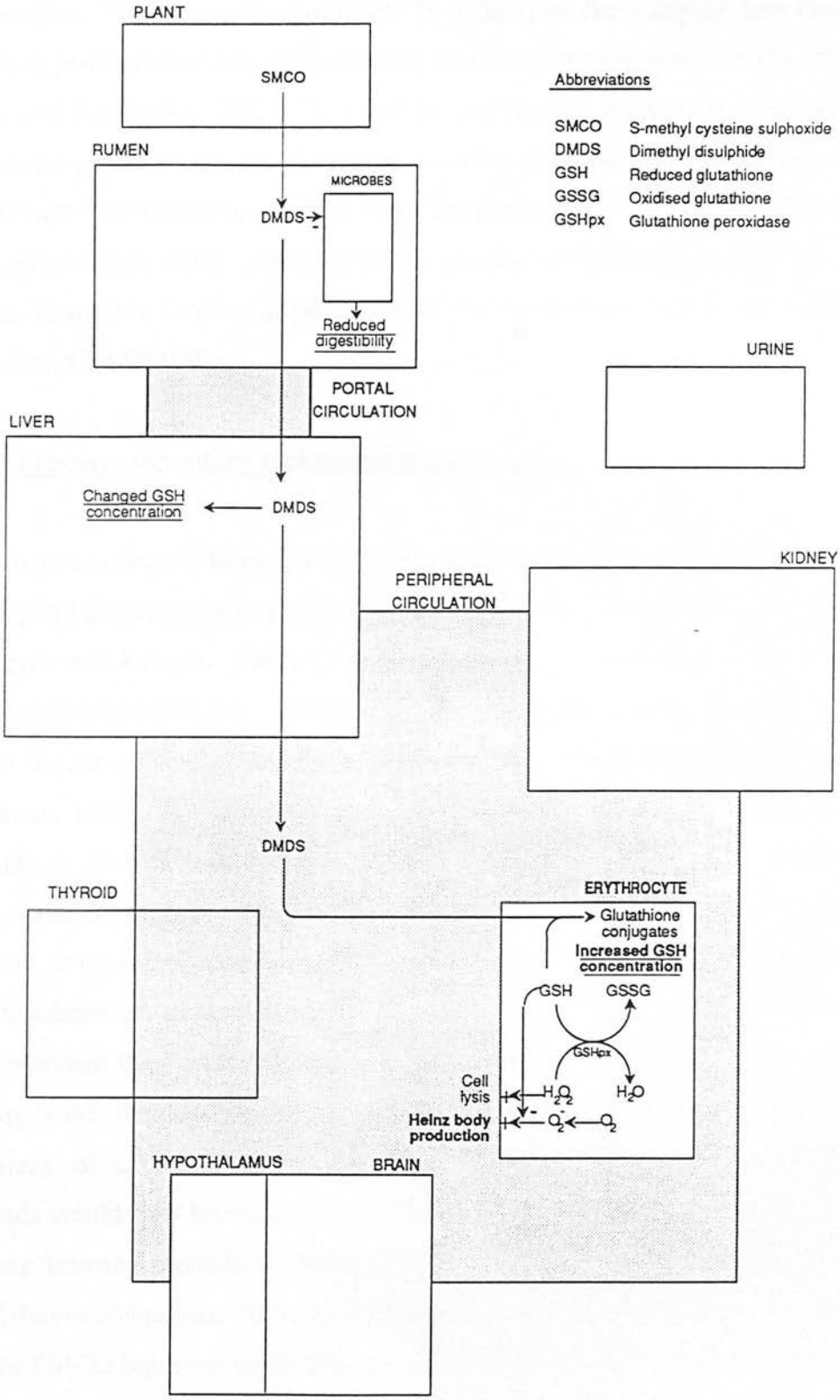


Figure 8.4 Potential metabolic fate and sites of action of dimethyl disulphide in sheep. Observed effects in bold text.

vitro techniques. The recent improvements in techniques for studying free radical-induced lipid peroxidation using spin-trapping techniques may be useful in this respect (Halliwell and Gutteridge, 1985). Thus free radical activity, as measured by electron spin resonance spectroscopy, could be used as an index of the peroxidative effects of DMDS *in vitro* and would be a useful basis for examining the influence of factors, such as erythrocyte GSH concentrations, glutathione peroxidase activity and superoxide dismutase activity in protection of the erythrocyte against the oxidative damage caused by DMDS.

Potential brassica secondary compound interactions

There is increasing evidence that plant secondary metabolites represent an evolved system of plant defence against potential herbivores (Kumar and Singh, 1984; Hruska, 1988; Meyer and Karasov, 1989). The particular defence strategy seen in different species presumably represent a balance between the energetic costs of maintaining a system of deterrent chemicals and the benefits in terms of reduced predation (Chew and Rodman, 1979). The reasons for maintaining two distinct groups of secondary compounds, as seen in *Brassica* species, are unclear; the two groups of compounds may be evolutionarily directed at different target herbivores, for example, mammals and insects or brassica specialist herbivores and generalist herbivores. Alternatively, successful adaptation of herbivores to the presence of one group of compounds may have necessitated the development of a second group of deterrent chemicals. A final possibility is that the different compounds act in concert and should be considered as one system of chemical protection. Interactions between different secondary compounds would thus be important in enhancing their toxicity.

Among brassica secondary compounds, there are a number of potential sites at which different compounds could interact in the animal. Most obviously, the anaemia caused by SMCO ingestion could limit oxygen supply to the tissues and so exacerbate the effects of ACN on oxidative phosphorylation. Although interactions between DMDS and ACN did not appear to increase their overall toxicity in the current experi-

ments (Experiment 5), such effects might have been more apparent, if a more severe anaemia had occurred.

Different secondary compounds could alter each other's metabolic fate and so increase, or decrease overall toxicity. This mechanism of interaction was observed in the present experiments (Experiment 5). Thus, urinary SCN^- excretion (indicative of free cyanide release in the tissues, with resulting effects on oxidative phosphorylation) was affected by the presence of other compounds; DMDS reduced SCN^- excretion, while AITC increased SCN^- excretion. This alteration to the catabolic route of ACN may have been due to the activity of the hepatic mixed function oxidase (MFO) system, an alternative route of ACN catabolism (Figure 8.5). Isothiocyanates have been shown to be MFO inhibitors (Plaa, 1970) and MFO inhibition by AITC may have slowed ACN catabolism resulting in greater free cyanide release. Dimethyl disulphide had the opposite effect on SCN^- excretion and may conceivably have caused MFO induction. Whether this was the mechanism by which DMDS and AITC influenced SCN^- excretion or not, interesting parallels were apparent in the interaction effects on SCN^- excretion and VFI. There was therefore a suggestion that free cyanide release was important in bringing about a reduction in VFI, possibly through its effects on oxidative phosphorylation. This hypothesis could be further investigated by direct determination of hepatic cytochrome oxidase activity as a measure of oxidative phosphorylation.

Mechanisms of voluntary food intake reduction

The processes by which a healthy animal maintains VFI at a level appropriate to requirements for maintenance and growth have been the subject of intense study (Forbes, 1986) and will not be discussed in detail here. The mechanisms by which compounds which disturb normal metabolism cause hypophagia, however, are still relatively unknown. The effects of deficiencies and excesses of minerals and vitamins have been considered to some extent, although the link between toxicity and inappetance is unclear (e.g. Moxon and Rhian, 1943; Ott, Smith, Harrington and

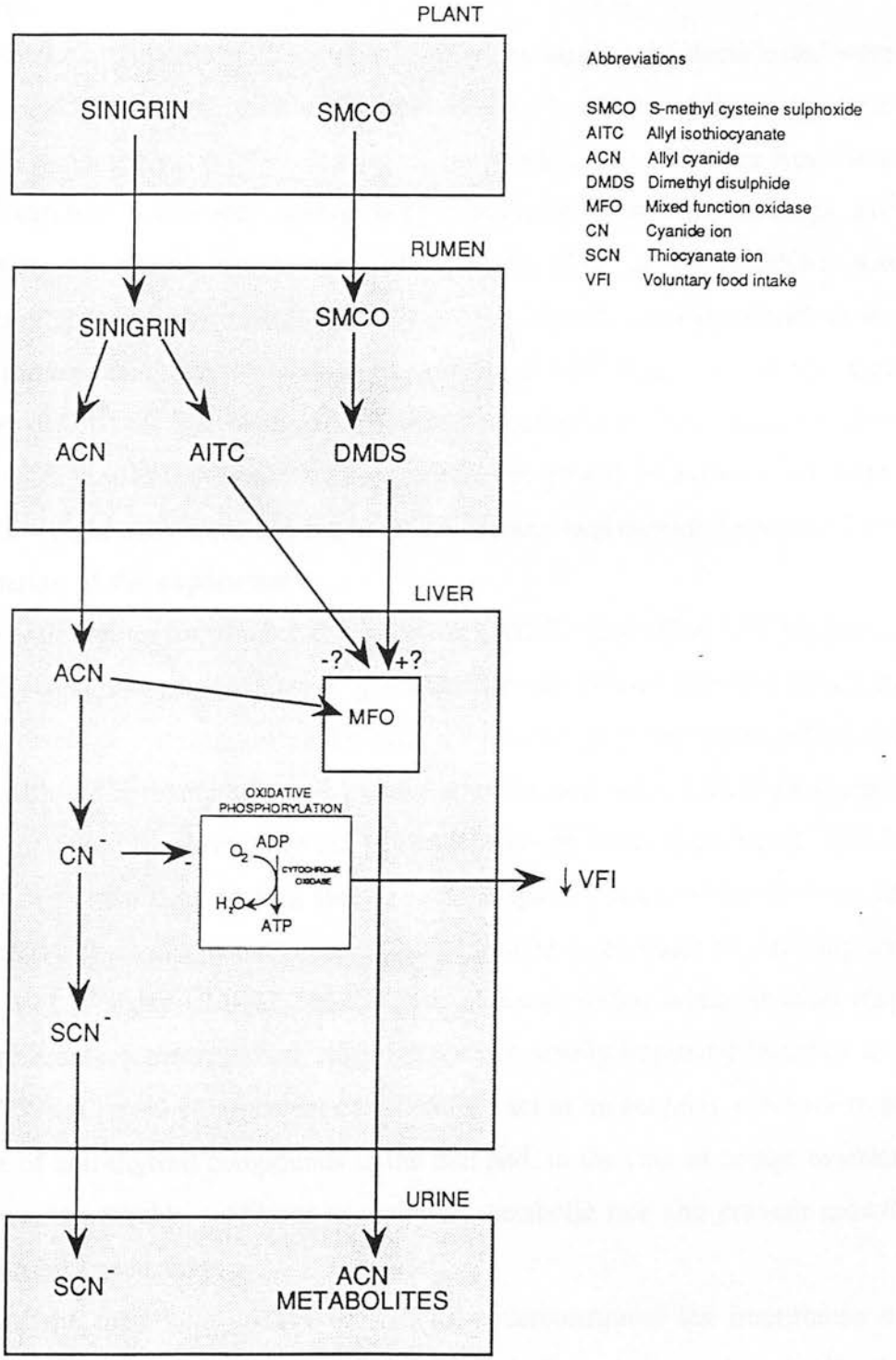


Figure 8.5 Potential effects of allyl isothiocyanate and dimethyl disulphide on the hepatic fate of allyl cyanide.

Beeson, 1966).

In the current experiments, both the glucosinolate breakdown products tested were shown to reduce VFI to some extent (Experiments 2a, 3 and 5) while, at the level tested, DMDS had no effect on VFI. Reference was made in the Literature Review to the "low and variable" VFI's seen among sheep consuming forage brassica crops. The current infusion experiments also showed VFI to be variable but this variability was not treatment related and was probably related to experimental procedures such as the presence of rumen cannulae. Although the magnitude of VFI reduction was not large (in the region of 0.10 - 0.20), when expressed as a proportion of food intake required for growth, such a reduction would seriously affect the growth of animals. Measurements of live-weight gain were not made in the current experiments because of the short-term nature of the experiments.

Some potential routes by which ACN, AITC or DMDS could affect VFI are shown in Figure 8.6. The goitrogenic effects of glucosinolates could have caused a reduction in VFI as a result of reduced metabolic rate. This route appeared to be of limited importance with the compounds tested in these experiments, since effects on thyroid function, as measured by plasma thyroid hormone concentrations, were small. This is in agreement with results of grazing studies with forage brassicas, which have found the anti-thyroid effects of glucosinolates to be of limited importance in affecting the growth and VFI of sheep (Russel, 1967). Thus, although iodine administration may prevent thyroid enlargement, growth rates are not necessarily improved (Sinclair and Andrews, 1959). Thyroid enlargement can therefore act as an adaptive mechanism to the presence of anti-thyroid compounds in the diet and, in the case of forage brassica glucosinolates, is probably sufficient to maintain metabolic rate and prevent growth reduction and VFI reduction.

Studies of the metabolic control of VFI have demonstrated the importance of hepatic concentrations of intermediates in fuel metabolism such as glucose (Booth, 1972) and propionate (Anil and Forbes, 1980). Inhibition of metabolic processes, such as glycolysis or oxidative phosphorylation, could lead to increasing levels of such metabolites and this information could be passed to the central nervous system, *via* the vagus nerve, invoking central nervous inhibition of VFI. Examples of the potential

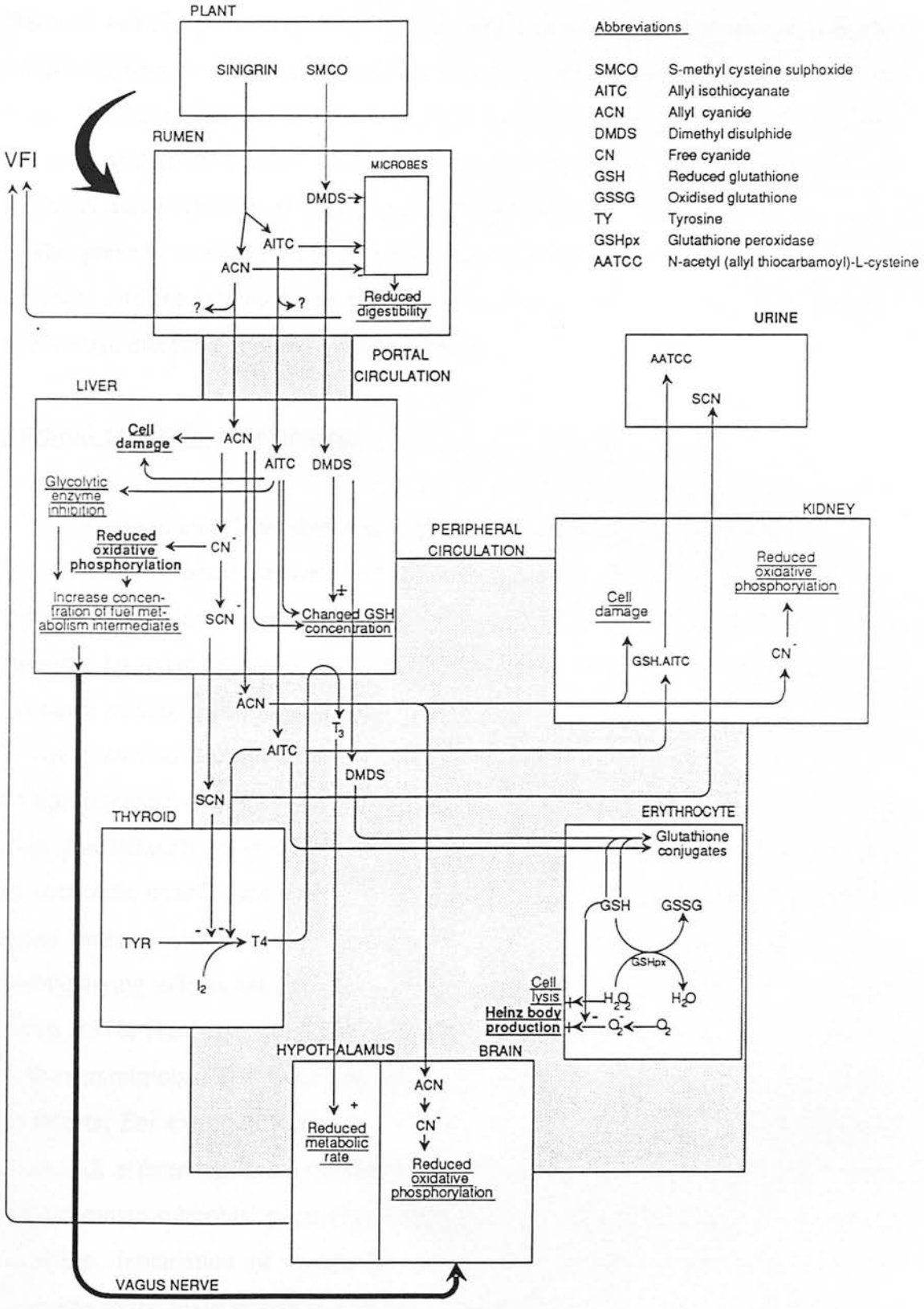


Figure 8.6 Potential routes of VFI reduction by brassica secondary metabolites in sheep.

effects of brassica anti-metabolites in this regard include the inhibition of oxidative phosphorylation by ACN, and inactivation of glycolytic enzymes by AITC. Alternatively, inhibition of glycolytic or oxidative phosphorylation pathways in the liver may lead to a build up of reducing equivalents and this may be the signal for reducing VFI *via* vagus nerve stimulation (Langhans, Egli and Scharrer, 1985).

The present results provide no experimental evidence for the operation of such processes and the intention has only been to indicate the potential mechanisms by which toxic effects and VFI could be linked.

Potential prophylactic measures

These experiments have shown that brassica secondary compounds may reduce the nutritive value of *Brassica* crops through their effects on VFI, and from an agricultural standpoint, ways of minimising their effects need to be considered. Effects could be alleviated by reducing ingestion or absorption of the compounds or by increasing the resistance of animals to their effects.

The glucosinolate content of the seeds of oilseed rape has been markedly reduced through intensive breeding over the last 20 years (Thomke, 1981). Similar reduction in the glucosinolate content of brassica herbage has not occurred, presumably because the economic benefits are much less clearly defined. Selection of low glucosinolate forage brassica cultivars could potentially improve their nutritive value, although accompanying effects on pest and disease resistance would need to be considered (Finch, 1978; Hardman and Ellis, 1978; Mithen et al., 1986).

Rumen microbial degradation of secondary compounds can reduce their physiological effects. For example, the ability of ruminants to consume *Leucaena* in Hawaii without ill effects has been attributed to the presence, in Hawaiian animals, of an adapted rumen microbial population which was able to degrade the toxic compound, mimosine. Inoculation of Australian cattle with Hawaiian rumen fluid conferred resistance to the toxic effects of *Leucaena* to the recipient animals (Jones, 1981). The role of rumen micro-organisms in ACN degradation was demonstrated in the current experiments (Experiments 4a and 4b). Adaptation to the presence of ACN in the

rumen occurred rapidly with a lag phase of less than six days and the potential for increasing resistance of animals to the effects of glucosinolates by prior inoculation with rumen microbes from adapted animals is therefore fairly limited. However, the organisms responsible for degradation were not identified and further work in this area could lead to the isolation of more efficient strains of "ACN-degraders". Rumen inoculation might then become a useful possibility for limiting the effects of brassica-derived nitriles in the animal.

Finally, the effects of brassica secondary metabolites could be minimised by increasing animal resistance to their effects. At present, the relative importance of the various toxic routes of brassica secondary compounds are not known with sufficient certainty to be able to suggest reliable ways of reducing their effects. Presumably the trace element status of the animals is important. For example, copper is a constituent of cytochrome oxidase, and copper deficiency might add to the inhibitory effects of free cyanide on cytochrome oxidase activity. Copper is also a constituent of superoxide dismutase, an important enzyme in erythrocyte protection. Sulphur status may also be important, since an adequate supply of sulphane sulphur is necessary for efficient cyanide detoxification (Westley, 1988), although the low release rates of cyanide from glucosinolate-derived nitriles are unlikely to overwhelm detoxification pathways. The potential involvement of erythrocyte glutathione in both the detoxification of glucosinolate breakdown products and the protection of red blood cells from the effects of DMDS have been highlighted in these experiments and increasing erythrocyte glutathione concentrations by nutritional or genetic means is a potential method of reducing the effects of all compounds. More information on modes of toxicity is required before potential prophylactic methods can be suggested and tested.

Conclusions

The physiological effects and metabolic fate in sheep of prominent representatives of both glucosinolate and SMCO breakdown products have been examined in a series of infusion experiments.

A method was developed to determine the *in vivo* fate of glucosinolates by quantifying urinary excretion of a metabolite of AITC. Refinement of this technique could yield useful information on the proportions of different glucosinolate breakdown products arising in the digestive tract. Preliminary results indicated isothiocyanates to account for approximately half of sinigrin hydrolysis products in the digestive tract of sheep.

The further rumen fate of glucosinolate breakdown products was examined *in vitro* and rumen micro-organisms were shown to degrade ACN to unknown products. Allyl isothiocyanate was also rapidly transformed in rumen fluid but the physiological significance of this is unclear and further work is required.

Both nitrile and isothiocyanate glucosinolate breakdown products (ACN and AITC respectively), were shown to reduce the VFI of ruminally-infused sheep. Allyl cyanide caused limited cellular damage to liver tissues at infusion levels similar to those arising following brassica ingestion. However, the effect of free cyanide release on oxidative phosphorylation is also an important route of ACN toxicity and may be significant with respect to VFI reduction.

AITC also caused VFI reduction at physiological levels but not, apparently, by the assumed mechanism of thyroid suppression. The mode of toxicity of isothiocyanates remains unclear but may relate to their capacity to inhibit key enzymes through their protein-binding properties.

The anaemia caused by DMDS administration was mild in comparison to previous studies, perhaps reflecting the influence of the basal diet on the digestive fate of DMDS. The absence of glutathione depletion in the erythrocytes of DMDS- dosed animals contradicts previous explanations of the mechanism leading to cell lysis and further work is necessary.

Interactions between the effects of the different brassica secondary compounds were investigated, and there was little evidence for more severe effects as a result of combined administration. Alterations to the metabolic fate of compounds as a result of their dual administration, however, emphasized the influence of the whole diet on the ultimate toxic effects of individual constituents.

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APPENDICES

Appendix 3.a Sample ANOVA Table from Experiment 1.

***** Analysis of variance *****

Variate: AATCC output over 24 hours (ug)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
animal stratum					
diet	1	0.5573	0.5573	4.98	0.067
Residual	6	0.6712	0.1119		
animal.period stratum					
treatment	3	10.9923	3.6641	34.06	<.001
period	3	1.1745	0.3915	3.64	0.037
period.diet	3	0.4651	0.1550	1.44	0.270
Residual	15	1.6138	0.1076		
Total	31	15.4742			

Appendix 3.b - Experimental design and allocation of treatments in experiment 1.

	SWEDE				GRASS			
ANIMAL	115	203	320	219	111	206	125	218
PERIOD I	C	D	A	B	A	B	C	D
PERIOD II	A	B	C	D	B	C	D	A
PERIOD III	D	A	B	C	D	A	B	C
PERIOD IV	B	C	D	A	C	D	A	B

Treatments

A. Control -empty gelatin capsule
B. Sinigrin - 1660 mg (4mmol) in gelatin capsule
C. Allyl isothiocyanate (AITC) - 396mg (4 mmol) in gelatin capsule
D. Allyl cyanide (ACN) - 268 mg (4 mmol) in gelatin capsule

Appendix 3.c - Individual urinary AATCC concentrations (ug/ml) in Experiment 1.

DIET - SWEDE
TREATMENT - SINIGRIN

PERIOD		I	II	III	IV
ANIMAL		219	203	320	115
TIME (h)	1	1.04	0.96	1.66	0.39
	2	0.91	2.65	5.64	NS
	4	0.62	1.34	1.62	0.65
	8	1.10	2.33	1.07	0.90
	24	0.10	0.17	0.27	0.28
	48	0.12	0.13	0.05	0.62
	72	0.25	0.01	0.03	0.25

DIET - SWEDE
TREATMENT - AITC

PERIOD		I	II	III	IV
ANIMAL		115	320	219	203
TIME (h)	1	0.12	16.12	0.00	2.62
	2	32.19	9.88	3.31	1.78
	4	7.84	10.92	1.34	0.95
	8	2.42	2.09	1.30	0.84
	24	0.74	0.39	0.56	0.30
	48	0.22	0.20	0.09	0.20
	72	0.15	0.07	0.00	0.35

DIET - SWEDE
TREATMENT - ACN

PERIOD		I	II	III	IV
ANIMAL		203	219	115	320
TIME(h)	1	0.53	0.44	0.00	0.35
	2	0.90	0.18	0.00	0.48
	4	0.46	0.55	0.18	0.27
	8	0.18	0.04	0.05	0.26
	24	0.16	0.17	0.43	0.00
	48	0.11	0.08	0.06	0.48
	72	0.19	0.06	0.00	0.11

DIET - SWEDE
TREATMENT - CONTROL

PERIOD		I	II	III	IV
ANIMAL		320	115	203	219
TIME (h)	1	0.00	0.11	0.00	0.55
	2	0.11	0.50	0.00	0.28
	4	0.46	0.39	0.01	0.11
	8	0.20	0.00	0.00	0.20
	24	0.08	0.02	0.08	0.00
	48	0.11	0.18	0.11	0.82
	72	0.19	0.05	0.00	0.20

Appendix 3.c continued

DIET -DRIED GRASS PELLETS
TREATMENT - SINIGRIN

PERIOD		I	II	III	IV
ANIMAL		206	111	125	218
TIME (h)	1	0.00	1.80	4.55	0.00
	2	15.96	2.24	12.45	6.95
	4	5.08	1.66	8.33	6.88
	8	1.74	1.51	3.06	3.08
	24	1.34	1.82	0.73	1.91
	48	0.30	1.62	0.48	0.56
	72	0.11	1.18	0.06	0.14

DIET - DRIED GRASS PELLETS
TREATMENT -AITC

PERIOD		I	II	III	IV
ANIMAL		125	206	218	111
TIME (h)	1	18.92	0.24	5.12	2.57
	2	15.91	7.67	9.65	0.95
	4	8.83	6.67	7.63	6.22
	8	3.86	7.65	13.16	4.41
	24	1.63	6.59	2.11	1.73
	48	0.23	1.90	0.11	0.36
	72	0.04	1.10	0.06	0.12

DIET - DRIED GRASS PELLETS
TREATMENT - ACN

PERIOD		I	II	III	IV
ANIMAL		218	125	111	206
TIME (h)	1	0.29	0.73	0.00	0.00
	2	0.61	1.22	0.12	0.00
	4	0.18	0.86	0.40	0.00
	8	0.53	1.05	0.00	0.00
	24	0.01	0.71	0.04	0.00
	48	0.00	0.63	0.24	0.00
	72	0.04	0.64	0.06	0.00

DIET - DRIED GRASS PELLETS
TREATMENT - CONTROL

PERIOD		I	II	III	IV
ANIMAL		111	218	206	125
TIME (h)	1	0.00	0.41	0.00	0.00
	2	0.27	0.67	0.00	0.07
	4	0.15	0.57	0.00	0.00
	8	0.01	0.68	0.18	0.00
	24	0.05	0.79	0.00	0.00
	48	0.03	0.00	0.16	0.00
	72	0.00	0.59	0.00	0.00

Appendix 3.d - Individual urinary outputs over each period in Experiment 1 (ml)

DIET - SWEDE
TREATMENT - SINIGRIN

PERIOD		I	II	III	IV
ANIMAL		219	203	320	115
TIME (h)	1	380	185	500	470
	2	305	145	105	0
	4	590	160	375	560
	8	960	140	1080	860
	24	2890	2320	2360	3100
	48	4430	3760	3740	5300
	72	4020	3740	3380	4790

DIET - SWEDE
TREATMENT - AITC

PERIOD		I	II	III	IV
ANIMAL		115	320	219	203
TIME (h)	1	210	210	300	170
	2	175	260	440	205
	4	500	210	365	370
	8	820	760	1000	880
	24	2650	2650	2285	1930
	48	4110	3590	4560	4100
	72	0.15	3840	3840	4020

DIET - SWEDE
TREATMENT - ACN

PERIOD		I	II	III	IV
ANIMAL		203	219	115	320
TIME(h)	1	320	215	640	510
	2	75	0	315	110
	4	95	690	220	75
	8	500	970	530	880
	24	2390	2780	2730	3000
	48	4110	4400	3995	5000
	72	3930	3950	3750	4480

DIET - SWEDE
TREATMENT - CONTROL

PERIOD		I	II	III	IV
ANIMAL		320	115	203	219
TIME (h)	1	645	470	250	480
	2	160	255	210	295
	4	910	490	495	535
	8	200	810	1040	975
	24	2350	2560	1750	1960
	48	3730	4010	2700	4990
	72	3700	4040	3040	4320

Appendix 3.d continued

DIET -DRIED GRASS PELLETS
TREATMENT - SINIGRIN

PERIOD		I	II	III	IV
ANIMAL		206	111	125	218
TIME (h)	1	0	195	100	85
	2	170	45	45	55
	4	240	500	90	85
	8	150	410	135	180
	24	1200	770	785	450
	48	1390	850	1160	590
	72	1040	860	1090	1350

DIET - DRIED GRASS PELLETS
TREATMENT -AITC

PERIOD		I	II	III	IV
ANIMAL		125	206	218	111
TIME (h)	1	130	0	50	145
	2	85	145	35	85
	4	200	125	45	70
	8	190	295	70	210
	24	1080	450	750	1350
	48	2510	830	1160	1570
	72	1970	1100	1250	1200

DIET - DRIED GRASS PELLETS
TREATMENT - ACN

PERIOD		I	II	III	IV
ANIMAL		218	125	111	206
TIME (h)	1	85	105	40	85
	2	90	55	135	0
	4	170	320	115	185
	8	230	465	325	470
	24	920	990	745	1690
	48	1320	1110	1660	1080
	72	1390	980	1910	970

DIET - DRIED GRASS PELLETS
TREATMENT - CONTROL

PERIOD		I	II	III	IV
ANIMAL		111	218	206	125
TIME (h)	1	85	60	95	45
	2	90	40	0	55
	4	170	130	190	140
	8	230	175	255	195
	24	920	440	565	590
	48	1320	780	1160	870
	72	1390	1120	1270	850

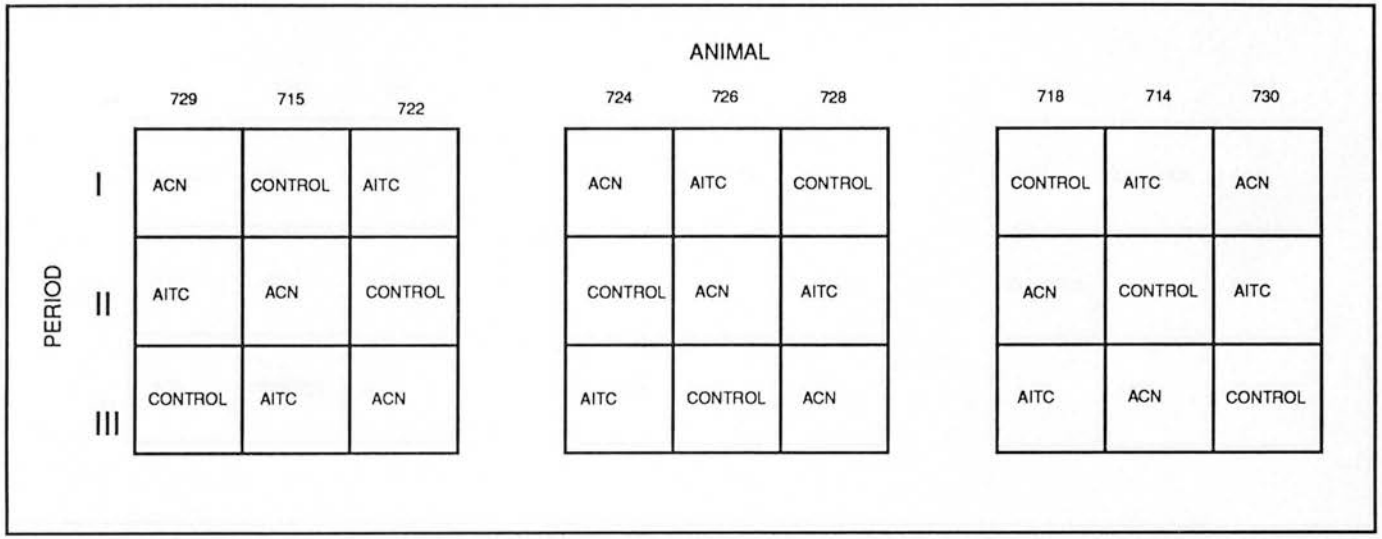
Appendix 4a. Sample ANOVA table from Experiment 2a.

***** Analysis of variance *****

Variate: Voluntary food intake (g DM /d)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
animal stratum	8		597359.	74670.		
animal.period stratum						
treatment	2		388185.	194093.	3.52	0.060
period	2		742056.	371028.	6.72	0.010
Residual	13	(1)	717625.	55202.		
Total	25	(1)	2427197.			

Appendix 4.b - Experimental design and allocation of treatments in Experiment 2a.

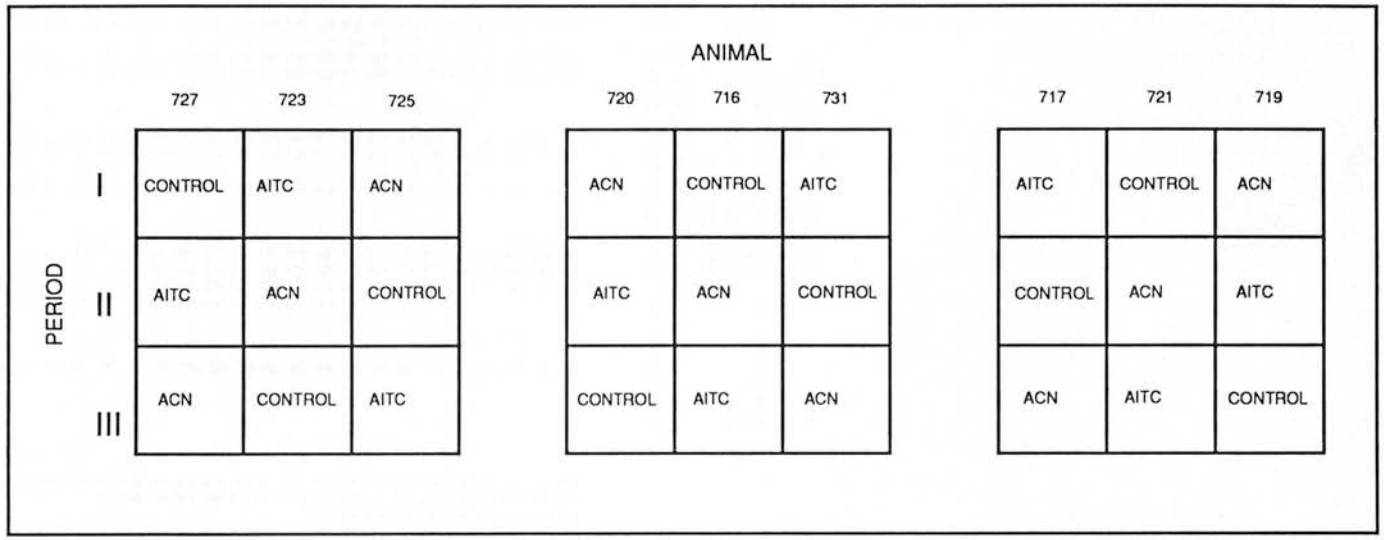


Treatments - ACN : 2.4 mmol/d allyl cyanide in 80 ml propylene glycol intra-ruminally.

AITC : 2.4 mmol/d allyl isothiocyanate in 80 ml propylene glycol intra-ruminally.

CONTROL : 80 ml propylene glycol /d intra-ruminally

Appendix 4.c - Experimental design and allocation of treatments in Experiment 2b.



Treatments - ACN : 4.8 mmol/d allyl cyanide in 80 ml propylene glycol intra-ruminally.

AITC : 4.8 mmol/d allyl isothiocyanate in 80 ml propylene glycol intra-ruminally.

CONTROL : 80 ml propylene glycol /d intra-ruminally.

Appendix 4.d - Data values used in analysis of variance in Experiment 2.a (forage rape diet)

treatm	anim	period	square	Intk	T3	T4	Haem	PCV
ACN	a729	I	x	361	1.13	61.8	14.3	38
CONTROL	a715	I	x	1227	1.51	108.0	12.6	34
AITC	a722	I	x	1086	1.06	70.3	12.4	34
ACN	a724	I	y	1063	1.78	86.3	15.1	37
AITC	a726	I	y	932	0.67	44.8	12.0	34
CONTROL	a728	I	y	919	1.13	60.9	11.9	33
CONTROL	a718	I	z	1331	0.94	46.0	13.5	36
AITC	a714	I	z	*	0.80	37.2	14.9	38
ACN	a730	I	z	1063	0.81	43.1	12.3	32
AITC	a729	II	x	633	1.90	68.1	13.1	34
ACN	a715	II	x	421	3.11	99.6	15.7	38
CONTROL	a722	II	x	1047	2.54	96.9	12.4	31
CONTROL	a724	II	y	1163	3.32	119.6	14.5	37
ACN	a726	II	y	1025	2.68	100.7	13.6	34
AITC	a728	II	y	1577	2.11	90.3	12.6	33
ACN	a718	II	z	1024	2.19	56.4	15.0	36
CONTROL	a714	II	z	1101	1.61	62.5	14.9	39
AITC	a730	II	z	1144	1.77	65.1	12.0	31
CONTROL	a729	III	x	563	1.16	74.2	13.0	33
AITC	a715	III	x	987	1.22	96.9	13.0	31
ACN	a722	III	x	493	1.60	81.2	14.2	35
AITC	a724	III	y	697	1.68	90.2	15.8	40
CONTROL	a726	III	y	683	2.11	91.0	15.5	40
ACN	a728	III	y	660	2.76	148.5	15.5	39
AITC	a718	III	z	640	1.55	93.2	14.2	35
ACN	a714	III	z	380	1.40	81.6	16.1	39
CONTROL	a730	III	z	804	1.49	73.8	13.7	34

Key

Intk = Voluntary food intake (g DM /d)
T3 = Plasma tri-iodothyronine conc. (umol/l)
T4 = Plasma thyroxine conc. (umol/l)
Haem = Blood haemoglobin conc. (g/dl)
PCV = Blood PCV (%)
* = Missing value

Appendix Table 4.e - Data values used in analysis of variance in Experiment 2.b (dried grass pellet diet)

treatm	anim	period	square	intk	T3	T4	Haem	PCV
CONTROL	a727	I	x	1631	1.65	58.1	14.1	38
AITC	a723	I	x	1994	1.34	53.6	12.3	33
ACN	a725	I	x	2218	1.43	52.0	14.5	37
ACN	a720	I	y	1407	1.41	85.0	15.0	39
CONTROL	a716	I	y	1596	0.24	65.4	15.1	39
AITC	a731	I	y	2232	1.41	55.3	12.5	35
AITC	a717	I	z	1311	1.55	68.3	14.1	37
CONTROL	a721	I	z	1943	1.02	57.9	14.3	37
ACN	a719	I	z	1691	1.35	53.3	13.2	37
AITC	a727	II	x	1332	2.16	75.6	14.5	38
ACN	a723	II	x	1639	1.85	96.2	13.2	32
CONTROL	a725	II	x	1806	1.81	84.4	12.4	33
AITC	a720	II	y	1153	2.15	98.3	15.7	39
ACN	a716	II	y	1039	1.45	66.6	14.9	35
CONTROL	a731	II	y	1851	1.73	101.6	14.5	38
CONTROL	a717	II	z	1159	3.43	92.8	16.1	39
ACN	a721	II	z	1780	2.29	89.7	14.3	34
AITC	a719	II	z	1345	1.68	89.1	14.7	36
ACN	a727	III	x	1389	2.16	85.8	15.9	39
CONTROL	a723	III	x	1643	1.86	102.9	14.9	39
AITC	a725	III	x	1410	1.93	108.1	15.0	37
CONTROL	a720	III	y	987	2.40	46.9	16.4	39
AITC	a716	III	y	1138	1.43	81.6	16.3	39
ACN	a731	III	y	1239	2.11	100.1	13.3	35
ACN	a717	III	z	897	2.64	97.3	16.7	40
AITC	a721	III	z	1850	2.15	12.9	16.1	40
CONTROL	a719	III	z	1491	1.67	98.0	14.6	35

Key

Intk = Voluntary food intake (g DM /d)
T3 = Plasma tri-iodothyronine conc. (umol/l)
T4 = Plasma thyroxine conc. (umol/l)
Haem = Blood haemoglobin conc. (g/dl)
PCV = Blood PCV (%)
* = Missing value

Appendix 5.a Sample ANOVA Table from Experiment 3.

***** Analysis of variance *****

Variate: Voluntary food intake (g DM /d)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
block stratum	6	8258044.	1376341.		
block.animal stratum					
treatment	2	2238112.	1119056.	4.65	0.046
Residual	8(4)	1923272.	240409.		
block.animal.*Units* stratum					
week	8	4236437.	529555.	13.74	<.001
treatment.week	16	619625.	38727.	1.00	0.458
Residual	112(32)	4318126.	38555.		
Total	152(36)	19251812.			

Appendix 5.b - Allocation of treatments in Experiment 3.

Block	Animal number	Treatment
I	2	C
	3	L
	19	H
II	14	L
	20	H
	21	C
III	7	C
	12	L
	16	H
IV	1	H
	8	L
	13	C
V	9	C
	11	L
	18	H
VI	4	C
	6	H
	17	L
VII	5	L
	10	C
	15	H

Appendix 5.c - Individual data values from Experiment 3; VFI and plasma parameters

animal	treatm	block	week	int	gsh	crt	ure	ggt
1	H	IV	w4	576	74	52.07	2.36	36.64
1	H	IV	w5	853	85	64.27	2.27	32.46
1	H	IV	w6	924	80	50.74	2.72	32.46
1	H	IV	w7	900	75	32.62	2.38	35.45
1	H	IV	w8	939	86	48.62	2.84	34.17
1	H	IV	w9	1023	90	54.54	3.52	36.60
1	H	IV	w10	1053	91	57.28	3.00	36.36
1	H	IV	w11	1036	93	55.69	3.22	39.08
1	H	IV	w12	1036	91	42.52	2.98	36.74
2	C	I	w4	1550	109	76.91	4.27	28.07
2	C	I	w5	1472	112	65.77	5.78	26.67
2	C	I	w6	1578	121	61.44	4.17	25.70
2	C	I	w7	1620	104	39.87	3.97	26.75
2	C	I	w8	1667	116	70.19	3.57	27.62
2	C	I	w9	1780	113	45.08	4.55	29.14
2	C	I	w10	1717	119	66.92	4.46	29.74
2	C	I	w11	1853	122	59.32	4.62	29.87
2	C	I	w12	1858	115	49.06	5.32	29.56
3	L	I	w4	1082	115	64.36	2.19	27.03
3	L	I	w5	1204	110	59.94	3.85	32.04
3	L	I	w6	1372	110	62.32	4.08	27.63
3	L	I	w7	1160	97	34.39	3.70	30.56
3	L	I	w8	1203	102	67.54	2.35	30.49
3	L	I	w9	1581	109	56.75	3.91	30.99
3	L	I	w10	2023	110	*	3.95	30.67
3	L	I	w11	1466	115	53.75	4.70	28.91
3	L	I	w12	1847	116	93.44	*	29.56
4	C	VI	w4	*	*	*	*	*
4	C	VI	w5	*	*	*	*	*
4	C	VI	w6	*	*	*	*	*
4	C	VI	w7	*	*	*	*	*
4	C	VI	w8	*	*	*	*	*
4	C	VI	w9	*	*	*	*	*
4	C	VI	w10	*	*	*	*	*
4	C	VI	w11	*	*	*	*	*
4	C	VI	w12	*	*	*	*	*
5	L	VII	w4	563	72	68.95	3.60	30.24
5	L	VII	w5	620	67	66.39	2.35	30.63
5	L	VII	w6	769	70	64.09	3.26	30.23
5	L	VII	w7	574	60	65.59	4.71	37.07
5	L	VII	w8	735	64	59.76	2.69	37.26
5	L	VII	w9	888	54	54.37	3.50	34.68
5	L	VII	w10	888	58	58.52	3.58	32.99
5	L	VII	w11	1016	65	54.37	3.45	34.26
5	L	VII	w12	996	58	80.45	2.93	34.03
6	H	VI	w4	492	107	65.77	5.38	31.68
6	H	VI	w5	605	82	61.35	1.99	33.64
6	H	VI	w6	1083	80	71.43	3.46	31.77
6	H	VI	w7	1094	91	65.24	3.55	35.18
6	H	VI	w8	1143	99	76.91	2.58	34.43
6	H	VI	w9	1254	97	54.10	4.07	35.93
6	H	VI	w10	1288	98	67.01	3.52	34.44
6	H	VI	w11	1305	99	53.75	3.97	35.32
6	H	VI	w12	1279	96	80.27	4.41	33.84

Appendix 5.c continued

animal	treatm	block	week	int	gsh	crt	ure	ggt
7	C	III	w4	1051	84	65.15	2.82	26.47
7	C	III	w5	1102	91	46.06	2.39	24.94
7	C	III	w6	1247	83	68.42	2.81	25.06
7	C	III	w7	1288	78	61.00	2.55	28.41
7	C	III	w8	1379	80	66.83	2.73	28.38
7	C	III	w9	1620	87	52.86	*	28.26
7	C	III	w10	1544	93	48.09	2.82	26.71
7	C	III	w11	1575	106	58.96	4.21	27.92
7	C	III	w12	1628	98	70.10	3.09	27.63
8	L	IV	w4	*	*	*	*	*
8	L	IV	w5	*	*	*	*	*
8	L	IV	w6	*	*	*	*	*
8	L	IV	w7	*	*	*	*	*
8	L	IV	w8	*	*	*	*	*
8	L	IV	w9	*	*	*	*	*
8	L	IV	w10	*	*	*	*	*
8	L	IV	w11	*	*	*	*	*
8	L	IV	w12	*	*	*	*	*
9	C	V	w4	211	86	56.40	4.70	26.67
9	C	V	w5	565	88	47.21	*	28.51
9	C	V	w6	1027	89	64.98	1.87	31.36
9	C	V	w7	849	73	49.15	2.97	35.33
9	C	V	w8	784	80	67.72	2.51	33.61
9	C	V	w9	862	82	50.57	3.32	33.89
9	C	V	w10	1124	82	60.38	2.60	32.80
9	C	V	w11	1251	88	63.74	3.38	30.41
9	C	V	w12	1470	91	51.54	3.53	31.64
10	C	VII	w4	*	*	*	*	*
10	C	VII	w5	*	*	*	*	*
10	C	VII	w6	*	*	*	*	*
10	C	VII	w7	*	*	*	*	*
10	C	VII	w8	*	*	*	*	*
10	C	VII	w9	*	*	*	*	*
10	C	VII	w10	*	*	*	*	*
10	C	VII	w11	*	*	*	*	*
10	C	VII	w12	*	*	*	*	*
11	L	V	w4	449	82	64.00	4.59	29.67
11	L	V	w5	661	76	58.96	3.11	30.61
11	L	V	w6	739	79	81.60	3.46	29.79
11	L	V	w7	839	70	53.13	2.92	32.71
11	L	V	w8	947	82	67.45	2.64	33.00
11	L	V	w9	1077	82	61.00	2.77	32.47
11	L	V	w10	1038	87	74.70	3.23	31.83
11	L	V	w11	1058	93	55.07	3.31	31.17
11	L	V	w12	1131	96	67.54	2.73	31.39
12	L	III	w4	514	110	68.25	6.05	19.02
12	L	III	w5	491	101	50.48	2.90	22.28
12	L	III	w6	648	108	72.75	2.10	20.11
12	L	III	w7	763	92	53.57	2.42	23.93
12	L	III	w8	490	102	77.97	3.09	24.94
12	L	III	w9	720	94	64.80	2.73	24.09
12	L	III	w10	763	106	73.64	2.50	21.79
12	L	III	w11	520	106	77.44	2.50	21.35
12	L	III	w12	928	103	69.40	2.78	23.05

Appendix 5.c continued

animal	treatm	block	week	int	gsh	crt	ure	ggt
13	C	IV	w4	1339	102	59.94	4.67	26.55
13	C	IV	w5	1387	96	56.75	4.93	26.23
13	C	IV	w6	1584	106	63.12	5.22	24.98
13	C	IV	w7	1218	101	56.40	4.26	27.74
13	C	IV	w8	354	105	59.76	3.83	28.97
13	C	IV	w9	699	99	62.15	2.89	28.45
13	C	IV	w10	671	99	76.82	3.23	29.99
13	C	IV	w11	753	98	68.60	2.95	30.19
13	C	IV	w12	1197	94	62.06	3.02	29.49
14	L	II	w4	1062	112	59.94	4.41	25.97
14	L	II	w5	1232	118	58.08	5.95	24.88
14	L	II	w6	1229	122	70.99	4.65	24.58
14	L	II	w7	1275	111	46.15	4.24	27.01
14	L	II	w8	1265	123	64.80	3.50	27.45
14	L	II	w9	1390	118	51.19	5.02	28.09
14	L	II	w10	1472	127	59.49	4.18	28.42
14	L	II	w11	1538	126	58.88	5.87	28.17
14	L	II	w12	1495	119	58.52	3.86	27.79
15	H	VII	w4	669	78	56.67	3.11	31.81
15	H	VII	w5	852	80	57.73	2.55	33.19
15	H	VII	w6	787	81	70.54	2.92	35.27
15	H	VII	w7	880	71	50.92	2.52	39.42
15	H	VII	w8	1114	86	65.59	3.91	37.75
15	H	VII	w9	1182	89	59.58	5.01	36.28
15	H	VII	w10	1315	89	62.68	2.94	36.96
15	H	VII	w11	1348	91	64.89	3.96	38.30
15	H	VII	w12	1420	98	55.78	3.41	37.30
16	H	III	w4	897	81	51.72	3.16	23.84
16	H	III	w5	976	83	56.67	2.91	24.05
16	H	III	w6	1226	87	67.80	2.03	23.60
16	H	III	w7	1237	75	42.70	2.57	24.68
16	H	III	w8	1227	85	68.33	3.97	26.49
16	H	III	w9	1353	86	50.48	4.66	25.29
16	H	III	w10	1392	95	67.63	3.62	25.57
16	H	III	w11	829	*	*	*	*
16	H	III	w12	509	*	*	*	*
17	L	VI	w4	557	94	60.02	3.63	30.95
17	L	VI	w5	613	94	73.99	2.91	29.31
17	L	VI	w6	805	90	69.31	1.81	30.59
17	L	VI	w7	824	82	52.60	3.10	30.65
17	L	VI	w8	909	88	74.88	2.42	31.47
17	L	VI	w9	978	89	57.02	3.32	34.35
17	L	VI	w10	1005	96	71.69	2.65	34.57
17	L	VI	w11	1028	103	66.04	3.41	33.12
17	L	VI	w12	982	99	77.09	2.54	29.72
18	H	V	w4	*	*	*	*	*
18	H	V	w5	*	*	*	*	*
18	H	V	w6	*	*	*	*	*
18	H	V	w7	*	*	*	*	*
18	H	V	w8	*	*	*	*	*
18	H	V	w9	*	*	*	*	*
18	H	V	w10	*	*	*	*	*
18	H	V	w11	*	*	*	*	*
18	H	V	w12	*	*	*	*	*

Appendix Table 5.c continued

animal	treatm	block	week	int	gsh	crt	ure	ggt
19	H	I	w4	1109	102	61.97	3.02	30.19
19	H	I	w5	1254	106	50.65	3.03	31.20
19	H	I	w6	1370	111	45.44	2.34	33.42
19	H	I	w7	1421	104	46.76	2.90	33.47
19	H	I	w8	1389	116	71.07	2.80	33.01
19	H	I	w9	1419	106	54.46	3.96	35.04
19	H	I	w10	1521	115	48.00	2.87	32.20
19	H	I	w11	1568	121	50.39	3.67	33.29
19	H	I	w12	1535	114	54.81	3.11	33.85
20	H	II	w4	1180	89	58.52	3.63	30.71
20	H	II	w5	1207	97	45.79	3.88	29.76
20	H	II	w6	1237	94	30.85	3.03	32.08
20	H	II	w7	1232	91	40.22	4.16	34.62
20	H	II	w8	1221	96	61.26	4.38	34.38
20	H	II	w9	1247	96	51.45	4.14	35.00
20	H	II	w10	1359	96	50.92	4.27	35.62
20	H	II	w11	1383	101	42.26	4.98	37.71
20	H	II	w12	1325	96	61.71	4.05	35.90
21	C	II	w4	960	92	55.16	2.18	26.90
21	C	II	w5	977	96	58.08	4.22	25.80
21	C	II	w6	1069	96	37.39	2.91	26.91
21	C	II	w7	1169	116	37.75	2.81	28.35
21	C	II	w8	1282	110	50.21	2.38	26.70
21	C	II	w9	1410	108	45.62	2.75	29.34
21	C	II	w10	1635	110	47.83	2.79	28.78
21	C	II	w11	1559	117	39.96	3.54	29.40
21	C	II	w12	1751	115	57.81	3.28	28.27

Key

int = Voluntary Food Intake (g DM/d)
gsh = Blood glutathione concentration (g/100 ml RBC)
crt = Plasma creatinine concentration (umol/l)
ure = Plasma Urea concentration (mmol/l)
ggt = Plasma GGTP concentration (IU/l)
* = missing value

Appendix 5.d - Individual data values from Experiment 3 ; tissue data

animal	treatm	block	animwt	kidwt	livrwt	kid-gsh	livr-gsh	kid-cytox	livr-cytox
1	H	IV	36.5	3.85	19.69	222.8	431.6	13.92	6.93
2	C	I	55.5	3.25	20.99	281.3	837.1	15.26	13.43
3	L	I	54.5	3.44	19.14	392.9	954.4	29.55	14.29
4	C	VI	*	*	*	*	*	*	*
5	L	VII	39.0	3.77	19.71	372.5	487.8	28.86	16.78
6	H	VI	48.0	3.34	18.78	327.1	952.0	21.42	9.08
7	C	III	49.5	3.67	18.71	366.9	514.1	21.69	12.66
8	L	IV	*	*	*	*	*	*	*
9	C	V	39.0	4.32	18.99	279.8	669.6	17.56	14.74
10	C	VII	*	*	*	*	*	*	*
11	L	V	43.5	4.06	17.04	368.6	1210.2	15.31	10.45
12	L	III	35.5	3.52	19.96	275.3	600.7	23.17	15.99
13	C	IV	42.5	3.68	17.26	348.6	845.1	13.77	8.53
14	L	II	50.0	3.41	17.73	235.8	841.8	16.83	10.44
15	H	VII	41.5	3.88	20.60	345.3	928.5	11.53	8.67
16	H	III	*	*	*	*	*	*	*
17	L	VI	38.5	3.61	19.99	354.6	394.7	21.86	9.59
18	H	V	*	*	*	*	*	*	*
19	H	I	48.5	3.44	22.60	427.1	876.7	28.34	8.31
20	H	II	46.0	3.73	18.59	342.4	812.3	17.42	9.38
21	C	II	49.0	3.92	19.78	315.9	701.2	21.06	12.97

Key

animwt = Liveweight of animal (kg)
kidwt = Fresh weight of kidney (g/kg W)
livrwt = Fresh weight of liver (g/kg W)
kidgsh = Kidney glutathione conc. (mg/100g)
livrgsh= Liver glutathione conc. (mg/100g)
kidcytox= Kidney cytochrome oxidase activity (delta log[ferrocytochrome c]/min for 1:100 tissue dilution)
livrcytox= Liver cytochrome oxidase activity (delta log[ferrocytochrome c]/min for 1:100 tissue dilution)

(delta log[ferrocytochrome c]/min for 1:100 tissue dilution)

Appendix 6.a - Individual ACN concentration data values from Experiment 4.b (umol/ml)

treatm	time	pretr	day1	day6	day8	day15	day22	day29	posttr
cont0	0	0.564	0.553	0.579	0.487	0.580	0.473	0.463	0.555
cont0	0	0.504	0.533	0.491	0.435	0.545	0.470	0.468	0.519
cont0	0	0.561	0.565	0.492	0.421	0.496	*	0.410	0.506
cont300	300	0.454	0.422	0.459	0.482	0.424	0.406	0.417	0.447
cont300	300	0.571	0.430	0.458	0.488	0.424	0.486	0.464	0.450
cont300	300	0.488	0.424	0.444	0.498	0.395	0.406	0.419	0.434
CA	0	0.530	0.552	0.594	0.561	0.626	0.454	0.527	0.541
CA	0	0.594	0.520	0.644	0.573	0.559	0.511	0.650	0.606
CA	0	*	0.516	0.517	0.527	0.556	0.484	*	0.534
CA	15	0.609	0.507	0.471	0.412	0.428	0.377	0.476	0.532
CA	15	0.827	0.484	0.455	0.469	0.460	0.343	0.443	0.568
CA	15	0.647	0.498	0.431	0.520	0.475	0.323	0.424	0.476
CA	60	0.588	0.585	0.365	0.430	0.386	0.310	0.396	0.550
CA	60	0.644	0.488	0.390	0.538	0.321	0.332	0.361	0.506
CA	60	0.594	0.494	0.385	0.401	0.322	0.322	0.357	0.531
CA	120	0.686	0.428	0.268	0.406	0.284	0.262	*	*
CA	120	0.617	0.440	0.312	0.378	0.282	0.272	0.322	0.547
CA	120	0.576	0.471	0.311	0.371	0.254	0.269	0.348	0.522
CA	300	0.629	0.343	0.100	0.246	0.146	0.201	0.283	0.512
CA	300	0.640	0.328	0.091	0.287	0.152	0.187	0.235	0.517
CA	300	0.579	0.327	0.095	0.282	*	0.179	0.285	0.471
DG	0	0.571	0.542	0.592	0.628	0.664	0.528	0.471	0.540
DG	0	0.622	0.589	0.649	0.567	0.591	0.535	0.528	0.551
DG	0	*	0.504	0.568	0.528	0.555	0.498	0.493	0.548
DG	15	0.509	0.536	0.533	0.523	0.679	0.473	0.475	0.604
DG	15	0.585	0.638	0.591	0.486	0.729	0.558	0.486	0.574
DG	15	0.537	0.596	0.552	0.493	0.588	0.460	0.436	0.561
DG	60	0.558	0.553	0.704	0.686	0.494	0.519	0.554	0.545
DG	60	0.540	0.557	0.618	0.580	0.501	0.504	0.520	0.605
DG	60	0.576	0.509	0.527	0.502	0.476	0.447	*	0.542
DG	120	0.564	0.519	0.630	0.576	0.441	0.454	0.476	0.562
DG	120	0.539	*	0.582	*	0.477	0.476	0.449	0.496
DG	120	0.508	0.507	0.699	0.479	0.530	0.461	0.476	0.504
DG	300	0.578	0.463	*	0.568	0.427	0.435	0.415	0.511
DG	300	0.523	0.483	0.520	0.535	0.436	0.498	0.432	0.478
DG	300	0.534	0.483	0.558	0.502	0.473	0.508	0.476	0.518

Key

treatm	=	Treatment
time	=	Incubation time (minutes)
pretr	=	Pre-treatment
posttr	=	Post-treatment
cont0	=	Buffer samples at 0 minutes
cont300	=	Buffer samples at 300 minutes
CA	=	Cabbage treatment
DG	=	Dried grass treatment
*	=	Missing value

Appendix 6.b - Individual AITC concentration data values from Experiment 4.b (umol/ml)

treatm	time	pretr	day1	day6	day8	day15	day22	day29	posttr
cont0	0	0.201	0.162	0.180	0.215	0.196	*	0.171	0.289
cont0	0	0.194	0.166	0.145	0.171	0.191	0.146	0.179	0.271
cont0	0	0.221	0.189	0.181	0.163	0.189	*	0.120	0.201
cont300	300	0.097	0.109	0.062	0.124	0.073	0.066	0.060	0.078
cont300	300	0.143	0.112	0.077	0.156	0.070	0.139	0.074	0.036
cont300	300	0.116	0.133	0.078	0.160	0.063	0.071	0.069	0.074
CA	0	0.103	0.024	0.006	0.120	0.016	0.008	0.009	0.132
CA	0	0.133	0.013	0.011	0.040	0.007	0.008	0.022	0.124
CA	0	*	0.015	0.000	0.036	0.008	0.008	0.065	*
CA	15	0.008	0.007	0.009	0.056	0.008	0.013	0.000	0.071
CA	15	0.018	0.056	0.008	0.027	0.000	0.014	0.006	0.094
CA	15	0.009	0.017	0.012	0.014	0.000	0.005	0.023	0.052
CA	60	0.004	0.013	0.009	0.047	0.000	0.000	0.027	0.042
CA	60	0.000	0.027	0.005	0.019	0.046	0.013	0.028	0.043
CA	60	0.006	0.011	0.007	0.012	0.022	0.015	0.030	0.064
CA	120	0.005	0.047	0.000	0.016	0.007	0.005	0.005	0.023
CA	120	0.004	0.004	0.000	0.036	0.000	0.017	0.011	0.010
CA	120	0.008	0.008	0.000	0.007	0.011	0.009	0.020	0.000
CA	300	0.043	0.022	0.000	0.035	0.000	0.008	0.015	0.011
CA	300	0.128	0.040	0.009	0.007	0.000	0.026	0.016	0.005
CA	300	0.007	0.023	0.000	0.030	*	0.011	0.023	0.022
DG	0	0.116	0.100	0.094	0.148	0.148	0.120	0.065	0.042
DG	0	0.098	0.104	0.115	0.128	0.140	0.139	0.101	0.135
DG	0	*	0.087	0.099	0.145	0.112	0.125	0.080	0.160
DG	15	0.005	0.023	0.013	0.028	0.029	0.011	0.011	0.021
DG	15	0.008	0.029	0.014	0.030	0.000	0.011	0.009	0.047
DG	15	0.047	0.031	0.006	0.015	0.000	0.008	0.005	0.070
DG	60	0.006	0.032	0.008	0.092	0.005	0.009	0.039	0.081
DG	60	0.000	0.015	0.014	0.027	0.013	0.010	0.006	0.140
DG	60	0.006	0.011	0.000	0.013	0.000	0.009	0.000	0.129
DG	120	0.004	0.007	0.006	0.080	0.000	0.023	0.015	0.018
DG	120	0.027	*	0.000	*	0.000	0.000	0.010	0.009
DG	120	0.029	0.037	0.000	0.009	0.000	0.013	0.007	0.007
DG	300	0.007	0.061	*	0.022	0.008	0.007	0.011	0.008
DG	300	0.005	0.000	0.006	0.049	0.009	0.009	0.006	0.013
DG	300	0.000	0.020	0.010	0.012	0.010	0.007	0.005	0.030

Key

time	=	Incubation time (minutes)
pretr	=	Pre-treatment
posttr	=	Post-treatment
cont0	=	Buffer samples at 0 minutes
cont300	=	Buffer samples at 300 minutes
CA	=	Cabbage treatment
DG	=	Dried grass treatment
*	=	Missing value

Appendix 7.a Sample ANOVA Table from Experiment 5.

***** Analysis of variance *****

Variate: Voluntary food intake (g DM /d)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
block stratum	4	9723684.	2430921.		
block.anim stratum					
acn	1	1287643.	1287643.	7.59	0.010
aitc	1	4403829.	4403829.	25.95	<.001
dmds	1	177288.	177288.	1.04	0.315
acn.aitc	1	588797.	588797.	3.47	0.073
acn.dmds	1	982715.	982715.	5.79	0.023
aitc.dmds	1	64319.	64319.	0.38	0.543
acn.aitc.dmds	1	324768.	324768.	1.91	0.177
Residual	28	4752013.	169715.		
block.anim.week stratum					
week	4	480921.	120230.	5.01	<.001
week.acn	4	375022.	93756.	3.91	0.005
week.aitc	4	1358422.	339605.	14.16	<.001
week.dmds	4	80454.	20113.	0.84	0.503
week.acn.aitc	4	179936.	44984.	1.88	0.119
week.acn.dmds	4	167362.	41840.	1.74	0.144
week.aitc.dmds	4	218244.	54561.	2.28	0.065
Residual	125(7)	2997318.	23979.		
Total	192(7)	26899388.			

Appendix 7.b - Allocation of animals to blocks and treatments in Experiment 5.

Animal number	Pre-treatment VFI (g DM/d)	Block	Treatment factors present		
38	2398.71	1	ACN	AITC	DMDS
35	2221.06	1			DMDS
5	2199.58	1			
3	2187.71	1	ACN		
33	2166.33	1	ACN	AITC	
19	2164.90	1		AITC	
8	2163.88	1		AITC	DMDS
18	2151.77	1	ACN		DMDS
25	2150.85	2	ACN		DMDS
36	2140.94	2		AITC	
13	2059.93	2	ACN	AITC	DMDS
23	2049.61	2			DMDS
12	2048.87	2			
30	2031.62	2		AITC	DMDS
34	2030.78	2	ACN	AITC	
14	2010.17	2	ACN		
16	1995.02	3	ACN		
1	1980.98	3		AITC	DMDS
32	1975.03	3		AITC	
27	1974.67	3			
11	1942.46	3			DMDS
2	1941.67	3	ACN	AITC	DMDS
37	1941.61	3	ACN		DMDS
15	1924.55	3	ACN	AITC	
28	1924.51	4			
24	1897.50	4		AITC	DMDS
22	1896.58	4		AITC	
6	1849.71	4	ACN	AITC	DMDS
39	1849.28	4			DMDS
7	1835.28	4	ACN	AITC	
21	1815.04	4	ACN		DMDS
29	1799.40	4	ACN		
9	1798.78	5		AITC	
31	1768.64	5			
26	1750.24	5		AITC	DMDS
4	1709.47	5			DMDS
10	1698.77	5	ACN		DMDS
20	1620.24	5	ACN		
17	1596.59	5	ACN	AITC	DMDS
40	1566.21	5	ACN	AITC	

Key

ACN = Allyl cyanide
AITC = Allyl isothiocyanate
DMDS = Dimethyl disulphide

Appendix 7c - Stability of a mixture of DMDS, ACN and AITC over time (units = peak area as a proportion of internal standard peak area)

Time (h)	DMDS	ACN	AITC
0	0.871	0.800	0.890
4	0.858	0.807	0.906
69	0.806	0.791	0.901
123	0.827	0.765	0.883
144	0.881	0.792	0.894

Appendix 7.d - Individual data values from Experiment 7 for voluntary food intake, blood reduced glutathione, blood haemoglobin concentration, blood PCV, Heinz body count and plasma tri-iodothyronine concentrations.

		treatment									
anim	bl	acn	aitc	dmds	wk	int	gsh	hbn	pcv	loghnz	t3
1	3	0	1	1	1	1981	111.3	11.10	31.0	0.262	2.78
1	3	0	1	1	2	1071	79.7	11.80	34.0	0.800	1.77
1	3	0	1	1	3	1558	69.7	12.30	32.0	1.055	1.53
1	3	0	1	1	4	1560	67.1	11.80	31.0	1.186	0.77
1	3	0	1	1	5	1352	80.0	11.50	32.0	1.138	2.37
2	3	1	1	1	1	1942	104.6	12.00	33.0	0.004	6.68
2	3	1	1	1	2	*	*	*	*	*	*
2	3	1	1	1	3	*	*	*	*	*	*
2	3	1	1	1	4	*	*	*	*	*	*
2	3	1	1	1	5	*	*	*	*	*	*
3	1	1	0	0	1	2188	91.8	12.50	34.0	0.248	2.52
3	1	1	0	0	2	2456	37.1	12.00	31.5	0.465	1.53
3	1	1	0	0	3	2597	27.0	10.70	28.5	0.721	1.74
3	1	1	0	0	4	2481	22.8	11.00	29.0	0.233	1.71
3	1	1	0	0	5	2493	28.0	11.60	32.5	0.130	2.28
4	5	0	0	1	1	1663	98.9	13.30	37.0	0.889	2.67
4	5	0	0	1	2	1822	111.8	13.40	34.0	1.227	5.78
4	5	0	0	1	3	1956	122.0	13.50	35.0	1.030	1.99
4	5	0	0	1	4	1948	91.6	13.30	35.5	1.027	2.73
4	5	0	0	1	5	1942	98.1	13.60	37.0	1.188	3.16
5	1	0	0	0	1	2200	111.3	11.90	31.0	0.336	3.83
5	1	0	0	0	2	2418	97.6	11.60	29.0	0.358	1.30
5	1	0	0	0	3	2667	76.7	12.30	33.0	0.360	1.95
5	1	0	0	0	4	2533	65.3	10.40	30.5	0.258	2.38
5	1	0	0	0	5	2484	79.6	9.60	25.5	0.772	1.66
6	4	1	1	1	1	1749	21.8	14.60	38.0	0.580	3.69
6	4	1	1	1	2	1539	28.3	13.40	35.0	1.313	3.36
6	4	1	1	1	3	1747	36.5	11.90	31.0	1.635	3.17
6	4	1	1	1	4	1864	33.2	10.20	29.5	1.093	3.23
6	4	1	1	1	5	1685	50.4	9.60	28.0	1.485	2.22
7	4	1	1	0	1	1835	90.4	14.30	38.5	0.430	1.50
7	4	1	1	0	2	1696	35.4	15.00	39.0	0.522	1.60
7	4	1	1	0	3	1386	33.9	14.20	34.5	0.587	1.57
7	4	1	1	0	4	1494	14.0	13.40	35.0	0.674	1.42
7	4	1	1	0	5	1621	19.7	12.60	33.0	0.584	2.38
8	1	0	1	1	1	2164	80.6	12.40	34.5	0.369	5.92
8	1	0	1	1	2	2133	64.2	12.50	31.0	1.206	2.14
8	1	0	1	1	3	2195	54.7	11.60	30.5	1.349	2.34
8	1	0	1	1	4	2085	46.0	11.00	30.0	1.281	1.69
8	1	0	1	1	5	2231	50.7	11.10	30.0	1.267	1.82
9	5	0	1	0	1	1799	108.2	11.30	33.0	0.526	3.72
9	5	0	1	0	2	2129	80.6	12.70	32.5	0.522	1.61
9	5	0	1	0	3	2182	63.6	13.20	33.0	0.403	1.91
9	5	0	1	0	4	2104	45.6	12.30	34.0	0.465	1.86
9	5	0	1	0	5	2133	52.0	11.70	32.5	0.396	1.79
10	5	1	0	1	1	1699	116.1	11.20	30.5	0.655	3.04
10	5	1	0	1	2	1940	66.3	11.40	30.0	1.158	2.26
10	5	1	0	1	3	2072	50.7	11.70	29.0	1.369	3.89
10	5	1	0	1	4	2118	56.2	10.50	26.0	1.286	4.35
10	5	1	0	1	5	2313	63.6	10.80	28.0	1.514	3.66

Appendix 7.d continued

anim	bl	acn	aitc	dmds	wk	int	gsh	hbn	pcv	loghnz	t3
11	3	0	0	1	1	1942	116.5	13.10	34.5	0.627	2.93
11	3	0	0	1	2	2243	109.6	13.40	35.5	0.827	2.76
11	3	0	0	1	3	2333	108.1	13.40	34.5	1.130	2.14
11	3	0	0	1	4	2153	101.2	11.80	34.0	0.841	2.00
11	3	0	0	1	5	2306	116.0	12.60	35.0	0.957	2.69
12	2	0	0	0	1	2049	113.7	11.40	33.5	0.207	3.06
12	2	0	0	0	2	1546	93.0	11.70	31.5	0.243	2.69
12	2	0	0	0	3	2465	88.7	12.30	33.5	0.543	1.69
12	2	0	0	0	4	2437	70.6	11.40	33.0	0.666	3.69
12	2	0	0	0	5	2545	89.7	11.70	32.0	0.805	2.29
13	2	1	1	1	1	2060	109.1	12.00	33.0	0.334	2.31
13	2	1	1	1	2	2237	72.3	11.70	30.0	1.042	2.68
13	2	1	1	1	3	2090	54.8	11.30	31.0	1.221	2.11
13	2	1	1	1	4	2082	45.2	11.00	30.5	1.331	4.04
13	2	1	1	1	5	2176	57.5	10.40	28.5	1.070	3.99
14	2	1	0	0	1	2010	119.1	11.00	31.5	0.134	3.26
14	2	1	0	0	2	2307	69.7	11.10	29.0	0.230	2.93
14	2	1	0	0	3	2447	45.7	10.80	30.0	0.401	3.05
14	2	1	0	0	4	2487	37.8	10.00	27.0	0.589	2.70
14	2	1	0	0	5	2981	42.8	10.60	29.0	0.561	1.70
15	3	1	1	0	1	1925	107.5	13.10	38.5	0.004	2.75
15	3	1	1	0	2	1553	46.8	13.40	34.0	0.241	5.82
15	3	1	1	0	3	*	20.0	12.50	35.0	0.340	1.61
15	3	1	1	0	4	*	*	*	*	*	*
15	3	1	1	0	5	*	*	*	*	*	*
16	3	1	0	0	1	1995	115.4	11.60	33.0	0.438	3.03
16	3	1	0	0	2	1271	48.1	12.80	32.0	0.004	3.38
16	3	1	0	0	3	1942	33.5	10.80	29.0	0.320	4.10
16	3	1	0	0	4	1739	25.7	11.50	30.0	0.854	5.57
16	3	1	0	0	5	1762	32.5	10.70	29.5	0.473	2.28
17	5	1	1	1	1	1597	116.8	13.80	38.0	0.654	5.20
17	5	1	1	1	2	1246	54.7	14.90	38.0	1.085	2.76
17	5	1	1	1	3	1034	41.3	11.90	31.5	1.520	4.65
17	5	1	1	1	4	823	34.9	11.90	27.5	1.760	2.93
17	5	1	1	1	5	524	44.9	9.60	26.5	1.499	2.29
18	1	1	0	1	1	2152	121.0	10.80	30.0	0.004	1.91
18	1	1	0	1	2	2386	73.9	12.50	31.0	0.497	2.48
18	1	1	0	1	3	2284	54.2	11.00	29.5	0.614	4.17
18	1	1	0	1	4	2393	44.0	10.40	27.5	1.207	1.95
18	1	1	0	1	5	2551	49.5	11.30	28.5	0.262	2.19
19	1	0	1	0	1	2165	123.9	11.50	30.5	0.330	2.81
19	1	0	1	0	2	2269	80.3	12.00	31.5	0.531	2.59
19	1	0	1	0	3	2100	65.6	11.20	30.5	0.629	2.42
19	1	0	1	0	4	2151	52.5	11.20	30.5	0.425	2.88
19	1	0	1	0	5	2373	57.4	11.30	31.0	0.258	2.23
20	5	1	0	0	1	1732	117.6	13.30	38.0	0.588	4.85
20	5	1	0	0	2	1887	52.1	13.20	34.0	0.695	6.39
20	5	1	0	0	3	2057	28.6	11.50	31.5	0.648	6.19
20	5	1	0	0	4	1978	22.9	11.90	31.0	0.117	3.84
20	5	1	0	0	5	2119	29.3	11.10	30.0	0.567	5.93
21	4	1	0	1	1	1815	128.4	13.10	35.5	0.459	4.51
21	4	1	0	1	2	2058	88.9	13.20	36.0	1.102	3.89
21	4	1	0	1	3	2025	75.8	12.10	33.0	1.135	5.11
21	4	1	0	1	4	1924	71.0	12.20	33.5	1.043	3.17
21	4	1	0	1	5	2035	81.0	12.00	31.0	1.260	4.62

Appendix 7.d continued

anim	bl	acn	aitc	dmds	wk	int	gsh	hbn	pcv	loghnz	t3
22	4	0	1	0	1	1897	121.0	10.70	30.5	0.458	3.33
22	4	0	1	0	2	2065	82.9	12.50	31.0	0.155	2.26
22	4	0	1	0	3	2175	63.3	10.60	30.5	0.435	2.56
22	4	0	1	0	4	2191	58.2	10.10	27.5	0.790	1.18
22	4	0	1	0	5	2305	45.5	9.60	36.5	0.551	1.86
23	2	0	0	1	1	2050	122.6	12.00	34.5	0.403	3.25
23	2	0	0	1	2	2212	105.8	13.20	34.5	0.841	2.18
23	2	0	0	1	3	2361	102.2	11.60	32.0	1.021	4.86
23	2	0	0	1	4	2441	98.7	10.80	31.5	0.782	2.98
23	2	0	0	1	5	2339	104.4	10.60	31.5	0.708	2.35
24	4	0	1	1	1	1898	117.4	12.40	35.0	0.215	4.96
24	4	0	1	1	2	1863	93.3	12.70	33.0	1.078	3.13
24	4	0	1	1	3	1962	80.0	10.80	30.0	1.207	2.72
24	4	0	1	1	4	1840	71.6	10.70	31.0	0.938	1.97
24	4	0	1	1	5	1774	81.5	8.90	27.0	0.840	2.31
25	2	1	0	1	1	2151	126.7	10.70	31.5	0.238	5.01
25	2	1	0	1	2	2582	93.3	12.10	33.0	0.585	4.57
25	2	1	0	1	3	2526	56.1	10.30	28.5	0.785	3.73
25	2	1	0	1	4	2492	54.2	10.60	29.5	0.410	5.50
25	2	1	0	1	5	2650	62.9	11.80	35.0	0.533	4.20
26	5	0	1	1	1	1750	146.1	12.20	32.5	0.425	4.72
26	5	0	1	1	2	1656	125.2	12.40	33.0	0.512	3.63
26	5	0	1	1	3	1554	115.2	11.50	33.0	0.614	3.00
26	5	0	1	1	4	1767	112.9	11.80	31.0	1.166	4.18
26	5	0	1	1	5	1941	116.2	12.00	31.5	1.121	4.32
27	3	0	0	0	1	1975	138.2	11.60	33.5	0.004	5.70
27	3	0	0	0	2	2229	118.3	12.70	36.0	0.243	4.05
27	3	0	0	0	3	2394	99.4	11.90	34.5	0.666	1.91
27	3	0	0	0	4	2283	84.0	11.50	35.0	0.817	6.23
27	3	0	0	0	5	2271	99.7	12.80	34.5	0.630	4.68
28	4	0	0	0	1	1925	111.9	11.00	33.5	0.004	6.24
28	4	0	0	0	2	2338	83.9	10.60	33.0	0.297	3.85
28	4	0	0	0	3	2477	79.1	11.60	32.5	0.146	4.07
28	4	0	0	0	4	2526	72.8	11.30	32.0	0.004	3.39
28	4	0	0	0	5	2610	81.9	11.80	32.0	0.318	3.29
29	4	1	0	0	1	1799	111.6	10.70	32.0	0.360	2.45
29	4	1	0	0	2	2269	75.5	11.00	33.5	0.420	3.85
29	4	1	0	0	3	2085	53.0	11.40	31.5	0.270	3.72
29	4	1	0	0	4	2049	48.4	11.20	32.0	0.417	3.75
29	4	1	0	0	5	2135	57.2	11.70	32.5	0.423	3.78
30	2	0	1	1	1	2032	109.6	11.90	34.5	0.004	3.38
30	2	0	1	1	2	2070	97.6	13.30	38.0	0.299	5.14
30	2	0	1	1	3	1947	90.6	12.10	35.0	0.577	4.59
30	2	0	1	1	4	1877	80.3	11.30	33.5	0.624	2.82
30	2	0	1	1	5	2094	92.9	11.70	34.0	0.830	4.47
31	5	0	0	0	1	1769	106.7	11.60	31.5	0.494	4.09
31	5	0	0	0	2	1837	92.8	10.70	33.5	0.348	2.22
31	5	0	0	0	3	1967	79.1	11.10	32.5	0.502	3.18
31	5	0	0	0	4	2056	74.2	11.20	31.0	0.367	2.78
31	5	0	0	0	5	2054	82.7	11.30	30.0	0.783	3.18
32	3	0	1	0	1	1975	93.1	11.60	33.5	0.127	5.42
32	3	0	1	0	2	2122	81.5	12.40	34.0	0.155	4.17
32	3	0	1	0	3	2073	74.0	11.60	31.5	0.290	2.90
32	3	0	1	0	4	2101	60.7	11.20	31.0	0.297	2.95
32	3	0	1	0	5	2184	64.4	11.40	32.0	0.579	2.75

Appendix 7.d continued

anim	bl	acn	aitc	dmds	wk	int	gsh	hbn	pcv	loghnz	t3
33	1	1	1	0	1	2166	99.7	11.90	34.0	0.594	4.47
33	1	1	1	0	2	2386	57.7	13.30	35.0	1.015	8.67
33	1	1	1	0	3	2236	37.8	12.10	32.5	0.863	5.30
33	1	1	1	0	4	2209	29.4	11.20	31.0	0.877	5.60
33	1	1	1	0	5	2206	29.6	12.80	34.5	0.354	5.45
34	2	1	1	0	1	2031	89.2	12.60	37.0	0.199	4.60
34	2	1	1	0	2	2003	35.7	12.80	37.0	0.442	5.48
34	2	1	1	0	3	1740	17.5	12.90	36.0	0.535	3.69
34	2	1	1	0	4	1510	12.4	13.10	37.0	0.880	3.98
34	2	1	1	0	5	1740	18.2	12.60	34.0	0.616	4.35
35	1	0	0	1	1	2221	104.1	11.50	32.0	0.939	4.84
35	1	0	0	1	2	2530	114.1	11.70	32.0	1.066	2.39
35	1	0	0	1	3	2304	111.1	11.30	31.5	1.224	2.49
35	1	0	0	1	4	2512	98.8	11.30	32.0	1.489	1.66
35	1	0	0	1	5	2586	113.7	11.80	31.5	1.271	2.70
36	2	0	1	0	1	2141	117.5	12.50	36.0	0.004	5.99
36	2	0	1	0	2	2308	107.2	13.10	36.0	0.233	4.91
36	2	0	1	0	3	2358	87.3	11.60	35.5	0.004	3.66
36	2	0	1	0	4	2464	78.0	12.30	34.5	0.348	3.64
36	2	0	1	0	5	2496	81.7	12.60	34.5	0.467	3.71
37	3	1	0	1	1	1942	94.7	11.80	32.0	0.569	6.02
37	3	1	0	1	2	2009	61.4	10.90	28.0	1.086	3.71
37	3	1	0	1	3	1908	44.1	10.30	29.5	1.046	4.80
37	3	1	0	1	4	1942	54.3	11.20	30.0	0.766	4.41
37	3	1	0	1	5	2197	59.7	12.10	34.0	0.799	3.75
38	1	1	1	1	1	2399	94.3	12.90	35.0	0.334	4.44
38	1	1	1	1	2	2395	64.6	12.70	34.5	0.874	6.31
38	1	1	1	1	3	2249	50.0	11.30	32.0	1.009	4.63
38	1	1	1	1	4	2362	44.1	10.90	29.5	1.168	5.50
38	1	1	1	1	5	2462	53.5	11.90	31.0	0.867	4.70
39	4	0	0	1	1	1849	118.2	13.10	32.5	0.348	4.47
39	4	0	0	1	2	1976	117.1	12.20	34.5	1.000	3.53
39	4	0	0	1	3	2107	112.5	11.50	33.5	0.931	3.98
39	4	0	0	1	4	2068	104.2	11.30	33.0	0.993	3.61
39	4	0	0	1	5	2109	111.8	12.10	34.0	1.048	3.69
40	5	1	1	0	1	1566	117.2	11.90	32.0	0.230	2.36
40	5	1	1	0	2	1217	80.9	11.90	33.5	0.410	4.69
40	5	1	1	0	3	881	44.7	10.90	32.0	0.513	2.10
40	5	1	1	0	4	959	32.0	10.20	27.5	0.408	3.39
40	5	1	1	0	5	1162	32.6	10.30	27.0	0.886	3.03

Key

int = voluntary food intake (g DM /d)
gsh = blood reduced glutathione concentration (g/100ml RBC)
hbn = blood haemoglobin concentration (g/dl)
pcv = blood packed cell volume (%)
loghnz = Heinz body count (log(% of red blood cells))
t3 = plasma tri-iodothyronine conentration (umol/l)
anim = animal number
bl = block number
wk = week number

Appendix Table 7.e - Individual data values from Experiment 7 for plasma thyroxine concentration , free thyroxine index, plasma albumin concentration, plasma urea concentration, plasma creatinine concentration, plasma gamma glutamyl transpeptidase activity and plasma alkaline phosphatase activity.

anim	bl	acn	aitc	dmds	wk	t4	fti	alb	ure	crt	ggt	alp
1	3	0	1	1	1	65.2	19.3	26.8	5.70	42.8	55.2	76.0
1	3	0	1	1	2	78.1	19.7	27.5	4.55	49.9	62.2	54.6
1	3	0	1	1	3	83.4	22.2	27.3	5.25	51.3	70.8	59.0
1	3	0	1	1	4	72.1	18.8	26.6	6.42	54.8	65.8	47.6
1	3	0	1	1	5	86.3	22.3	27.5	5.27	43.4	67.1	48.8
2	3	1	1	1	1	93.5	26.9	28.5	4.94	36.7	83.3	110.4
2	3	1	1	1	2	*	*	*	*	*	*	*
2	3	1	1	1	3	*	*	*	*	*	*	*
2	3	1	1	1	4	*	*	*	*	*	*	*
2	3	1	1	1	5	*	*	*	*	*	*	*
3	1	1	0	0	1	71.5	19.2	26.8	5.35	43.4	93.3	72.1
3	1	1	0	0	2	64.5	17.1	25.5	5.06	44.7	99.9	64.5
3	1	1	0	0	3	69.4	19.1	26.8	6.71	53.7	106.1	55.9
3	1	1	0	0	4	66.8	18.5	27.7	5.93	47.6	102.1	46.1
3	1	1	0	0	5	77.1	22.0	27.3	6.42	49.1	104.4	41.6
4	5	0	0	1	1	82.7	22.8	28.2	4.30	46.5	88.8	62.3
4	5	0	0	1	2	98.4	28.2	27.6	4.56	51.5	87.3	57.9
4	5	0	0	1	3	78.9	21.9	28.2	3.87	42.0	55.5	60.1
4	5	0	0	1	4	77.9	22.9	29.2	5.19	43.7	57.8	52.3
4	5	0	0	1	5	75.0	22.3	29.6	6.81	45.2	56.5	51.9
5	1	0	0	0	1	56.5	18.0	24.0	4.62	40.3	83.4	83.2
5	1	0	0	0	2	49.3	15.4	24.4	5.96	44.9	83.6	81.3
5	1	0	0	0	3	59.0	19.3	25.4	5.93	48.4	92.1	78.7
5	1	0	0	0	4	54.9	18.9	26.3	6.16	45.0	80.5	78.5
5	1	0	0	0	5	51.7	17.7	24.1	6.77	40.0	72.0	62.6
6	4	1	1	1	1	90.4	24.5	28.3	4.16	53.0	79.7	59.1
6	4	1	1	1	2	74.7	20.8	28.1	5.37	48.8	80.4	41.4
6	4	1	1	1	3	70.3	19.2	27.3	6.55	54.3	72.3	45.4
6	4	1	1	1	4	74.7	20.8	28.5	6.92	53.1	71.0	36.7
6	4	1	1	1	5	85.5	23.1	29.6	6.82	56.1	69.6	32.4
7	4	1	1	0	1	74.0	21.8	29.4	5.16	54.2	61.3	132.3
7	4	1	1	0	2	80.2	21.9	26.8	4.53	47.0	84.0	85.0
7	4	1	1	0	3	88.1	24.6	25.6	4.05	52.9	109.6	75.0
7	4	1	1	0	4	86.9	24.4	26.2	4.31	58.4	129.2	83.7
7	4	1	1	0	5	81.8	23.3	25.5	5.01	53.3	133.4	86.8
8	1	0	1	1	1	63.2	17.2	26.6	4.95	45.2	69.6	74.5
8	1	0	1	1	2	62.7	16.6	25.7	4.27	34.0	89.0	59.8
8	1	0	1	1	3	81.7	20.9	26.5	4.56	43.4	88.2	50.6
8	1	0	1	1	4	81.4	21.8	27.4	4.85	45.2	89.4	49.1
8	1	0	1	1	5	74.8	19.9	27.4	5.89	45.1	100.5	51.9
9	5	0	1	0	1	52.6	15.7	28.7	5.71	43.5	66.7	101.2
9	5	0	1	0	2	48.6	14.6	27.4	5.01	35.2	75.0	96.7
9	5	0	1	0	3	54.9	16.2	26.7	6.28	43.1	78.7	99.7
9	5	0	1	0	4	57.1	18.1	28.4	6.32	52.5	77.3	92.2
9	5	0	1	0	5	54.8	17.7	27.6	7.22	45.2	75.1	85.2
10	5	1	0	1	1	86.8	23.5	26.5	4.54	47.5	90.0	101.0
10	5	1	0	1	2	74.1	18.7	26.1	4.87	44.2	117.2	68.6
10	5	1	0	1	3	98.2	25.6	26.0	4.68	46.4	119.7	69.1
10	5	1	0	1	4	93.1	24.8	26.5	5.61	50.4	113.6	64.1
10	5	1	0	1	5	94.7	26.4	26.5	5.70	43.6	107.3	61.5

Appendix 7.e continued

anim	bl	acn	aitc	dmds	wk	t4	fti	alb	ure	crt	ggt	alp
11	3	0	0	1	1	74.8	22.8	26.2	4.25	38.4	61.8	136.9
11	3	0	0	1	2	76.5	22.2	26.9	4.46	42.7	66.8	141.8
11	3	0	0	1	3	77.9	22.8	28.0	5.22	45.3	69.8	124.2
11	3	0	0	1	4	80.6	24.0	25.8	5.30	47.8	64.5	101.0
11	3	0	0	1	5	75.8	23.6	27.5	5.85	49.8	63.2	118.2
12	2	0	0	0	1	77.9	22.6	26.2	3.12	36.0	75.4	72.0
12	2	0	0	0	2	62.2	17.7	25.2	3.62	38.9	83.1	61.0
12	2	0	0	0	3	72.7	22.0	26.3	4.26	47.5	83.3	60.8
12	2	0	0	0	4	53.0	15.6	27.1	5.15	48.5	74.9	51.4
12	2	0	0	0	5	79.4	23.7	26.9	5.12	45.6	76.1	54.1
13	2	1	1	1	1	67.9	19.2	26.2	5.07	38.6	61.6	100.5
13	2	1	1	1	2	77.2	21.5	25.5	3.74	36.1	84.1	87.7
13	2	1	1	1	3	82.1	23.4	25.7	4.81	39.6	93.7	85.7
13	2	1	1	1	4	88.4	25.9	26.7	5.25	48.6	98.7	85.0
13	2	1	1	1	5	74.7	21.7	26.4	5.94	44.0	92.9	92.9
14	2	1	0	0	1	63.8	18.8	25.5	5.99	41.7	61.9	100.7
14	2	1	0	0	2	67.4	19.4	25.1	4.86	39.8	74.2	111.9
14	2	1	0	0	3	68.1	20.3	26.0	6.09	42.3	82.1	120.7
14	2	1	0	0	4	69.2	22.2	26.5	7.28	45.8	82.1	117.7
14	2	1	0	0	5	67.1	21.5	27.7	7.96	47.7	85.9	108.0
15	3	1	1	0	1	72.0	19.9	27.1	4.44	38.6	38.6	70.8
15	3	1	1	0	2	78.7	19.9	24.2	4.68	44.1	51.0	47.8
15	3	1	1	0	3	80.4	19.7	22.7	4.15	45.8	48.7	14.5
15	3	1	1	0	4	*	*	*	*	*	*	*
15	3	1	1	0	5	*	*	*	*	*	*	*
16	3	1	0	0	1	97.5	31.4	27.6	5.85	43.2	48.9	79.0
16	3	1	0	0	2	91.5	25.5	26.6	4.54	47.8	56.4	73.1
16	3	1	0	0	3	101.4	30.2	27.0	4.62	48.1	68.6	66.0
16	3	1	0	0	4	100.2	31.2	28.5	6.12	53.3	73.6	73.5
16	3	1	0	0	5	84.0	26.0	28.0	6.32	54.9	71.7	65.6
17	5	1	1	1	1	95.5	25.5	27.4	5.35	43.3	75.8	80.8
17	5	1	1	1	2	95.1	21.3	25.7	4.48	53.8	97.7	68.5
17	5	1	1	1	3	90.9	21.0	26.5	3.54	49.9	106.4	57.8
17	5	1	1	1	4	71.1	15.7	25.7	4.51	54.1	100.3	33.9
17	5	1	1	1	5	72.2	15.6	25.9	3.29	50.7	101.3	39.1
18	1	1	0	1	1	45.3	13.0	24.6	4.20	37.4	63.8	65.1
18	1	1	0	1	2	80.2	21.3	25.7	4.44	42.1	78.0	68.0
18	1	1	0	1	3	55.0	15.4	26.7	4.08	39.6	78.4	55.8
18	1	1	0	1	4	52.1	15.4	26.5	5.00	44.6	82.1	46.8
18	1	1	0	1	5	57.0	16.8	28.5	6.98	43.1	86.6	45.4
19	1	0	1	0	1	77.1	22.1	25.4	4.80	35.0	77.0	48.9
19	1	0	1	0	2	66.1	17.7	24.9	4.12	36.3	94.2	52.9
19	1	0	1	0	3	72.1	19.7	25.3	4.00	38.4	101.5	54.6
19	1	0	1	0	4	86.9	24.5	27.4	4.90	44.5	108.2	55.8
19	1	0	1	0	5	75.3	22.4	26.4	5.05	43.9	105.0	59.1
20	5	1	0	0	1	112.4	29.6	29.5	4.60	39.7	72.5	57.9
20	5	1	0	0	2	115.1	28.9	27.3	4.08	44.5	84.4	60.6
20	5	1	0	0	3	97.2	25.9	27.5	4.77	45.9	90.9	52.3
20	5	1	0	0	4	111.7	29.9	29.4	5.09	49.8	94.5	45.3
20	5	1	0	0	5	103.3	30.0	27.6	5.95	49.2	94.3	43.9
21	4	1	0	1	1	94.9	29.1	29.4	5.39	40.8	78.1	98.9
21	4	1	0	1	2	83.4	24.0	27.3	4.64	43.7	86.6	103.5
21	4	1	0	1	3	96.8	29.2	29.0	4.57	38.7	91.5	85.8
21	4	1	0	1	4	99.8	29.6	30.2	4.99	46.4	94.0	82.5
21	4	1	0	1	5	96.3	30.1	29.1	5.47	40.5	93.1	79.2

Appendix 7.e continued

anim	bl	acn	aitc	dmds	wk	t4	fti	alb	ure	crt	ggt	alp
22	4	0	1	0	1	61.2	16.7	26.5	4.94	39.6	75.4	57.4
22	4	0	1	0	2	60.6	16.1	26.6	4.53	45.3	82.4	57.1
22	4	0	1	0	3	65.5	18.9	26.5	5.35	48.8	84.2	50.8
22	4	0	1	0	4	64.3	18.8	26.5	4.19	50.3	81.2	43.9
22	4	0	1	0	5	57.4	17.5	25.5	6.40	49.0	83.6	37.9
23	2	0	0	1	1	84.0	21.4	24.1	3.85	36.9	77.2	57.8
23	2	0	0	1	2	70.6	19.0	23.3	4.07	37.3	76.1	62.6
23	2	0	0	1	3	75.1	21.5	25.8	4.57	37.7	77.6	53.6
23	2	0	0	1	4	77.9	22.5	27.6	4.71	38.5	77.1	49.8
23	2	0	0	1	5	66.0	18.3	27.2	5.36	45.9	78.2	48.4
24	4	0	1	1	1	73.8	21.8	25.4	3.70	35.4	61.3	64.3
24	4	0	1	1	2	96.4	22.6	24.5	3.42	33.8	98.4	55.6
24	4	0	1	1	3	94.0	22.9	25.9	4.52	39.3	101.3	52.4
24	4	0	1	1	4	87.2	21.5	24.0	4.25	36.5	91.8	36.6
24	4	0	1	1	5	79.5	19.0	23.6	5.06	40.7	95.9	37.9
25	2	1	0	1	1	76.7	22.6	26.4	4.20	40.4	77.4	79.0
25	2	1	0	1	2	81.5	22.1	25.0	3.33	36.5	103.6	84.8
25	2	1	0	1	3	76.2	22.2	25.1	4.32	41.4	104.6	71.3
25	2	1	0	1	4	72.6	22.0	26.4	4.16	39.7	114.6	72.8
25	2	1	0	1	5	83.5	25.1	26.9	5.04	41.5	110.7	67.0
26	5	0	1	1	1	52.7	15.9	24.9	5.02	51.7	65.9	89.3
26	5	0	1	1	2	53.4	14.9	23.5	4.17	51.4	78.8	86.5
26	5	0	1	1	3	59.4	16.5	24.2	4.76	53.9	79.5	103.4
26	5	0	1	1	4	59.1	17.4	25.1	4.68	51.2	79.1	102.7
26	5	0	1	1	5	58.7	17.5	25.3	6.46	53.6	79.2	98.5
27	3	0	0	0	1	73.9	20.1	26.2	5.06	33.4	43.0	56.8
27	3	0	0	0	2	83.7	21.5	25.7	4.76	31.3	51.1	60.6
27	3	0	0	0	3	68.4	18.0	26.2	5.80	33.8	55.7	60.3
27	3	0	0	0	4	75.6	21.3	27.8	6.10	38.2	60.4	58.6
27	3	0	0	0	5	65.5	18.5	27.3	6.25	37.8	58.0	53.6
28	4	0	0	0	1	69.7	20.2	25.4	4.72	43.7	52.4	97.0
28	4	0	0	0	2	71.0	19.0	24.2	4.35	42.0	54.8	93.2
28	4	0	0	0	3	71.7	20.8	25.5	5.21	44.8	55.7	97.2
28	4	0	0	0	4	75.5	22.3	27.1	5.45	40.8	56.9	103.0
28	4	0	0	0	5	69.7	21.8	26.3	5.82	44.6	52.6	109.6
29	4	1	0	0	1	59.0	17.7	26.2	6.66	48.8	64.9	126.1
29	4	1	0	0	2	66.5	18.2	25.8	5.68	49.6	84.9	121.2
29	4	1	0	0	3	73.9	21.6	26.8	5.93	51.5	86.4	104.4
29	4	1	0	0	4	84.4	25.0	27.8	6.97	49.6	88.4	93.7
29	4	1	0	0	5	70.5	21.4	27.9	7.37	49.8	87.5	87.5
30	2	0	1	1	1	74.5	22.2	25.9	3.90	41.8	62.7	103.7
30	2	0	1	1	2	77.3	20.9	25.0	3.40	35.4	83.2	75.2
30	2	0	1	1	3	85.3	24.7	24.8	3.86	41.3	82.4	73.1
30	2	0	1	1	4	85.4	24.0	24.6	3.67	39.5	85.9	61.6
30	2	0	1	1	5	83.8	25.5	25.4	4.52	40.3	88.5	78.7
31	5	0	0	0	1	67.7	21.0	25.9	4.06	46.6	63.8	72.8
31	5	0	0	0	2	55.4	15.8	27.6	4.30	44.0	74.2	97.0
31	5	0	0	0	3	65.5	21.1	26.7	4.65	51.1	70.1	83.3
31	5	0	0	0	4	67.3	20.1	28.8	5.29	48.9	78.8	82.2
31	5	0	0	0	5	60.9	20.8	26.3	5.83	50.6	74.0	74.1
32	3	0	1	0	1	92.1	28.0	25.9	3.98	39.6	77.2	50.4
32	3	0	1	0	2	91.7	24.5	24.0	4.09	40.5	90.5	45.9
32	3	0	1	0	3	86.2	25.7	25.0	5.04	39.6	89.2	41.6
32	3	0	1	0	4	90.3	27.5	25.4	4.73	43.7	89.4	48.4
32	3	0	1	0	5	94.7	28.4	25.9	5.90	46.3	91.3	38.9

Appendix 7.e continued

anim	bl	acn	aitc	dmds	wk	t4	fti	alb	ure	crt	ggt	alp
33	1	1	1	0	1	74.0	20.5	24.9	5.12	47.1	80.4	110.4
33	1	1	1	0	2	70.1	16.8	25.0	3.91	47.2	115.4	88.2
33	1	1	1	0	3	63.9	17.2	25.2	5.24	47.7	122.4	92.0
33	1	1	1	0	4	68.1	18.7	26.3	5.33	43.0	130.4	78.0
33	1	1	1	0	5	69.4	19.6	25.3	6.11	47.1	118.4	86.3
34	2	1	1	0	1	91.2	22.2	25.1	5.20	39.3	55.5	65.0
34	2	1	1	0	2	101.4	23.0	24.2	3.64	39.3	78.9	54.6
34	2	1	1	0	3	93.2	20.9	25.3	4.65	39.3	88.8	43.1
34	2	1	1	0	4	97.2	27.1	26.3	3.95	41.2	93.0	43.6
34	2	1	1	0	5	89.2	21.9	24.7	5.07	42.3	88.7	36.7
35	1	0	0	1	1	67.0	17.7	26.2	4.71	34.7	56.9	67.8
35	1	0	0	1	2	69.1	17.7	24.6	3.79	32.1	62.2	69.3
35	1	0	0	1	3	66.9	16.7	27.7	4.55	38.4	67.9	63.3
35	1	0	0	1	4	61.7	16.8	28.2	4.46	37.9	68.6	63.5
35	1	0	0	1	5	65.9	18.6	26.8	6.33	40.8	64.1	60.3
36	2	0	1	0	1	65.1	17.9	25.3	5.20	43.8	58.1	53.8
36	2	0	1	0	2	74.4	21.2	24.4	3.98	40.6	69.4	47.3
36	2	0	1	0	3	62.4	19.1	26.0	4.92	47.5	76.0	44.4
36	2	0	1	0	4	66.4	19.8	27.0	4.61	44.8	79.5	34.7
36	2	0	1	0	5	60.0	18.8	26.2	6.16	45.6	78.3	36.2
37	3	1	0	1	1	104.9	27.1	26.9	7.07	49.5	85.8	98.7
37	3	1	0	1	2	100.4	25.0	23.0	3.35	39.9	84.4	42.1
37	3	1	0	1	3	104.4	25.2	25.7	4.62	49.0	95.9	63.6
37	3	1	0	1	4	106.5	26.4	28.0	5.51	45.9	100.3	81.7
37	3	1	0	1	5	101.2	27.0	27.2	6.92	50.8	91.3	81.2
38	1	1	1	1	1	91.9	22.1	25.2	3.95	42.9	97.3	63.8
38	1	1	1	1	2	97.4	23.0	23.1	3.51	40.3	126.1	54.8
38	1	1	1	1	3	92.3	23.9	24.3	3.96	47.9	129.8	85.8
38	1	1	1	1	4	103.2	25.0	24.8	4.12	44.5	139.6	45.1
38	1	1	1	1	5	96.7	23.4	24.7	5.13	44.4	136.6	43.9
39	4	0	0	1	1	89.3	24.3	28.1	5.32	52.6	54.8	71.8
39	4	0	0	1	2	98.6	27.2	24.2	5.04	45.5	55.1	79.5
39	4	0	0	1	3	96.3	27.2	27.7	5.28	54.1	59.4	58.6
39	4	0	0	1	4	80.3	23.1	27.9	4.84	46.2	58.0	72.6
39	4	0	0	1	5	91.2	26.4	28.4	7.07	50.0	56.3	85.0
40	5	1	1	0	1	91.1	25.4	25.1	3.52	49.2	58.1	73.8
40	5	1	1	0	2	115.8	30.5	22.5	1.98	49.9	61.0	47.3
40	5	1	1	0	3	86.5	23.0	25.1	3.30	53.7	77.4	48.8
40	5	1	1	0	4	90.6	25.0	24.3	2.64	48.4	75.3	53.6
40	5	1	1	0	5	70.7	19.3	26.0	3.90	55.2	74.9	56.1

Key

t4 = plasma thyroxine concentration (umol/l)
fti = free thyroxine index (%)
alb = plasma albumin concentration (g/l)
ure = plasma urea concentration (umol/l)
crt = plasma creatinine concentration (umol/l)
ggt = plasma gamma glutamyl transpeptidase activity (IU/l)
alp = plasma alkaline phosphatase activity (IU/l)
anim = animal number
bl = block number
wk = week number

Appendix Table 7.f Two-way interactions between allyl cyanide (ACN), allyl isothiocyanate (AITC) and dimethyl disulphide (DMDS); effects on blood glutathione concentration , blood haemoglobin concentration, and Heinz body count

BLOOD GLUTATHIONE CONCENTRATION (g/100ml RBC)

	AITC		DMDS		DMDS	
	0	1	0	1	0	1
ACN 0	100.1	83.6	84.7	99.0	73.3	91.2
ACN 1	64.4	52.2	50.4	66.3	61.8	74.0
level of significance	NS		NS		NS	
SED	5.73		5.73		5.73	

BLOOD HAEMOGLOBIN CONCENTRATION (g/dl)

	AITC		DMDS		DMDS	
	0	1*	0	1	0	1
ACN 0	11.89	11.70	11.58	12.00	11.45	11.87
ACN 1	11.43	12.10	11.97	11.56	12.10	11.69
Level of significance	NS		NS		NS	
SED	0.314		0.314		0.314	

HEINZ BODY COUNT (log(% of red blood cells))

	AITC		DMDS		DMDS	
	0	1	0	1	0	1
ACN 0	0.680	0.595	0.391	0.883	0.410	0.884
ACN 1	0.614	0.760	0.462	0.912	0.443	0.911
Level of significance	NS		NS		NS	
SED	0.0828		0.0828		0.0828	

* 0 = Absence of compound
1 = Presence of compound

Appendix Table 7.g Two-way interactions between allyl cyanide (ACN), allyl isothiocyanate (AITC) and dimethyl disulphide (DMDS); effects on plasma tri-iodothyronine (T3) concentrations, plasma thyroxine (T4) concentrations and free thyroxine index (FTI)

PLASMA T3 CONCENTRATION (umol/ml)

	AITC 0	AITC 1*
ACN 0	3.23	3.07
ACN 1	3.67	3.97
Level of significance	NS	
SED	0.523	

	DMDS 0	DMDS 1
ACN 0	3.17	3.13
ACN 1	3.57	4.06
Level of significance	NS	
SED	0.523	

	DMDS 0	DMDS 1
AITC 0	3.40	3.50
AITC 1	3.34	3.69
Level of significance	NS	
SED	0.523	

PLASMA T4 CONCENTRATION (umol/ml)

	AITC 0	AITC 1
ACN 0	72.3	72.1
ACN 1	83.4	84.4
Level of significance	NS	
SED	5.47	

	DMDS 0	DMDS 1
ACN 0	67.9	76.6
ACN 1	82.3	85.5
Level of significance	NS	
SED	5.47	

	DMDS 0	DMDS 1
AITC 0	74.2	81.5
AITC 1	76.0	80.6
Level of significance	NS	
SED	5.47	

FREE THYROXINE INDEX (%)

	AITC 0	AITC 1
ACN 0	20.9	20.3
ACN 1	23.6	22.3
Level of significance	NS	
SED	1.38	

	DMDS 0	DMDS 1
ACN 0	20.0	21.2
ACN 1	22.7	23.1
Level of significance	NS	
SED	1.38	

	DMDS 0	DMDS 1
AITC 0	21.7	22.8
AITC 1	21.1	21.5
Level of significance	NS	
SED	1.38	

* 0 = Absence of compound
1 = Presence of compound

Appendix Table 7.h Two-way interactions between allyl cyanide (ACN), allyl isothiocyanate (AITC) and dimethyl disulphide (DMDS); effects on plasma albumin concentration, plasma urea concentration and plasma creatinine concentration.

PLASMA ALBUMIN CONCENTRATION (g/l)

	AITC 0	AITC 1*
ACN 0	26.56	25.93
ACN 1	26.96	25.97
Level of significance	NS	
SED	0.469	

	DMDS 0	DMDS 1
ACN 0	26.16	26.33
ACN 1	26.23	26.69
Level of significance	NS	
SED	0.469	

	DMDS 0	DMDS 1
AITC 0	26.63	26.89
AITC 1	25.76	26.14
Level of significance	NS	
SED	0.469	

PLASMA UREA CONCENTRATION (mmol/l)

	AITC 0	AITC 1
ACN 0	5.04	4.89
ACN 1	5.40	4.62
Level of significance	NS	
SED	0.277	

	DMDS 0	DMDS 1
ACN 0	5.12	4.82
ACN 1	5.12	4.91
Level of significance	NS	
SED	0.277	

	DMDS 0	DMDS 1
AITC 0	5.49	4.95
AITC 1	4.74	4.77
Level of significance	NS	
SED	0.277	

PLASMA CREATININE CONCENTRATION (umol/l)

	AITC 0	AITC 1
ACN 0	42.97	43.67
ACN 1	45.30	46.24
Level of significance	NS	
SED	1.828	

	DMDS 0	DMDS 1
ACN 0	42.99	43.65
ACN 1	47.13	44.42
Level of significance	NS	
SED	1.828	

	DMDS 0	DMDS 1
AITC 0	44.96	43.30
AITC 1	45.15	44.77
Level of significance	NS	
SED	1.828	

* 0 = Absence of compound
1 = Presence of compound

Appendix Table 7.i Two-way interactions between allyl cyanide (ACN), allyl isothiocyanate (AITC) and dimethyl disulphide (DMDS); effects on plasma gamma glutamyl transpeptidase activity and plamsa alkaline phosphatase activity

PLASMA GAMMA GLUTAMYL TRANSPEPTIDASE ACTIVITY (IU/l)

	AITC 0	AITC 1*
ACN 0	67.4	81.1
ACN 1	88.2	90.8

	DMDS 0	DMDS 1
ACN 0	75.4	73.1
ACN 1	83.3	95.6

	DMDS 0	DMDS 1
AITC 0	75.3	80.2
AITC 1	83.4	88.5

level of significance	NS	NS	NS
SED	6.07	6.07	6.07

PLASMA ALKALINE PHOSPHATASE ACTIVITY (IU/l)

	AITC 0	AITC 1
ACN 0	75.1	62.5
ACN 1	76.5	66.8

	DMDS 0	DMDS 1
ACN 0	66.4	71.2
ACN 1	72.5	70.8

	DMDS 0	DMDS 1
0	77.4	74.2
1	61.5	67.9

level of significance	NS	NS	NS
SED	10.49	10.49	10.49

* 0 = Absence of compound
1 = Presence of compound