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Nitrogen Assimilation by Rumen Microorganisms. A Study of the Assimilation of Ammonia by Rumen Bacteria *in vivo* and *in vitro*.

by

Nicholas John Edwards, B.Ag.Sc.(HONS.)

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Doctor of Philosophy

Department of Animal Sciences,

Waite Agricultural Research Institute,

The University of Adelaide.

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A1.10 Specific activities of GS, AsS, GOGAT, GDH and AlaDH, and intracellular levels of glutamic acid, aspartic acid and alanine, before, and at 30, 60, 120 and 240 mins after, bolus additions of "synthetic saliva" only and "synthetic saliva" plus 0.5 moles NH_4Cl to the rumen of diet E (experiment 2). 255

A1.11 Specific activities of GS, AsS, GOGAT, GDH and AlaDH, and intracellular levels of glutamic acid, aspartic acid and alanine, before, and at 30, 60, 120 and 240 mins after, bolus additions of "synthetic saliva" plus 0.5 moles glucose and "synthetic saliva" plus 0.5 moles NH_4Cl and 0.5 moles glucose to the rumen of diet E (experiment 2). 256

A2.1 Effects of MSX and azaserine on growth of cultures of *B. ruminicola* BA25, *S. ruminantium* MP72, *S. ruminantium* ME13, *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 in NFMS media supplemented with 8 mM NH_4Cl. 257

ABSTRACT

The work presented in this thesis was undertaken to investigate nitrogen assimilation and metabolism in rumen bacteria with the object of understanding the basic processes and their controls. Knowledge of nitrogen assimilation in these organisms is scant, in contrast to the situation for many aerobic bacteria. This investigation will help develop an understanding of one of the limitations to the attainment of maximum productive potential (i.e. of growth, reproduction, lactation and wool production) in ruminant animals, namely their inability to fully utilise dietary nitrogen. This research project aims to elucidate mechanisms of ammonia assimilation and utilisation in particular rumen bacterial species.

A range of activities of four enzymes, glutamine synthetase (GS : EC. 6.3.1.2), glutamate synthase (GOGAT : EC. 1.4.1.13 & 1.4.1.14), glutamate dehydrogenase (GDH : EC. 1.4.1.2, 1.4.1.3 & 1.4.1.4) and alanine dehydrogenase (AlaDH : EC. 1.4.1.1), responsible for nitrogen (as ammonia) assimilation in rumen microorganisms are reported for mixed rumen bacterial samples in the first series of experiments. These experiments also examined the effects of short term changes in rumen ammonia and glucose (energy) levels on the activities of the enzymes in cell-free extracts of mixed rumen bacterial samples from sheep maintained on two extreme diets - low nitrogen / low energy and high nitrogen / high energy. GS was the only enzyme to respond to the sudden environmental changes, but only when its activity was high, as a consequence of very low ammonia levels. GS, GOGAT and GDH were the major nitrogen-assimilating enzymes in these rumen bacterial mixtures, while AlaDH was present at much lower levels. Asparagine synthetase (EC. 6.3.1.1 & 6.3.1.4) was also studied, but its activity appeared to be insignificant and was not investigated further.

Cofactor requirements of GOGAT, GDH and AlaDH were investigated in the mixed rumen bacterial samples. The NADH-dependent activities were up to 100 times greater than their NADPH-dependent counterparts, contrary to some previous reports. Microorganisms have been shown to possess GDH's with specificity for one of the nicotinamide adenine dinucleotides, or a non-specific requirement. Given the plethora of bacterial species present in the mixed rumen bacterial samples it is unlikely that a single form of GDH was present. The same can also be said for GOGAT and AlaDH specificities. In aerobic microorganisms, NADH-specific GDH is involved primarily in oxidation of glutamate to 2-oxoglutarate at low

ammonia concentrations or when amino acids are present, while NADPH-specific GDH is responsible for glutamate biosynthesis at low ammonia concentrations. The ratio of NADH to NADPH isozymes of GDH in the mixed rumen bacterial samples appears to be consistent with this designation, since glutamate is consistently the most abundant amino acid present in feedstuffs, however the results may indicate a biosynthetic role for NADH-specific GDH in the rumen. GOGAT is considered to catalyse a unidirectional reaction from glutamine to glutamate.

Subsequent experiments were carried out using pure cultures of individual rumen bacterial species to eliminate variations between species, as well as those caused by rumen factors. In addition, enzyme studies of the ammonia-assimilatory pathways enabled a comparison to be made between the rumen bacterial species grown in pure cultures.

The effects of L-methionine DL-sulphoximine (MSX), a competitive inhibitor of GS, and O-diazoacetyl-L-serine (azaserine), a competitive inhibitor of GOGAT, on the growth of batch cultures of *Bacteroides ruminicola* subsp. *brevis*, *Selenomonas ruminantium* subsp. *lactilytica* and *Selenomonas ruminantium* subsp. *ruminantium* were studied with ammonia as the sole nitrogen source. Results show that GS and GOGAT are key enzymes in nitrogen assimilation at ammonia concentrations up to 128 mM in these species, with only a few exceptions. In particular, GS apparently was not important at ammonia levels below 1 mM in *B. ruminicola*. More detailed studies of the ammonia-assimilatory enzymes of *B. ruminicola* showed that both the GS / GOGAT and GDH pathways were quantitatively important based on relative enzyme activity measurements. The GS / GOGAT pathway was apparently especially important at media ammonia concentrations of less than 8 mM. For GS the apparent K_m-value for hydroxylamine was 4.5 mM when ammonia was the nitrogen source for growth. For NADPH-specific GDH the K_m-value for NH_4Cl was 8.1 mM, and for NADH-specific GDH it was 24.3 mM. The NADPH-specific GDH may therefore be the major assimilatory enzyme in *B. ruminicola* at media ammonia concentrations between 8 and 128 mM, since its level was relatively independent of ammonia concentration in the growth media, compared with a 10-fold reduction in GS activity values. Nevertheless, GS and GOGAT apparently maintained vital roles in growth of this species even at high ammonia levels. Possible reasons for these results are discussed.

Transferase activity of GS was not detected in *S. ruminantium* subsp. *lactilytica*, however inhibitor studies indicated that the enzyme is present. NADPH-specific GDH activity in this species was fairly constant over a wide range of media ammonia concentrations (0.5 to 128 mM). GOGAT appeared to be a vital enzyme for cell growth in these species at all media ammonia concentrations and its activity was largely independent of ammonia levels. Nevertheless, the specific activity of the NADPH-dependent enzyme was relatively low in both species, as was the NADPH-specific AlaDH activity which may, however, contribute to ammonia assimilation at high ammonia levels, at least in *S. ruminantium*. When amino acids and peptides were available as nitrogen sources for growth by *B. ruminicola* and *S. ruminantium* neither the GS / GOGAT nor the GDH pathways appeared to be important, and ammonia was not readily utilised as a nitrogen source for growth.

Unlike *B. ruminicola* subsp. *brevis* and *S. ruminantium*, *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* did not grow with NH_4Cl as a sole nitrogen source. Nevertheless, growth of *B. ruminicola* subsp. *ruminicola* was still inhibited by up to 50 % by both MSX and azaserine, whereas growth of *Butyrivibrio fibrisolvens* was virtually unaffected at various ammonia concentrations when amino acids or peptides were also present. In addition, GOGAT, GDH and AlaDH activities were low for *Butyrivibrio fibrisolvens*, but transferase activity for GS was not detected. These data support the suggestion that *Butyrivibrio fibrisolvens* may use a pathway of nitrogen assimilation other than those studied in this thesis.

The significance of various NADH / NADPH ratios for GDH activity in *B. ruminicola*, *S. ruminantium* and *Butyrivibrio fibrisolvens* is discussed in this thesis. Of particular interest is that the NADH / NADPH ratio for GDH in *B. ruminicola* subsp. *brevis* was 0.015 for all media ammonia concentrations, but changed to 0.152 when amino acids and peptides were also available as nitrogen sources. A similar 10-fold change in ratio (from 0.036 to 0.350) was evident for GOGAT activity in this species, and the NADPH-specific activity was greater than or equal to the NADH-specific activity for AlaDH. Higher NADPH- than NADH-specific activities were also recorded in *S. ruminantium* for GOGAT and GDH, in contrast to the results for mixed rumen bacterial samples, as demonstrated in the first series of experiments. The cofactor preferences for these enzymes were variable in *Butyrivibrio fibrisolvens*. Clearly, significant differences exist between the activities of the various enzymes in the species of

rumen bacteria studied, indicating the variety of ammonia-assimilating processes present in rumen bacterial species.

Early experiments in this study established the importance and quantitative significance of GS, GOGAT and GDH, and the relative unimportance of AlaDH, in the assimilation of ammonia in *B. ruminicola* subsp. *brevis* and *S. ruminantium* subsp. *lactilytica*. When cells were grown with amino acids and peptides as nitrogen sources the enzyme activities were far less than were found with ammonia as a sole nitrogen source, and growth rates on amino acid / peptide media were also greater than on ammonia-based media. This observation led to the final series of experiments in which it was shown that *B. ruminicola* subsp. *brevis* and *S. ruminantium* subsp. *lactilytica* preferentially utilize peptide nitrogen, but are also able to grow with proteins, amino acids or ammonia as nitrogen sources. In addition, two glucose levels were superimposed on these treatments to investigate the effect of energy level on growth. *B. ruminicola* subsp. *brevis* had a shorter lag period and achieved a higher cell density and maximum growth rate when Tryptone (peptide nitrogen) was the nitrogen source than when ammonia, casein (protein nitrogen), Casamino Acids (free amino acids) and mixtures of these nitrogen sources were supplied. These results indicate a preference for peptide nitrogen by this species, followed by ammonia, proteins, and free amino acids. Growth on Casamino Acids was, in fact, vastly different in the time between inoculation and logarithmic growth, maximum cell density and growth rate *per se*. *S. ruminantium* subsp. *lactilytica* also had a preference for Tryptone as a nitrogen source, followed by ammonia and then casein. No growth was evident with Casamino Acids as sole nitrogen source. In contrast, *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* showed an absolute requirement for peptide or amino acid nitrogen and did not grow with ammonia as the sole nitrogen source. Without exception, growth of *B. ruminicola* subsp. *brevis* and *S. ruminantium* subsp. *lactilytica* was restricted on media containing 0.05 % [w/v] glucose compared with 0.5 % [w/v] glucose. Thus, neither species was able to utilise one of the nitrogen sources as an alternative energy source.

These experiments show that, in pure cultures, some rumen bacterial species are capable of utilising a wide range of nitrogen sources. The data raise the question of what actually happens in the rumen. If one extrapolates from the *in vitro* work to the rumen environment it is tempting to speculate that for some bacteria, such as *B. ruminicola* and *S. ruminantium*, a

variety of nitrogen-assimilatory routes exist to facilitate bacterial growth, initially utilising peptide nitrogen (as feed is degraded) and subsequently ammonia, peptides and free amino acids resulting from the breakdown of proteins, peptides and amino acids. In contrast, other organisms such as *Butyrivibrio fibrisolvens* are not capable of assimilating ammonia nitrogen directly and thus may not compete effectively in all microenvironments. This is a clear example of the great diversity in the metabolism of nitrogen compounds in the rumen microbial ecosystem, indicating mechanisms for microbial selection *in vivo*.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

If accepted for the award of a PhD degree, this thesis will be available for loan and photocopying.

Nick Edwards

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This thesis is dedicated to the memory of Bill Edwards

(1957 - 1989)

NOMENCLATURE

The major enzymes mentioned in this thesis are listed below with their numbers and systematic names, as recommended by the Enzyme Commission (Enzyme Nomenclature, 1984).

Trivial Name	Systematic Name, Number and Reaction
Glutamine synthetase (GS)	L-Glutamate : ammonia ligase (ADP-forming) E.C. 6.3.1.2 ATP + L-glutamate + NH_3 = ADP + orthophosphate z+ L-glutamine
Glutamate synthase (GOGAT) - an iron-sulphur flavoprotein. In the reverse reaction ammonia can act instead of glutamine, but more slowly.	L-Glutamate : $NADP^+$ oxidoreductase (transaminating) E.C. 1.4.1.13 (formerly 2.6.1.53) 2 L-Glutamate + $NADP^+$ = L-glutamine + 2-oxoglutarate + NADPH
	L-Glutamate : NAD^+ oxidoreductase (transaminating) E.C. 1.4.1.14 2 L-Glutamate + NAD^+ = L-glutamine + 2-oxoglutarate + NADH
Glutamate dehydrogenase (GDH)	L-Glutamate : NAD^+ oxidoreductase (deaminating) E.C. 1.4.1.2 L-Glutamate + H_2O + NAD^+ = 2-oxoglutarate + NH_3 + NADH
	L-Glutamate : $NAD(P)^+$ oxidoreductase (deaminating) E.C. 1.4.1.3 L-Glutamate + H_2O + $NAD(P)^+$ = 2-oxoglutarate + NH_3 + NAD(P)H
	L-Glutamate : $NADP^+$ oxidoreductase (deaminating) E.C. 1.4.1.4 L-Glutamate + H_2O + $NADP^+$ = 2-oxoglutarate + NH_3 + NADPH
Alanine dehydrogenase (AlaDH)	L-Alanine : NAD^+ oxidoreductase (deaminating) E.C. 1.4.1.1 L-Alanine + H_2O + NAD^+ = pyruvate + NH_3 + NADH
Asparagine synthetase (AsS)	L-Aspartate : ammonia ligase (AMP-forming) E.C. 6.3.1.1 ATP + L-aspartate + NH_3 = AMP + pyrophosphate + L-asparagine
	L-Aspartate : ammonia ligase (ADP-forming) E.C. 6.3.1.4 ATP + L-aspartate + NH_3 = ADP + orthophosphate + L-asparagine

ABBREVIATIONS

The abbreviations for chemicals, symbols and units in general follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature (Biochem. J. (1966) **101** : 1 - 7), or the Instructions for Authors for the Journal of Biological Chemistry (1990).

Chemicals

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin (Fraction V)
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-[2-Hydroxyethyl]-1-piperazineethanesulfonic acid
mRNA	Messenger Ribonucleic Acid
MSX	L-Methionine DL-sulphoximine
N	Nitrogen
Na-ADP	Adenosine 5'-diphosphate (sodium salt)
Na-ATP	Adenosine 5'-triphosphate (sodium salt)
NAD^+	ß-Nicotinamide adenine dinucleotide
NADH	ß-Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	ß-Nicotinamide adenine dinucleotide phosphate
NADPH	ß-Nicotinamide adenine dinucleotide phosphate (reduced form)
Ninhydrin	2,2-Dihydroxyl-1,3-indanedione
P_i	Organic phosphate
RNA	Ribonucleic Acid
SSA	Sulpho-salicylic acid
THM mixture	20 µl 1.0 M Tris-HCl; 60 µl Hydroxylamine Solution (2.0 M hydroxylamine-monohydrochloride neutralised with 2.0 M NaOH); 1.5 µl 0.1 M $MnCl_2$ (pH 6.8)
TKD buffer	50 mM Tris-HCl; 1 % [w/v] KCl; 1 mM dithiothreitol, [pH 7.4]
Tris-HCl buffer	Tris(hydroxymethyl)aminomethane

Others

DMD	Dry matter digestibility
γ-GT	γ-Glutamyl transferase
"Hungate" tube	16 x 125 mm Pyrex® screw-capped culture tubes with butyl rubber septa inserted into the caps
"*in vitro* " tube	100 ml glass centrifuge tube

log	Logarithmic (i.e. early, mid and late log; of bacterial culture growth)
NFMS	Nitrogen Free Mineral Salts (medium)
RGM	Routine Growth Medium
R.O. water	Reverse osmosis water (alternative to distilled water)
SS	Synthetic Saliva (see McDougall, 1948)

Symbols and Units

℃	degree centrigade or Celcius
c.c.	cubic centrimetre(s)
g	unit of gravitational field (acceleration of gravity)
g	gram
hr(s)	hour(s)
kg	kilogram
K_m	Michaelis constant
K_s	Ammonia saturation constant
M	molar
mg	milligram
min.(s)	minute(s)
ml	millilitre
mmol	millmole(s)
mM	millimolar
mV	millivolt(s)
µg	microgram
µl	microlitre
µmol	micromole(s)
µM	micromolar
N	normal
nm	nanometre
nmole	nanomole(s)
%	percent
p.s.i.	Pounds per square inch
rpm	revolutions per minute
UV	ultraviolet
V_{max}	rate of enzyme catalysed reaction at infinite concentration of substrate
W	watt(s)

Others

&	and
approx.	approximately
cf.	compared with
conc^n.	concentration

e.g.	for example
et al.	*et alia* (and others)
etc.	*et cetera* (and so on)
i.e.	*id est* (that is)
m-, p-, o-	*meta-, para-, ortho*
n	number of (samples)
p. (pp.)	page(s)
per se	by or in itself; intrinsically
P	probability
vit.	vitamin(s)
v/v	volume : volume
w/v	weight : volume
<	less than
>	greater than
/	per

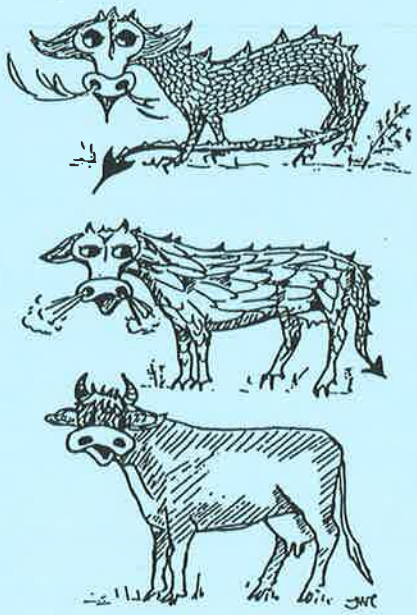
CHAPTER 1

Review of the Literature (to 30/6/1986)

"Sans les animaux, la nature de l'homme serait encore plus incompréhensible "

(Without animals, human nature would be even more incomprehensible)

(Georges Louis Leclerc, comte de Buffon : Histoire Naturelle, 1779)



Development of the rumen (1)

Czerkawski (1986)

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Chapter 1

Review of the Literature

**** This Review covers literature up to June 30th, 1986, the date when experimental **** **** work for this project commenced. References to literature after that date, and up to **** **** the end of the project, are included in the subsequent Discussions. ****

1.1 THE RUMINANT - A Digestive Success Story!

A number of animal production systems are used worldwide, however the cheapest and most common, and the one which predominates in Australia, is that involving grazing lands (Jarrige, 1980; Morley, 1981b). In fact, nearly all of the agriculturally-important animals in Australia (and worldwide), such as sheep, cattle, horses, goats and deer, are herbivores, well adapted to life in grazing environments. Ruminants, as the most successful of herbivorous animals, supply the majority of meat and milk consumed by humans, as well as large quantities of textiles and leather goods, and much of the draught power in many areas of the world (Church, 1979a; Jarrige, 1980; Morley, 1981a; Orskov, 1982).

The success of ruminants in grazing systems is largely due to the superiority of their digestive system, which is the most advanced herbivore strategy (Williams, 1981; Hume, 1984). The rumen, reticulum and omasum are unique to the ruminant animal, and the population of microorganisms (bacteria, protozoa, phycomycetous fungi, mycoplasmas, bacteriophages, viruses), which are in a symbiotic relationship with the animal in the reticulo-rumen, enable it to largely degrade ingested food by fermentation prior to the digesta reaching the true stomach, or abomasum. This is one of the major advantages of rumen fermentation since, not only is the fermentation superior to that which occurs in the caecum of other herbivores, but the products of this fermentation are able to pass through the intestines for further digestion and absorption, in contrast to the situation in "simple-stomached" herbivores, such as horses (Hume & Warner, 1980; Hobson & Wallace, 1982a; Orskov, 1982; Leng, 1985; Thomas, 1985).

The unique digestive system of ruminants also makes them adaptable to a wide range of feeds, and is the reason ruminants are the best equipped herbivores for maximal fibre digestion, since fibrous plant material can be retained in the reticulo-rumen for prolonged periods, often exceeding 60 hours, thereby ensuring that rumination by the animal has sufficient opportunity to mechanically break the food into degradable components, and that the resident microflora also have sufficient opportunity to degrade the cellulosic cell-wall constituents (Hobson & Wallace, 1982a; Hume, 1984). Although an advantage in many situations, prolonged retention time can also be a problem with high-fibre diets because retention time may be extreme, to the point where feed intake will be limited by rumen distention (Hume, 1984).

Another attribute of the ruminant digestive system is the synthetic ability of rumen microorganisms, effectively rendering the adult ruminant completely independent of a dietary source of the B-group of vitamins. In addition, the ruminal microflora can degrade a wide variety of plant secondary compounds such as alkaloids, caffeic acid, gossypol, mycotoxins, oxalates and cyanogenic glycosides, thus protecting ruminants from these potentially harmful compounds which occur in some plants. Nevertheless, the detoxification mechanisms only appear to be effective when the compounds are present at low concentrations (James *et al.*, 1975; Hegarty, 1982; Hume, 1984).

1.2 THE RUMEN MICROFLORA

As mentioned above, fermentation of feedstuffs by the microbial population in the rumen provides the majority of the metabolisable nutrients for the ruminant. The diversity of this population, in which each organism tends to occupy it own niche, increases if one considers the variety of growth forms of the microorganisms (Orskov, 1982; Baker, 1985; Leng, 1985). Ruminal bacteria, for example, may exist free in the rumen fluid (approx. 30 % of the resident bacteria), attached to feed particles (60 - 70 %), other bacteria (syntrophs) and protozoa (1 % ?), or adhering to the rumen epithelial lining (1 %) (Leng, 1985). Similarly, protozoa may be found free in solution, attached to feed particles, or adhering to the rumen or reticulum epithelium, and phycomycetous fungi may occur as mycelia in fibre or other digesta, or as fungal zoospores free in the rumen and reticulum fluids (Cheng & Costerton, 1980; Baker, 1985; Leng, 1985).

Thus, there are basically three different populations within the rumen, namely the rumen fluid (or "free") organisms (of particular importance because it is mainly these organisms that enter the lower digestive tract), the microorganisms adhering to feed particles ("particle-bound" : these cause most of the fermentation of roughage), and the microorganisms adhering to the rumen epithelium. The species present in the latter population appear to be independent of the substrates being fermented in the rumen (Cheng & Costerton, 1980; Orskov, 1982).

Rumen bacteria are numerically the predominant component of the microbial population (Orskov, 1982; Czerkawski, 1986) and can be subdivided into groups based on morphological and histochemical divisions (cocci, rod, spiral, oval, etc. forms or Gram positive and Gram negative forms) or, more appropriately in terms of the niche (or niches) they fill in the rumen, on substrate specificity. Thus, there are ruminal species which digest fibrous material (cellulolytic), starch (amylolytic), fats (lipolytic), or soluble proteins, peptides and amino acids (proteolytic), as well as others which ferment soluble sugars (e.g. xylose, glucose, fructose, sucrose), or acids (e.g. lactate, succinate, propionate). There are also ruminal species which produce methane (methanogenic), however many species utilise more than one substrate and are therefore adapted to a number of niches within the rumen (Bryant, 1959; Bryant, 1963; Hungate *et al.*, 1964; Hungate, 1966; Ogimoto & Imai, 1981; Orskov, 1982; Russell, 1984).

Bacteroides ruminicola and *Selenomonas ruminantium* are probably the best examples of species which utilise a wide range of substrates (they ferment arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, starch, sucrose and xylose, and are proteolytic). *B. ruminicola* can represent 6 to 19 % of the total culturable carbohydrate fermenters present in the rumen (Joyner & Baldwin, 1966; Howlett *et al.*, 1976), whereas *S. ruminantium* represents up to 40 % of the total colony count from the rumen of steers fed cracked corn and urea (Hungate, 1966). Both species are maintained at relatively high levels under a wide variety of feeding conditions (Bryant, 1959; Hungate *et al.*, 1964; Hungate, 1966; Howlett *et al.*, 1976; Hespell & Bryant, 1981; Ogimoto & Imai, 1981; Russell & Hespell, 1981; Russell, 1984).

In contrast, the primary role of *Fibrobacter succinogenes* subsp. *succinogenes* (formerly *Bacteroides succinogenes* - Montgomery *et al.*, 1988) is the fermentation of cellulose and it is the most active cellulolytic species, digesting the more resistant cellulose (i.e. cotton fibres and the more mature hay) to a far greater extent than the other cellulolytic species, such as

Ruminococcus flavefaciens, R. albus and *Butyrivibrio fibrisolvens* (Bryant, 1973). Despite its primary role as a cellulose digester, *F. succinogenes* can also ferment starch (Hungate, 1966; Russell & Hespell, 1981; Ogimoto & Imai, 1981; Russell, 1984).

Butyrivibrio fibrisolvens, although not adapted to the same range of substrates as *B. ruminicola* and *S. ruminantium*, is also "*one of the most numerous (and) versatile . . . rumen bacteria* " (Hungate *et al.*, 1964), fermenting a wide range of carbohydrates (Bryant, 1959; Hungate, 1966; Bryant, 1973; Ogimoto & Imai, 1981; Russell, 1984). Although this species has cellulolytic activity, Hungate (1966) considers it doubtful whether it actively decomposes cellulose in the rumen. The species is generally classified as a "starch-digester", but is often also listed as being widely adapted.

A numerically important bacterial species in the rumen of animals fed high-grain diets (up to 10 % of the total bacteria present) is *Ruminobacter amylophilus* (formerly *Bacteroides ruminicola* - Stackebrandt & Hippe, 1986)(Hungate, 1966; Russell & Hespell, 1981; Hespell, 1984). As for *F. succinogenes, Ruminobacter amylophilus* mainly ferments starch and its hydrolytic products, but not glucose (Bryant, 1959; Hungate, 1966; Ogimoto & Imai, 1981). In addition, it may be one of the principal proteolytic organisms in the rumen, although its significance in the *in vivo* degradation of protein in the rumen is not clear (Hobson & Wallace, 1982b; Russell, 1984). Despite these features, *Ruminobacter amylophilus* has a high affinity for ammonia, its major nitrogen source (Hungate, 1966; Mahadevan *et al.*, 1980; Russell & Hespell, 1981; Hespell, 1984).

Another bacterial species which can constitute numerically significant proportions of the rumen microbial population is *Streptococcus bovis*. This starch-digesting bacterium proliferates when rations containing a large quantity of soluble starch (e.g. wheat grain) are fed to an animal, resulting in lactic-acidosis due to its production and release of lactate (Hungate, 1966; Ogimoto & Imai, 1981; Russell & Hespell, 1981; Akin, 1982; Russell, 1984).

Although this list is by no means an exhaustive summary of the bacteria found in the rumen (see Bryant, 1959; Hungate *et al.*, 1964; Hungate, 1966; Ogimoto & Imai, 1981 for more), it does represent most of the numerically important species of rumen bacteria, and serves to illustrate the great variety of bacterial species, and their roles, in the rumen.

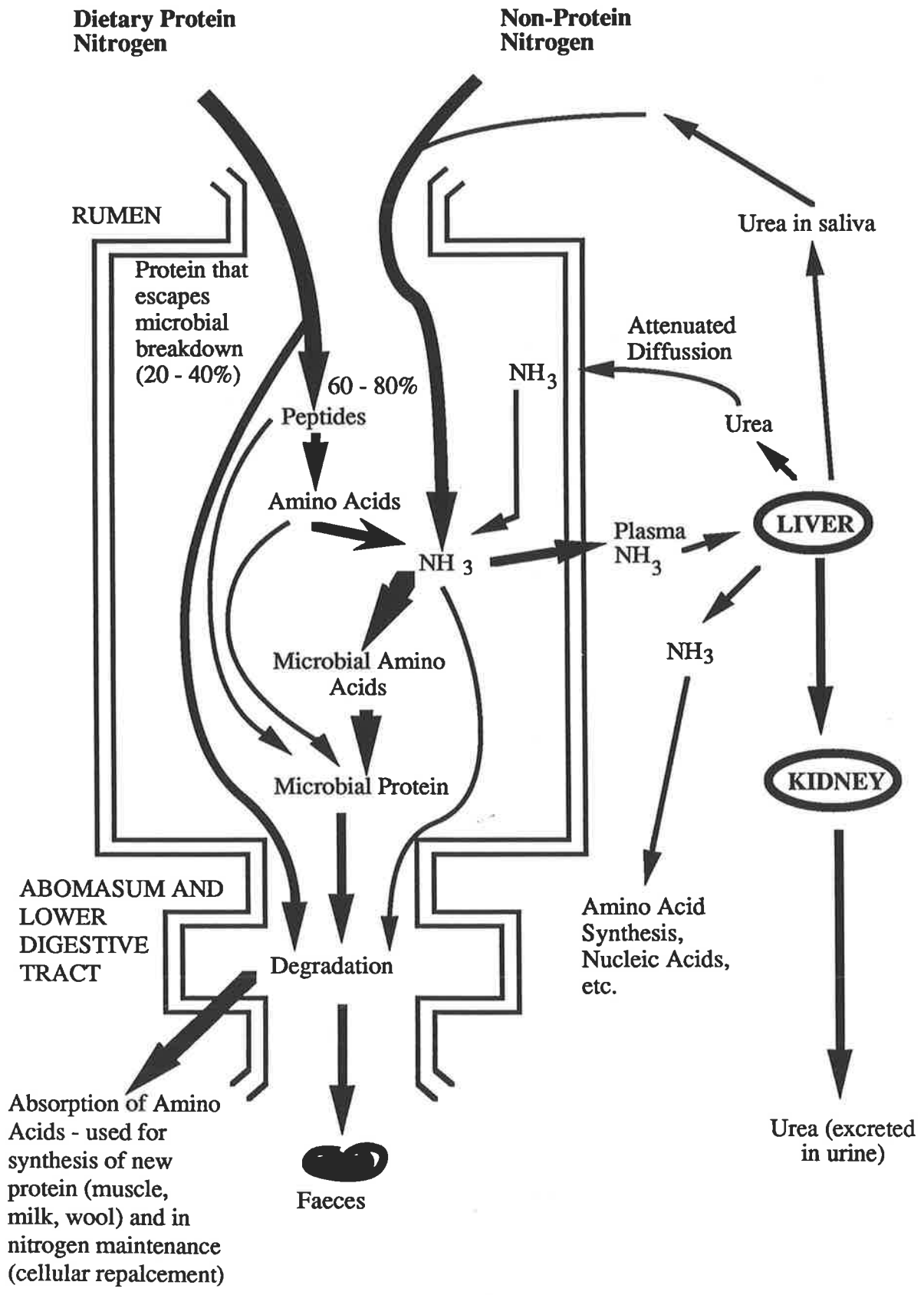
1.3 NITROGEN AND THE RUMINANT

Nitrogen, carbon, hydrogen, oxygen, phosphorous, and sulphur make up about 99 % of all living tissues, despite the fact that they are only six of the 92 naturally occurring elements (Curtis, 1980). Nitrogen, in particular, is a fundamental building block in the synthesis of many biological macromolecules, for example amino acids and proteins, purines, pyrimidines, nucleic acids, porphyrins, alkaloids and vitamins (Conn & Stumpf, 1976).

Ruminant animals, especially under grazing conditions, derive virtually all of their protein and non-protein nitrogen from digestion of plant material, whereas more intensive production systems (e.g. feedlots) also rely on protein and non-protein nitrogen derived from other sources (Thomas, 1985). The fate of this protein and non-protein nitrogen in the ruminant is presented in Figure 1.1. Some of the dietary protein nitrogen, which constitutes 80 - 90 % of herbage nitrogen, escapes breakdown in the rumen (20 - 40 % of the protein), while the rest is broken down to varying degrees by microbial action to yield peptides, amino acids, and ultimately ammonia (Annison, 1965; Chalupa, 1972; Smith, 1975; Buttery, 1977; Tamminga, 1979; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Hume, 1984; Leng & Nolan, 1984; Czerkawski, 1986). Non-protein nitrogen, which can constitute 10 - 20 % of the total nitrogen content of herbage, consists of free amino acids, peptides, nucleic acids, purine and pyrimidine bases, amines, amides, alkaloids, choline, urea, nitrates and ammonia (Barnett & Reid, 1961; Church, 1979d; Nikolic *et al.*, 1980; Hobson & Wallace, 1982b). These compounds are largely broken down to ammonia before being assimilated by the rumen microflora (Annison, 1965; Allison, 1969; Chalupa, 1972; Smith, 1975; Buttery, 1976, 1977; Russell & Hespell, 1981; Owens & Bergen, 1983; Hume, 1984; Leng & Nolan, 1984).

The microbial population of the rumen degrades most of the feed components entering the rumen and the breakdown products are utilised by the microorganisms for synthesis of their own cell components (Buttery, 1976; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Owens & Bergen, 1983; Leng & Nolan, 1984). This process of degradation of feed proteins and resynthesis of microbial proteins seems somewhat wasteful (Nolan, 1975), but ultimately it supplies 40 - 80 % of the nitrogen requirement of the animal by partial flushing of the rumen microflora into the abomasum and small intestine, where the microbial amino acids and peptides are absorbed. The remaining dietary nitrogen originates from digestion of undegraded feed proteins in the intestinal tract, and from direct absorption of rumen ammonia into the

Figure 1.1 : A scheme for nitrogen utilisation by the ruminant animal (compiled from Buttery, 1976; Satter & Roffler, 1977; Church, 1979d).



bloodstream (Hungate, 1966; Allison, 1970; Buttery, 1976; Owens & Bergen, 1983; Leng & Nolan, 1984). Thus, ammonia has a central role in the nutrition of the animal, since 60 - 80 % of the microbial nitrogen passes through the rumen ammonia "pool", after which it is synthesised into microbial protein (Figure 1.1 : Chalupa, 1972; Johnson, 1976; Buttery, 1977; Hespell & Bryant, 1979; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Baldwin & Allison, 1983; Hume, 1984; Leng & Nolan, 1984).

1.3.1 AMMONIA TERMINOLOGY

In keeping with reviews of microbial nitrogen metabolism by Brown *et al.* (1974) and Brown (1980), ammonia (or NH_3) is used throughout this thesis to identify both the NH_3 and the NH_4^+ forms of the compound. This imprecise terminology is often used although, with a pK (dissociation) of 9.25 at 24 °C, and under the conditions which occur in the rumen (pH 5.0 - 7.5 according to Czerkawski, 1986), more than 99 % of any ammonia would be present as NH_4^+ (Allison, 1970; Stevenson & Silver, 1977; Brown, 1980; Stewart, 1980). Therefore, although NH_3 can rapidly pass through membranes by non-specific diffusion, very little is present in the rumen at pH 7.0 (and even less as the pH decreases). In contrast, the ionic form of the molecule (i.e. NH_4^+) is present in abundance, but requires a specific transport mechanism for rapid entry into bacterial cells (Stewart, 1980; Kleiner, 1985).

Despite this overwhelming evidence that NH_4^+ is the form of ammonia which is transported across rumen bacterial cell membranes (and subsequently assimilated), some doubt remains as to which of the forms is actually transported into the rumen (Brown *et al.*, 1974; Brown, 1980; Kleiner, 1985). Nevertheless, the fact remains that ammonia, in whatever form, is the most important source of nitrogen in the rumen.

1.3.2 ALTERNATIVE FATES OF RUMEN AMMONIA

Although ammonia is the major source of nitrogen for microbial protein synthesis in the rumen (Blackburn, 1965; Allison, 1969; Chalupa, 1972; Smith, 1975; Buttery, 1976; Johnson, 1976; Hobson & Wallace, 1982b; Baldwin & Allison, 1983; Owens & Bergen, 1983; Leng & Nolan, 1984), its concentration is sometimes in excess to the needs of the microbes. Some of the excess ammonia is absorbed through the rumen epithelium into the

venous blood which drains the rumen, whilst the rest may be flushed from the rumen into the omasum, abomasum and small intestines, where it is absorbed into the bloodstream of the animal. Some dietary nitrogen, however may be excreted in the faeces, especially when high ammonia levels occur in the rumen. Ammonia which is absorbed into the bloodstream is transported to the liver where it is detoxified to urea and is largely excreted in the urine (Barnett & Reid, 1961; Annison, 1965; Blackburn, 1965; Buttery, 1976, 1977; Satter & Roffler, 1977; Hobson & Wallace, 1982b; Owens & Bergen, 1983; Hume, 1984). Alternatively, the urea produced in the liver may be utilised as a nitrogen source by body tissues, via conversion to glutamine, or to nucleotides (Barnett & Reid, 1961; Armstrong & Hutton, 1975).

Under "normal" nutrition, in which energy and nitrogen supply is relatively balanced, the liver is able to effectively detoxify and eliminate all of the free ammonia transported to it (Barnett & Reid, 1961; Buttery, 1976; Satter & Roffler, 1977; Owens & Bergen, 1983; Hume, 1984). Under some nutritional conditions a proportion of the ammonia may be recycled to the rumen as urea by transfer from blood plasma to the saliva and then to the rumen (Barnett & Reid, 1961; Annison, 1965; Satter & Roffler, 1977; Hobson & Wallace, 1982b; Hume, 1984). Another pathway of recycling, which is generally considered to be of minor importance, is attenuated (or facilitated) diffusion through the rumen epithelium. In this pathway urea diffuses from the bloodstream into the surrounding mucosa, and the urease-producing bacteria within the tissue-adherent population of the rumen (approx. 10 - 15 % of this special population) hydrolyse this urea. The liberated ammonia then rapidly diffuses through the rumen epithelium into the rumen, where it is trapped by conversion to ammonium ions at the neutral pH of the rumen. The bacterial urease activity, and ultimately nitrogen recycling to the rumen via this pathway, is therefore regulated by the concentration of ammonia in the rumen (Annison, 1965; Church, 1979d; Cheng & Costerton, 1980; Hobson & Wallace, 1982b; Owens & Bergen, 1983).

Although the liver is normally able to detoxify and eliminate all the free ammonia transported to it, the concentration of ammonia can become too high for this to occur effectively under some feeding conditions. Thus, when diets are supplemented with urea the ammonia circulating in the animal may reach toxic levels (James *et al.*, 1975; Huber & Kung, 1981). Acute ammonia toxicity is associated with rumen ammonia concentrations above 50 mM at pH 8 and above, and blood plasma concentrations above about 1 mM. The only effective treatment for acute toxicity is acidification of the rumen (e.g. with 5 - 10 % [v/v] acetic acid), or removal

of the ruminal contents, however these treatments are only useful before onset of tetany in the animal (Huber & Kung, 1981; Owens & Bergen, 1983). Subclinical ammonia toxicity may also occur, resulting in an altered carbohydrate metabolism in the liver and reducing the energetic efficiency of lactation, however urea *per se*, and urea feeding when care is taken, are normally harmless to the animal (Owens & Bergen, 1983).

Although ammonia recycling is clearly possible in ruminants, the amount of ammonia (as urea) actually recycled to the rumen is very variable, depending upon energy supply, feed intake and the microbial population, in addition to the ruminal ammonia, and plasma urea concentrations (Buttery, 1977; Satter & Roffler, 1977; Owens & Bergen, 1983; Hume, 1984). Nevertheless, despite the mechanisms for recycling dietary nitrogen that is not utilised during its first passage through the rumen, a considerable proportion of the unused nitrogen is not recycled, and is therefore wasted by the animal (Barnett & Reid, 1961).

1.4 LIMITATIONS OF THE RUMINANT DIGESTIVE SYSTEM

There are inherent limitations associated with a ruminant digestive system which limits the maximum productive potential of the animal (i.e. of growth, reproduction, lactation and wool production). For example, on high fibre diets when digesta move slowly out of the rumen there is a restricted intake (Hume, 1984). In addition, limitations result from the indirect pathways taken by nutrients before becoming available to the animal. Microbial protein, which supplies most of the protein-nitrogen for the ruminant, is not synthesised directly from plant amino acids, peptides and proteins, but from ammonia largely produced by degradation of this dietary nitrogen (Annison, 1965; Hungate, 1966). As a consequence, there is a mixture of proteins in the abomasum and small intestines which are often very different from that of the original feed protein (Annison, 1965; Thomas, 1985). Nevertheless, the amino acid composition and biological value of this protein varies little between diets (Weller, 1957; Barnett & Reid, 1961; Hungate, 1966; Chalupa, 1972; Williams & Dinusson, 1973; Beever & Thomson, 1977; Church & Fontenot, 1979; Smith, 1979; Huber & Kung, 1981; Orskov, 1982; Owens & Bergen, 1983; Thomas, 1985).

Thus, although rumen microorganisms are apparently able to synthesise all the amino acid requirements of the ruminant, including the ten essential amino acids (i.e. arginine, histidine,

isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan) (Hungate, 1966; Sauer *et al.,* 1975), there are conditions where the amino acid composition of the microbial protein leaving the rumen is not ideal for maximal productivity (Buttery, 1976; Beever & Thomson, 1977). This is largely due to the requirement for higher concentrations of some specific amino acids, such as methionine, threonine and lysine, which are essential for wool and milk production. Although elevated levels of these amino acids are often present in high quality feeds and supplements, these additional amino acids are lost to the animal as a result of ruminal activity (Barnett & Reid, 1961; Buttery, 1976, 1977; Owens & Bergen, 1983).

Manipulation of the ruminant digestive system to overcome these limitations has focussed on protection of dietary proteins from rumen degradation, the principal objective being to enhance the supply of essential amino acids to the productive animal (Chalupa, 1972, 1975; Beever & Thomson, 1977; Tamminga, 1979; Hobson & Wallace, 1982b; Orskov, 1982; Owens & Bergen, 1983; Hume, 1984). Heat and chemical treatment of dietary proteins have achieved this objective, however practical and economical procedures have not been devised as yet (Allison, 1970; Ferguson, 1975; Church, 1979d; Tamminga, 1979; Huber & Kung, 1981; Owens & Bergen, 1983; Thomas, 1985). Certain plant proteins which are naturally protected from bacterial attack in the rumen may also be used to enhance the amino acid supply to the animal, and are a more practical means of doing so. Unfortunately, these proteins are deficient in some amino acids and therefore do not markedly increase production (Barnett & Reid, 1961; Beever & Thomson, 1977; Oldham, 1980). In contrast, hydroxymethyl-DL-methionine is an example of the successful use of a specific amino acid protected by chemical or physical treatment to enhance productivity, in particular wool growth (Chalupa, 1972, 1975; Beever & Thomson, 1977; Reis, 1979; Tamminga, 1979; Oldham, 1980; Simpson & Stobbs, 1981).

In addition to the restriction that diets of both low and high fibre content place on animal productivity, and the limitations resulting from microbial action on dietary proteins, absolute nitrogen supply to the animal may be a limiting factor in the attainment of optimum growth and production in grazing animals (Coombe, 1981; Simpson & Stobbs, 1981). This is particularly evident during the dry period in Mediterranean and tropical climates, where sub-optimal performance is commonly related to low levels of protein (i.e. nitrogen) in the pastures, and is especially evident during periods of high nutrient demand, such as pregnancy, lactation and in young, rapidly growing grazing animals (Schwartz & Gilchrist, 1975; Orskov, 1980; Preston

& Leng, 1980; Allden, 1981, 1982; Mannetje, 1982; Reid & Jung, 1982). The result of such restrictions are reduced rates of growth and decreased production of milk and wool (Schwartz & Gilchrist, 1975; Simpson & Stobbs, 1981; Thomas, 1985).

1.5 NITROGEN SUPPLEMENTS TO OVERCOME NITROGEN DEFICIENCY

A common practice to overcome nitrogen deficiency is to supplement feed with cheap, non-protein nitrogen, such as organic and inorganic ammonium salts, ammoniated feeds and byproducts, dicyanodiamide, isobutylidene diurea, biuret or urea. These supplements are supplied either alone or in combination with an energy source, such as molasses (Chalupa, 1972; Johnson, 1976; Bartley & Deyoe, 1977; Church & Fontenot, 1979; Nikolic *et al.*, 1980; Huber & Kung, 1981; Simpson & Stobbs, 1981; Orskov, 1982; Thomas, 1985). The most widely used non-protein nitrogen supplement is urea, however, contrary to popular belief, its use does not re-establish optimal weight gains and productivity in animals. In fact, it is usually only effective in maintaining supplemented animals (i.e. preventing weight loss) and does not result in a major gain in weight, wool growth or milk production. This is because the nitrogen is released (in the form of ammonia) too rapidly, resulting in considerable quantities of ammonia being absorbed through the rumen epithelium and transported to the liver where it is detoxified (Barnett & Reid, 1961; Chalupa, 1972; Johnson, 1976; Bartley & Deyoe, 1977; Nikolic *et al.*, 1980; Allden, 1981; Coombe, 1981; Huber & Kung, 1981; Simpson & Stobbs, 1981; Hynd & Radcliffe, 1985). A similar situation often arises in stock grazing high-quality pasture (Thomas, 1985).

A high concentration of ammonia in the rumen, as a result of a bolus in supplemented animals, or grazing of high-quality pasture, usually results in a large loss of nitrogen to the animal through excretion, but may also result in ammonia toxicity. This occurs when the liver is unable to effectively detoxify the large quantity of ammonia. Bartley & Deyoe (1977) report that varied results have been achieved from attempts to reduce the rate of conversion of non-protein nitrogen to ammonia in the rumen, thereby reducing the production of excess ammonia. It appears that ammonia released from non-protein nitrogen cannot be converted to microbial protein unless a carbon skeleton and source of energy are also available (Johnson, 1976; Huber & Kung, 1981). It is therefore likely that conversion of non-protein nitrogen to microbial protein

would be maximised (and excess ammonia minimised) when the conversion of a feed component to ammonia occurs at a similar rate to the metabolism of carbohydrates (Johnson, 1976; Bartley & Deyoe, 1977; Corse, 1977; Coombe, 1981; Huber & Kung, 1981; Hobson & Wallace, 1982b). The efficient use of non-protein (i.e. supplementary) nitrogen by ruminants may also be limited by the supply of vitamins, minerals (especially sulphur, for the synthesis of the sulphur-containing amino acids, and phosphorus, for the synthesis of nucleic acids) and trace elements (Bartley & Deyoe, 1977; Buttery, 1977; Corse, 1977; Orskov, 1982; Thomas, 1985).

Many, largely unsuccessful, attempts have been made to alleviate the problems of supplementary nitrogen for grazing animals (Johnson, 1976; Bartley & Deyoe, 1977; Coombe, 1981; Huber & Kung, 1981). Successful use of non-protein nitrogen as a supplement for ruminants at pasture is a worthwhile goal, since not only would pasture nitrogen deficiencies be compensated for effectively, thereby ensuring continuing weight gains throughout the year, but animals would also be able to more effectively utilise the pasture nitrogen normally available to them. Feed urea is rapidly degraded in the rumen, yielding high ammonia concentrations, however the bulk of this ammonia is not utilised effectively by either the microorganisms or the animal. In the microorganisms it is assumed that the main limitation is the rate at which the bacterial enzymes can metabolise large amounts of ammonia, since high levels of ammonia apparently are not harmful to the bacteria (Smith, 1975; Bartley & Deyoe, 1977; Buttery, 1977; Harrison & McAllan, 1980). In the animal, large amounts of ammonia which are not metabolised into protein are absorbed through the rumen epithelium and transported to the liver, where it is detoxified and often excreted (Barnett & Reid, 1961; Chalupa, 1972; Buttery, 1976; Bartley & Deyoe, 1977; Allden, 1981; Coombe, 1981; Simpson & Stobbs, 1981). These metabolic barriers therefore appear to be the major factors restricting the use of supplementary nitrogen feeding programmes and the effective utilisation of high-quality pasture protein for animal production.

Clearly, the formation, and excretion, of excess nitrogen by ruminants is wasteful and expensive, directly in the case of supplementary nitrogen, or indirectly in the case of high quality pastures. It would therefore be of great benefit to animal productivity and profitability if this wastage could be eliminated. For this to occur it is necessary to understand fully how the nitrogen is utilised, and in the whole animal this is reasonably well understood (Bartley & Deyoe, 1977; Buttery, 1977; Corse, 1977; Simpson & Stobbs, 1981; Orskov, 1982; Thomas,

1985). In contrast, relatively little is known of how various species of rumen microorganisms utilise nitrogen and, in particular, how this affects nitrogen utilisation by the whole animal.

More information is therefore required about the way rumen microorganisms assimilate and metabolise ammonia and other nitrogen sources so that a better regulation can be achieved of the nitrogen losses, possibly through genetic manipulation. Before analysing the state of knowledge of nitrogen utilisation by rumen bacteria it is pertinent to first consider *in vivo* versus *in vitro* studies of the rumen and its bacteria.

1.6 METHODS FOR STUDYING RUMEN BACTERIA

Although only one of the components of the rumen ecosystem, rumen bacteria are numerically the major component of the microbial population (10^{10} - 10^{11} cells per ml of digesta compared to 10^5 - 10^6 protozoa per ml of digesta : Czerkawski, 1986). Fungi are also important components of the rumen microbial population, however both microbial crude protein synthesis and outflow (from the rumen) depend primarily upon the bacterial component. In fact, they supply up to 90 % of the microbial crude protein flowing from the rumen, in addition to many end products of metabolism, such as acetic, propionic and butyric acids. Nevertheless, fungi and protozoa are active in the rumen (Buttery, 1976; Church, 1979d; Owens & Bergen, 1983; Leng & Nolan, 1984; Czerkawski, 1986). The bacterial component of the rumen ecosystem was chosen for study of nitrogen metabolism and assimilation.

1.6.1 *In vivo* STUDIES

Ruminal bacteria can be studied using a variety of methods (Hobson, 1976). One such method is to analyse samples of rumen fluid (i.e. *in vivo* samples), however there are a number of problems associated with these experiments. Firstly, the sample taken from the rumen must be representative of the total microbial population (Hobson & Wallace, 1982a). This is only possible for animals fitted with permanent rumen fistulae, but it is still essential to sample from several regions of the rumen, since the microbial population is stratified with feed particles within the rumen (Barnett & Reid, 1961; Hungate, 1966; Orpin, 1982; Russell & Hespell, 1981). In less sophisticated experiments where permanent fistulae are not employed, representative rumen samples are much more difficult to obtain (Hungate, 1966; Clarke, 1977; Orpin, 1982).

A second major problem with *in vivo* studies is the influence of the animal on the microbial population (Hobson, 1976). For example, if one animal eats, drinks or ruminates at different times during the experiment, or takes in quantities of feed or water prior to sampling, the rumen population may be affected by the feed and water intake rather than as a consequence of the quality of the feed or any other treatment (Warner, 1965; Hungate, 1966; Hobson, 1976; Church, 1979c; Leng, 1985). Similarly, animals under the same dietary and environmental conditions do not necessarily have identical rumen microbial populations (Warner, 1965; Hungate, 1966; Church, 1979c; Leng, 1985), hence repetitive sampling in different animals may not reduce the amount of variation that appears when samples are taken from only one animal on different occasions.

A further problem with *in vivo* experiments is that even repeated sampling through a rumen fistula (the only effective means of continuous sampling - Hungate, 1966; Orpin, 1982) is not consistently representative of the rumen, given that it is virtually impossible to repeat sample from exactly the same region of the rumen. Moreover, rumen movements and metabolism continuously change the microbial mixture so that serial samples would be affected (Hungate, 1966; Church, 1979b). Nevertheless, Hobson & Wallace (1982a) believe that, if samples are taken in the same way for one animal, or for a number of animals sustained on the same kind of feed, although absolute values may be in error, relative values may be meaningful.

Another limitation of *in vivo* studies is that, even if a "consistent" and "representative" sample were prepared from the rumen, the processing methods employed to separate the bacterial fraction from the protozoal, fungal and plant fractions introduce further sources of error. This is particularly the case for those bacteria attached to plant material (50 - 90 % of the total bacteria in the rumen : Cheng & Costerton, 1980; Merry & McAllan, 1983; Leng, 1985), which often are not readily released during sample preparation, thus resulting in samples of predominantly "free" from rumen fluid, rather than a representative mixture of all bacteria in the rumen (Cheng & Costerton, 1980; Harrison & McAllan, 1980; Merry & McAllan, 1983; Leng, 1985). It has been tacitly assumed that the chemical composition of the bacteria from both "attached" and "free" populations are identical and that values derived from mixed bacteria separated from the liquid phase of the rumen digesta are representative of the

population leaving the rumen, if not of the total population *per se* (Merry & McAllan, 1983). This assumption, however, may not be tenable (Hobson, 1976; Russell, 1984).

1.6.2 *In vitro* MIXED CULTURES

Although the rumen is effectively a continuous culture system, with a continual inflow of saliva, or food mixed with saliva, and an outflow of digesta to the omasum (Hungate, 1966; Orpin, 1982), the system is too complicated for detailed analysis of the microbial population *in vivo* (Barnett & Reid, 1961; Hobson, 1976; Czerkawski, 1986). Attempts have been made to develop *in vitro* systems of rumen fermentation which duplicate the rumen environment whilst allowing some degree of control over the fermentation processes (Barnett & Reid, 1961; Hobson, 1976; Orpin, 1982; Russell & Hespell, 1981; Czerkawski, 1986). One such system, referred to as "Rusitec" (short for **Ru**men **si**mulation **tec**hnique), has a number of advantages over *in vivo* studies, since a stable microbial population, resembling that of the rumen, can be developed and maintained for up to a few weeks. This enables a study to be made of a carefully controlled "rumen" fermentation and analysis of the effects of various manipulations on the population and the fermentation (Hobson, 1976; Czerkawski, 1986; Orpin, 1982).

Other, less sophisticated, continuous culture systems can also be used to study components of the rumen system *in vitro*, although they also suffer from one of the major drawbacks of *in vivo* studies, namely the presence of a complex mixture of microorganisms having different metabolic activities so that the data are difficult to interpret (Allison, 1965; Hobson, 1976). Similarly, less sophisticated *in vitro* systems, involving batch cultures of rumen fluid, and which are generally used for forage evaluation (i.e. digestibility appraisal - Tilley & Terry, 1963), are also difficult to interpret because some microorganisms grow rapidly in this artificial mixed culture system compared with the way they would grow in their natural environment (Barnett & Reid, 1961; Allison, 1965; Russell & Hespell, 1981). These systems, although more rapid and repeatable than trials with animals, are difficult to relate to the *in vivo* situation, since they cannot duplicate the animal exactly (Barnett & Reid, 1961; Hobson, 1976; Russell & Hespell, 1981).

1.6.3 *In vitro* PURE CULTURES

Both continuous and batch culture systems can be used for pure cultures of rumen bacteria, the former possibly being closer to a rumen simulation because sub-samples contain cells at all phases of growth, as is the case in mixed culture *in vitro*, and in *in vivo*, experiments. In contrast, batch cultures contain cells which are relatively close to the same stage of growth (e.g. early log, mid log, late log or stationary phase). In addition to the problems of other *in vitro* experiments, analysis of selected species of rumen bacteria in pure culture may not be relevant to the natural system. In batch cultures the growth conditions can be more easily manipulated and are simpler to operate than continuous culture systems. This is an important consideration, since rumen microorganisms are either obligate or facultative anaerobes (Hungate, 1966).

1.6.4 SELECTION OF BACTERIAL SPECIES FOR PURE CULTURE STUDIES

Baldwin & Allison (1983), in a review of rumen metabolism, considered a three stage development of knowledge of rumen ecology. The first has involved the isolation, taxonomic identification and enumeration of the microbes present in the rumen. The second stage involves metabolic studies on predominant rumen microbes, and the third stage involves quantitation and dynamic analyses of all aspects of the microbial population in mixed culture, or *in vivo*.

The second stage of this study program requires a prudent choice of bacterial species for determining the major pathways of nitrogen assimilation and metabolism occurring in rumen bacteria, since these pathways are likely to reflect the metabolic activity of the numerically predominant species in the rumen population. The following rumen bacterial species were initially chosen for investigation in pure culture because they are representative of the important nutrient niches in the rumen, and often comprise significant proportions of the rumen community (Hungate, 1966) - *Bacteroides ruminicola* subsp. *brevis*, *B. ruminicola* subsp. *ruminicola*, *Butyrivibrio fibrisolvens*, *Fibrobacter (Bacteroides) succinogenes* subsp. *succinogenes*, *Ruminobacter (Bacteroides) amylophilus*, *Selenomonas ruminantium* subsp. *lactilytica* and *S. ruminantium* subsp. *ruminantium*. For example, *B. ruminicola* subsp. *brevis*, *B. ruminicola* subsp. *ruminicola* and *S. ruminantium* subsp. *ruminantium* are adapted to a wide range of substrates and are common constituents of the rumen microbial

community. *S. ruminantium* subsp. *lactilytica* also is adapted to a wide range of substrates, and is one of the few rumen bacteria to ferment lactate, however *Butyrivibrio fibrisolvens* is amylolytic, but can also metabolise many other substrates, including cellulose (at least in pure culture). *F. succinogenes* subsp. *succinogenes* is a cellulolytic organism, and *Ruminobacter amylophilus* is both proteolytic and amylolytic (Bryant, 1959; Bryant, 1963; Hungate, 1966; Kingsley & Hoeniger, 1973; Hespell & Bryant, 1981; Russell & Hespell, 1981; Ogimoto & Imai, 1981; Krieg & Holt, 1984). These species include some of those that greatly influence nitrogen assimilation and metabolism in the rumen.

1.7 NITROGEN SOURCES FOR RUMEN BACTERIAL GROWTH

It is known that ammonia is the major nitrogen source for the synthesis of microbial protein in the rumen, with 60 to 80 % of microbial nitrogen passing through the rumen ammonia "pool" (Blackburn, 1965; Allison, 1969; Chalupa, 1972; Smith, 1975; Johnson, 1976; Buttery, 1976, 1977; Church, 1979d; Hespell & Bryant, 1979; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Baldwin & Allison, 1983; Owens & Bergen, 1983; Hume, 1984; Leng & Nolan, 1984), however a number of other nitrogen sources are available for microbial growth in the rumen (Barnett & Reid, 1961; Allison, 1969; Chalupa, 1972; Church, 1979d; Patterson & Hespell, 1979; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Owens & Bergen, 1983). Feed and microbial proteins, as well as peptides and free amino acids from partial protein degradation, are the most abundant "alternative" nitrogen sources for microbial growth in the rumen, however some other non-protein nitrogen sources present include free amino acids, peptides, nucleic acids, purine and pyrimidine bases, amines, amides, alkaloids, choline, urea and nitrates (Barnett & Reid, 1961; McAllan & Smith, 1973; Smith, 1975; Church, 1979d; Patterson & Hespell, 1979; Nikolic *et al.*, 1980; Russell & Hespell, 1981; Hobson & Wallace, 1982b).

1.7.1 NITROGEN SOURCE PREFERENCES

Given the large number of nitrogen sources available for microbial growth in the rumen it is important to establish which of these is preferred for growth by the microorganisms.

Barnett & Reid (1961) state that non-protein nitrogen sources are largely degraded to ammonia before being assimilated by the rumen microflora. Hobson & Wallace (1982b), however, indicate that nucleic acids are degraded to purine and pyrimidine bases, some of which are metabolised by protozoa. Nitrate is used as a nitrogen source by some bacteria, or is reduced via nitrite to ammonia (Hobson & Wallace, 1982b). Urea also is hydrolysed to ammonia, which is then utilized for microbial growth (Chalupa, 1972; Johnson, 1976; Hobson & Wallace, 1982b).

Of the major nitrogen sources, protein is not utilised by rumen microorganisms *per se*, but much of it is degraded by the proteolytic activity in the rumen to form peptides, free amino acids, and ultimately ammonia (Blackburn, 1965; Chalupa, 1975; Tamminga, 1979; Russell & Hespell, 1981; Brock *et al.*, 1982; Hobson & Wallace, 1982b; Russell, 1984). Peptides are utilised as nitrogen sources by some rumen microorganisms (Allison, 1970; Mercer & Annison, 1976; Hespell & Bryant, 1979; *B. ruminicola, B. melaninogenicus* : Pittman & Bryant, 1964; Pittman *et al.*, 1967; Hobson & Wallace, 1982b; Russell, 1983 ; *Ruminobacter amylophilus* - Hullah & Blackburn, 1971), and by crude rumen samples *in vitro* even more efficiently than free amino acids (Wright, 1967; Allison, 1969; Allison, 1970; Maeng *et al.*, 1976; Cotta & Russell, 1982; Russell, 1984). Thus, Hobson & Wallace (1982b) speculate that this preference for oligopeptides may be a more general property of rumen microorganisms. The preference of some ruminal bacteria for preformed (peptide), over free, amino acids indicates that they have a preferential capacity to transport peptides compared with amino acids, and that there are separate transport systems for each nitrogen form (Pittman & Bryant, 1964; Allison, 1970; Mercer & Annison, 1976; Payne & Gilvarg, 1978; Becker & Naider, 1980; Payne, 1980a, b, c; Snell, 1980; Hobson & Wallace, 1982b).

Peptide transport into rumen bacterial cells, which clearly must be an active process due to the size of the molecules, has been attributed to a relatively non-specific system, since there is a wide variety of peptides which may be transported (Pittman & Bryant, 1964; Pittman *et al.*, 1967; Allison, 1970; Tamminga, 1979; Payne & Gilvarg, 1978). Although such a system normally results in accumulation of peptides within the cells, this has not been observed in *B. ruminicola*. This may be due to the rapid degradation of transported peptides to their constituent amino acids within the cell (Payne & Gilvarg, 1978; Wolfinbarger, 1980b). Alternatively, it has been suggested that peptide utilisation may involve non-specific binding of the peptide to the

bacterial cell-wall, followed by conversion into its constituent amino acids, which are transported into the cell (Pittman *et al.*, 1967; Allison, 1970; Mercer & Annison, 1976; Payne & Gilvarg, 1978; Payne, 1980a, b, c; Snell, 1980; Wolfinbarger, 1980; Cotta & Russell, 1982; Hobson & Wallace, 1982b). A further possibility is that of "coupled hydrolytic uptake", in which the hydrolysis of peptides occurs as they are transported (Payne & Gilvarg, 1978; Payne, 1980a, b, c; Snell, 1980;). Thus, although the actual mechanism of peptide use by rumen bacteria is not clear, the amino acids which the cell derives from these compounds are assimilated into cell material or are excreted. It is also not clear whether the assimilation of peptides is direct from amino acids or via ammonia (Pittman *et al.*, 1967; Hobson & Wallace, 1982b).

In contrast, evidence of uptake systems for free amino acids have also been found in *B. ruminicola* and *Ruminobacter amylophilus* (Hullah & Blackburn, 1971; Stevenson, 1979; Hobson & Wallace, 1982b). Most amino acids apparently are transported across cell membranes by H^+-symport systems (active), in which the amino acid enters the cell along with one or more protons, by means of a specific carrier protein (Anraku, 1980; Booth & Hamilton, 1980; Eddy, 1980; Wolfinbarger, 1980; Alberts *et al.*, 1983). This proton gradient from the inside (low) to the outside of the cell is maintained by ATP-dependent pumping of protons out of the cell (Anraku, 1980; Booth & Hamilton, 1980; Alberts *et al.*, 1983). Nevertheless, systems which utilise ATP, or a metabolite derived from ATP, as a direct source of energy also occur (Booth & Hamilton, 1980). In *B. ruminicola* it has been suggested that such uptake provides a supplementary supply of amino acids (i.e. to those from peptides) for immediate use in biosynthesis, rather than a major source of nitrogen (Stevenson, 1979; Hobson & Wallace, 1982b). At least six distinct uptake systems have been described (Stevenson, 1979; Anraku, 1980), which is consistent with the chemical structures of the various amino acid groups involved. Nevertheless, it is generally thought that "*The fate of (free) amino acids in the rumen is predominantly to be broken down rather than to be incorporated intact into cell material, although some direct incorporation does occur* " (Church, 1979d; Hobson & Wallace, 1982b). This assertion is largely based on the low concentration of amino acids in ruminal fluid (Allison, 1970; Mercer & Annison, 1976; Church, 1979d; Hobson & Wallace, 1982b), which may, in fact, be too low to allow for good transport rates, resulting in low or suboptimal growth rates of the bacteria (Hespell & Bryant, 1979). Nevertheless, free amino acid nitrogen is apparently preferred over ammonia nitrogen by a small number of ruminal species *in vitro*

(Russell, 1984), and some preformed amino acids or peptides may be required for maximal microbial growth in the rumen (Blake *et al.*, 1983).

Peptides are therefore the preferred source of nitrogen for growth by some rumen bacteria. Free amino acids may not generally be used directly as a nitrogen source, however ammonia is clearly the most important and abundant nitrogen source for rumen microbial growth, and it can be utilised by most (about 80 %) rumen bacteria (Blackburn, 1965; Allison, 1969; Chalupa, 1972; Buttery, 1976; Russell & Hespell, 1981; Cotta & Russell, 1982; Hobson & Wallace, 1982b; Owens & Bergen, 1983; Russell, 1984). In the context of problems associated with non-protein nitrogen supplementation and its inefficiencies, it is therefore important to study ammonia assimilation in rumen bacteria.

1.7.2 FERMENTATION OF NITROGEN SOURCES FOR ENERGY

In addition to nitrogen sources, free amino acids and peptides can be fermented for energy production, however these processes have not been studied in detail (Tamminga, 1979; Russell *et al.*, 1983; Russell, 1984). Bladen *et al.* (1961) demonstrated that most rumen bacteria are unable to grow on amino acids or peptides alone (i.e. as nitrogen and energy source), a fact confirmed more recently for *B. ruminicola* by Russell (1983, 1984). Nevertheless, Russell *et al.* (1983) report that *Megasphaera elsdenii* is apparently one of the few rumen bacteria able to grow with peptides or amino acids both as a nitrogen and energy source.

Amino acids or peptides cannot be used as energy sources by many rumen bacteria, since the energy yield from amino acid fermentation is poor (Russell, 1984). Nevertheless, Russell (1984) has shown that *B. ruminicola* is able to use the carbon skeletons of amino acids for maintenance with about the same efficiency as it uses glucose, but this species cannot ferment peptides at a rate fast enough to meet its maintenance energy requirement. Thus, although the theoretical energy yield from amino acid fermentation may be sufficient to suggest that they can be used as an energy source by rumen bacteria, the limiting factor, in practice, may be the rate at which the cells can ferment amino acids and peptides (proteolysis, deamination and phosphorylation of carbon skeletons contribute to overall fermentation).

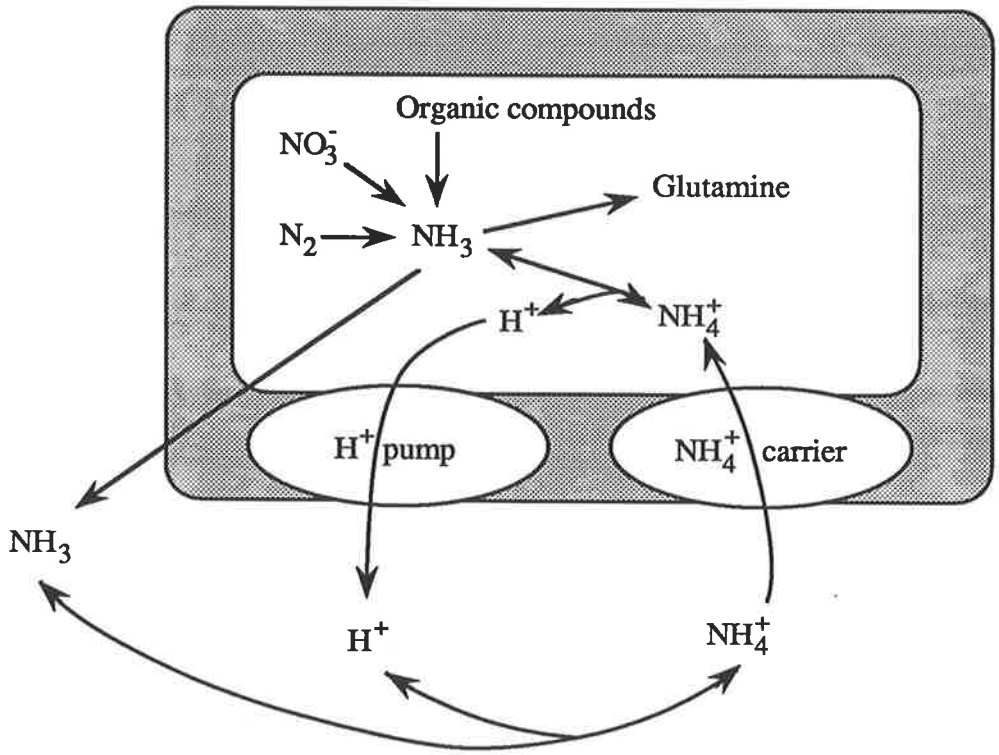
1.8 AMMONIA UPTAKE BY RUMEN BACTERIA

Hobson & Wallace (1982b) point out that the first stage in the synthesis of microbial amino acids (and ultimately proteins) from rumen ammonia is its uptake from the extracellular fluid. Little is known about the transport of ammonia across the cell membranes of rumen microorganisms, such as whether it is active or passive, specific or non-specific (Smith, 1979; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Hespell, 1984). Hobson & Wallace (1982b) state that knowledge of the transport systems of rumen bacteria have "... *important implications in interpreting kinetic data from the study of ammonia assimilating enzymes* ..." Should active transport be involved, the intracellular ammonia concentrations of the rumen bacteria may exceed the extracellular levels, thus making interpretation of these results difficult.

Kleiner (1985) lists more than 30 bacterial species, including some anaerobes (e.g. *Clostridium pasteurianum*), possessing ammonia transport systems, and states that many more species can be assumed to contain similar ammonia carriers under certain conditions. Similarly, Brown (1980) states that "*There is clear evidence that bacteria are able to retain ammonia intracellularly at concentrations higher than in the culture medium* ". Thus, ammonia transport appears to be fairly common in bacteria and may also occur in many rumen bacterial species. Possession of an active ammonia transport system would be an undoubted advantage for bacteria in the rumen, since it would enable the cells to regulate uptake so that adequate ammonia levels could be ensured in the cells under nitrogen limiting conditions (Kleiner, 1985). This type of regulation, which is often important for the first step in the metabolism of a compound, would not operate if ammonia uptake occurred by non-specific diffusion (Kleiner, 1985).

Although ammonia transport systems appear to be fairly common in bacteria and other microorganisms (Brown, 1980; Stewart, 1980; Kleiner, 1985), the exact mechanism of uptake is still not clear. Stevenson & Silver (1977) were the first to provide evidence for an NH_4^+ transport process in bacterial cells, based on data for an energy-dependent uptake of methylammonium in *Escherichia coli*. Subsequently Kleiner (1985) confirmed that ammonia uptake occurs via an energy-dependent NH_4^+ carrier. As a consequence of the high permeability of bacterial membranes to NH_3, Kleiner (1985) also suggests that the maintenance of intracellular NH_4^+ pools by bacteria living on N_2, NO_3^- or organic nitrogen sources involves energy-dependent cyclic NH_3 / NH_4^+ retention (Figure 1.2). That is, because the cellular membranes are so permeable to NH_3 it continuously diffuses out of the cell. Nevertheless,

Figure 1.2 : Proposed cyclic NH_3/NH_4^+ retention in the bacterial cell (after Kleiner, 1985).



when the external NH_4^+ concentration is high, diffusion of NH_3 through the membrane may be fast enough to support the cell's demand for nitrogen (Smith, 1979; Kleiner, 1985).

Definitive studies have not been carried out to determine the presence or otherwise of ammonia transport systems in rumen bacteria, however it is likely that they do occur since more than 99 % of the ammonia in the rumen is present as NH_4^+ and very little uptake of NH_3 is therefore likely to occur (section 1.3.1). A specific transport mechanism is therefore necessary for rapid entry of ammonia into the cells under the relatively low ammonia concentrations (< 10 mM) found in the rumen (Stewart, 1980; Russell & Hespell, 1981; Kleiner, 1985).

1.9 BIOCHEMISTRY AND ENZYMOLOGY OF THE ASSIMILATION AND METABOLISM OF NITROGEN COMPOUNDS

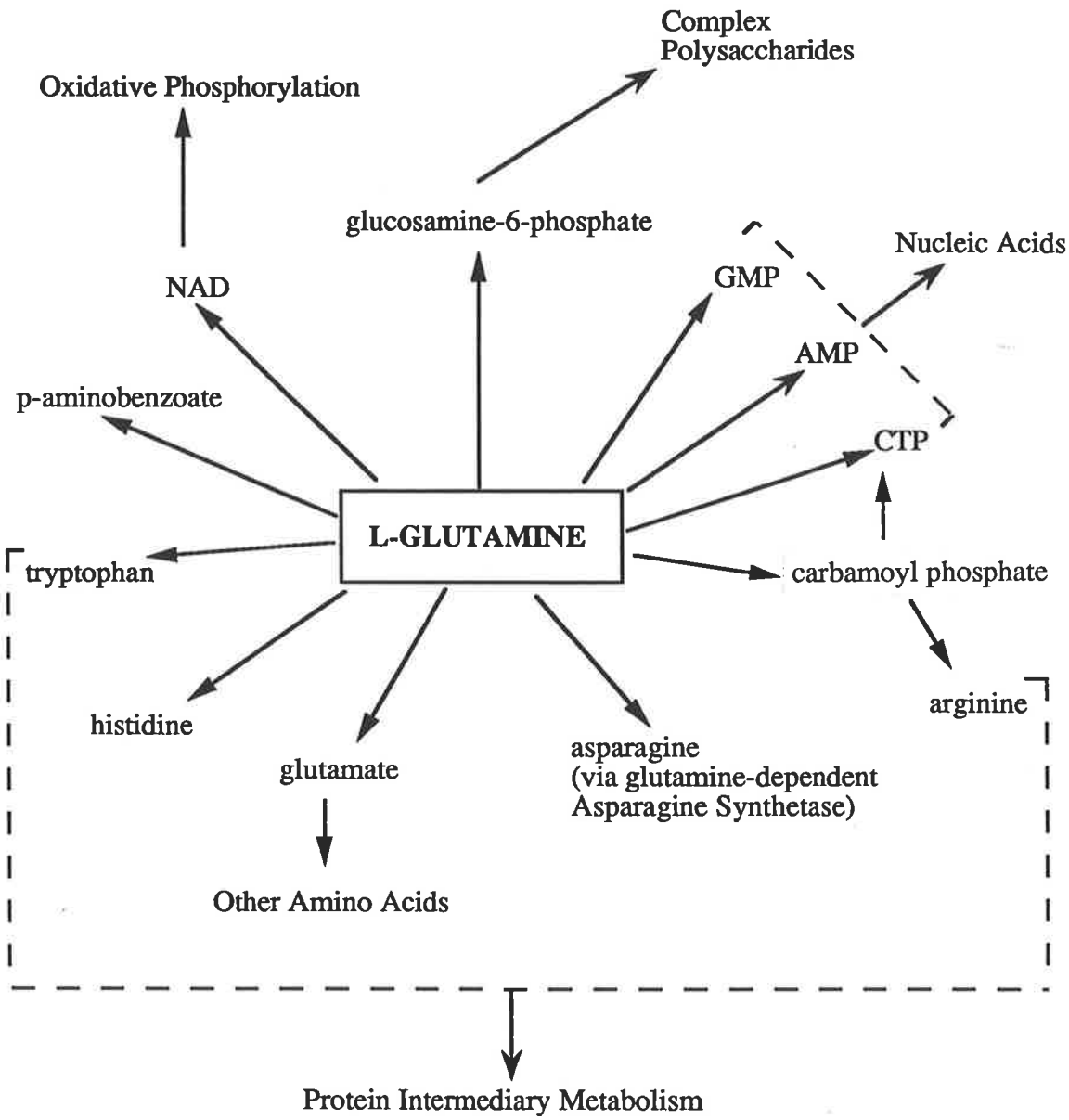
The assimilation of ammonia has been studied extensively in aerobic bacteria, particularly *E. coli*, and the enzymes have been characterised. These results have also been shown to apply to a variety of other organisms, including fungi and algae. In contrast, very few definitive studies have been made on ammonia assimilation in rumen bacteria, and these processes have therefore been assumed to follow the classical mechanisms described for aerobic bacteria.

1.9.1 THE ENZYMES OF AMMONIA ASSIMILATION

Glutamate and glutamine are central molecules in nitrogen metabolism in many bacteria, since most of the nitrogen assimilated via ammonia enters metabolism as the α-amino group of glutamate, and thence either the α-amino group of most amino acids, or as the amide group of glutamine. The latter is a nitrogen donor for certain steps in the biosynthesis of some amino acids, purines, pyrimidines, and certain other key compounds (Figure 1.3 : Stadtman, 1973; Stadtman & Ginsburg, 1974; Senior, 1975; Tyler, 1978; Umbarger, 1978; Stryer, 1981; Magasanik, 1982; Meister, 1984). As a consequence of the synthesis of most amino acids by transamination from glutamate, bacteria such as *E. coli* can synthesise all of the 20 amino acids required for growth (Senior, 1975; Cohen, 1976; Umbarger, 1978; Anraku, 1980; Stryer, 1981).

Glutamine is synthesised by the condensation of ammonia with glutamate in a reaction catalysed by glutamine synthetase (GS : E.C. 6.3.1.2), whereas glutamate can be produced in enteric bacteria by a number of reactions (Shapiro & Stadtman, 1970b; Senior, 1975; Tyler,

Figure 1.3 : Metabolic fates of the amide nitrogen of L-glutamine (compiled from Prusiner & Stadtman, 1973; Conn & Stumpf, 1976; Tyler, 1978).



1978; Brown, 1980; Magasanik, 1982; Meister, 1984). Ammonia can be assimilated either by a reaction catalysed by glutamate dehydrogenase (GDH : E.C. 1.4.1.2, 1.4.1.3 & 1.4.1.4), or usually as a result of a coupled reaction catalysed by glutamine synthetase and glutamate synthase (GOGAT : E.C. 1.4.1.13 & 1.4.1.14) (see Figure 1.4 : Stadtman, 1973; Brown *et al.*, 1974; Stadtman & Ginsburg, 1974; Senior, 1975; Tyler, 1978; Brown, 1980; Magasanik, 1982).

1.9.1.1 Glutamine Synthetase

This enzyme is widely distributed in nature and is found in most bacteria studied (Brown, 1980), as well as in plant and mammalian tissues (Meister, 1980; Miflin *et al.*, 1980; Meister, 1984), fungi, yeasts and algae (Brown, 1980; Stewart, 1980). It catalyses the ATP-dependent production of glutamine from ammonia and glutamate shown in Figure 1.4 (Equation 1), and requires Mg^{++} or Mn^{++} for maximal activity (Woolfolk *et al.*, 1966; Shapiro & Stadtman, 1970b; Stadtman, 1973; Stadtman & Ginsburg, 1974; Tyler, 1978; Brown, 1980; Meister, 1980; Magasanik, 1982; Meister, 1984). The enzyme has a K_m-value for ammonia of < 1 mM (high affinity for ammonia) and is thus able to scavenge ammonia at low concentrations (below 1 mM : Stadtman, 1973; Brown *et al.*, 1974; Brown, 1980). As the first enzyme in a highly branched biosynthetic pathway (see Figure 1.3 for compounds synthesised from glutamine), it regulates the flow of nitrogen in an organism, and is therefore subject to metabolic control (Stadtman, 1973; Stadtman & Ginsburg, 1974; Woolfolk *et al.*, 1966; Hubbard & Stadtman, 1967; Senior, 1975; Brown, 1980; Stadtman *et al.*, 1980).
Although widely distributed, the enzyme is not identical in various species of enteric bacteria and other organisms, varying in amino acid composition, the number and arrangement of subunits, as well as in some catalytic properties (Hubbard & Stadtman, 1967; Tyler, 1978; Meister, 1980; Magasanik, 1982; Meister, 1984). Thus, GS from most prokaryotic and eukaryotic cells has subunits with molecular weights between 44,000 and 55,000. The bacterial enzyme consists of 12 identical subunits arranged in two hexameric groups (Meister, 1974a; Stadtman & Ginsburg, 1974; Tyler, 1978; Meister, 1980; Magasanik, 1982). Thus, the total molecular weight of the enzyme varies from the 600 kDa figure described by Stadtman & Ginsburg (1974), Tyler (1978) and Brown (1980) (Magasanik, 1982). The enzymes from *Klebsiella aerogenes* and *E. coli* are similar, differing only in some kinetic properties, such as

isoactivity point (Bender *et al.*, 1977). In contrast, GS from brain, liver, peas and certain microorganisms is composed of only eight identical subunits arranged as two tetramers (Meister, 1974b, 1980, 1984; Wedler & Denman, 1984). The reaction catalysed by these variants of GS is basically the same as that catalysed by the common form of the enzyme (Meister, 1980).

Metabolic control operates at both the biosynthesis and activity levels, although the complexity of these systems varies between bacteria and between types of organisms (Brown, 1980). In the following discussion the full complement of control mechanisms will be discussed, with the understanding that they are not all present in all organisms.

The most basic control of GS in *E. coli* is the repression and derepression of enzyme biosynthesis in response to nitrogen source in the immediate environment. Growth on glutamate represses the enzyme, as does a high ammonia level (Woolfolk *et al.*, 1966; Shapiro & Stadtman, 1970b; Stadtman *et al.*, 1970; Stadtman & Ginsburg, 1974; Miflin & Lea, 1976; Brown, 1980; Magasanik & Rothstein, 1980; Magasanik, 1982). Once the enzyme has been synthesised, its kinetic characteristics are controlled by the level of divalent cations present, via interconversion of relaxed (inactive) and taut (active) enzyme forms, and by the ratio of the concentrations of ATP to divalent cation (Shapiro & Stadtman, 1970b; Stadtman *et al.*, 1970; Stadtman & Ginsburg, 1974; Brown, 1980).

In addition, GS is subject to cumulative feedback inhibition by CTP, AMP, glucosamine-6-phosphate, histidine, tryptophan, carbamyl-phosphate, alanine, glycine and serine. The amide nitrogen of glutamine is utilised directly in the biosynthesis of all of these compounds (Figure 1.3), except alanine, glycine and serine, and these are indirectly derived from glutamine via transamination. This feedback inhibition is probably mediated by each of these inhibitors binding to specific sites on the enzyme, such that its activity is almost completely switched off when the inhibitors are bound to it (Woolfolk *et al.*, 1966; Shapiro & Stadtman, 1970b; Stadtman *et al.*, 1970; Stadtman, 1973; Senior, 1975; Cohen, 1976; Conn & Stumpf, 1976; Brown, 1980; Stadtman *et al.*, 1980; Stryer, 1981). Glutamate is not a feedback inhibitor, although it is a product of glutamine metabolism. The regulatory effects of alanine and glycine may be a mechanism by which cells achieve the optimal balance between glutamate and glutamine, since these amino acids are in dynamic equilibrium with glutamate, via transamination. In this scheme, inhibition of GS would initially favour ammonia assimilation

by GDH to form glutamate and amino acids, but by decreasing the glutamine pool, GS synthesis would be induced to re-establish glutamine synthesis (Stadtman & Ginsburg, 1974; Hernández *et al.*, 1986).

The most complex control mechanism of GS activity is the reversible covalent modification of the enzyme, known as adenylylation/deadenylylation. In this mechanism AMP is covalently attached to the hydroxyl group of a specific tyrosine residue in each of the twelve subunits of the bacterial enzyme, rendering the enzyme less active *per se*, but also making it more susceptible to the cumulative feedback inhibition outlined above. Adenylylation therefore provides a fine control of the enzyme activity in addition to cumulative feedback inhibition and repression/derepression. Adenylylation itself is also subject to fine metabolite control, since the enzymes catalysing the adenylylation and deadenylylation reactions are stimulated by ATP and 2-oxoglutarate, and inhibited by glutamine. Thus, GS activity is altered by a regulatory cascade so that adenylylation is inhibited, and deadenylylation is stimulated. When the supply of glutamine is low, GS becomes less susceptible to cumulative feedback inhibition. This cascade therefore amplifies regulatory signals and increases the potential for allosteric control of the enzyme, since each enzyme in the cascade becomes an independent target for regulation, and finer tuning of the flow of nitrogen in the cell is therefore possible (Shapiro & Stadtman, 1970b; Stadtman *et al.*, 1970; Stadtman & Ginsburg, 1974; Cohen, 1976; Conn & Stumpf, 1976; Miflin & Lea, 1976; Tyler, 1978; Brown, 1980; Stadtman *et al.*, 1980; Stryer, 1981; Magasanik, 1982).

The result of these controls in many microorganisms is that cells grown with an excess of ammonia contain highly adenylylated, and therefore inactive, GS, whereas growth on limiting ammonia results in a non-adenylylated GS (Senior, 1975; Magasanik & Rothstein, 1980). It has been suggested that adenylylation/deadenylylation control of GS functions to prevent the depletion of the ATP pool in cells, and to prevent the accumulation of glutamine (Kustu *et al.*, 1984), however it is unlikely that there is an ATP pool in anaerobic bacteria (Hobson & Wallace, 1982b; Russell, 1984). Kustu *et al.* (1984), however, believe that the major function for enzyme inactivation by adenylylation is to conserve the glutamate pool in the cell for growth of cells when excess ammonia appears in the environment.

1.9.1.2 Glutamate Synthase

Glutamate occupies a central position in bacterial amino acid metabolism, acting as a reservoir of α-amino nitrogen which is used in the synthesis of other amino acids and their derivatives. Unlike glutamate dehydrogenase, which catalyses the formation of glutamate directly from ammonia (section 1.9.1.3), GS cannot catalyse a direct synthesis of glutamate (Tempest *et al.*, 1973; Brown *et al.*, 1974; Brown, 1980; Stryer, 1981). GS is coupled to the enzyme glutamate synthase (GOGAT), which has been detected in many prokaryotes (Tempest *et al.*, 1970; Buchanan, 1973a; Miller, 1973; Stadtman, 1973; Tempest *et al.*, 1973; Stadtman & Ginsburg, 1974; Tyler, 1978; Miflin *et al.*, 1980; Stryer, 1981).

This widely-distributed enzyme catalyses the formation of two molecules of glutamate from 2-oxoglutarate and glutamine, with NADH and/or NADPH as reductants (Equation 2; Figure 1.4), effectively rendering the GS catalysed reaction irreversible, since GOGAT utilises the glutamine formed by GS (Tempest *et al.*, 1970; Buchanan, 1973a; Miller, 1973; Stadtman, 1973; Tempest *et al.*, 1973; Brown *et al.*, 1974; Conn & Stumpf, 1976; Tyler, 1978; Brown, 1980; Kang & Titus, 1981; Stryer, 1981; Magasanik, 1982). GOGAT enzymes are either NADH- or NADPH-dependent, but in plants, reduced ferredoxin is the electron donor (Buchanan, 1973a; Brown *et al.*, 1974; Miflin & Lea, 1976; Herbert *et al.*, 1978; Stewart, 1980; Kenealy *et al.*, 1982; Bhatnagar *et al.*, 1986a). In addition, Brown *et al.* (1974) consider this catalytic reaction to be unidirectional. The GOGAT isoenzymes are flavoproteins composed of 8 subunits of 2 dissimilar types, 32 Fe atoms, labile sulphur and 8 non-covalently linked flavin molecules (i.e. iron-sulphur flavoproteins : Dainty, 1972; Miller & Stadtman, 1972; Buchanan, 1973a; Miller, 1973; Brown *et al.*, 1974; Miflin & Lea, 1976; Tyler, 1978; Miflin *et al.*, 1980; Bernlohr *et al.*, 1984). The enzyme is also highly specific for 2-oxoglutarate and glutamine, and generally has a K_m-value < 0.5 mM for each (as low as 7.3 μm for 2-oxoglutarate in *E. coli* : Meers *et al.*, 1970; Miller & Stadtman, 1972; Buchanan, 1973a; Brown *et al.*, 1974; Miflin & Lea, 1976; Brown, 1980).

Relatively little is known about the regulation of the biosynthesis of GOGAT, although it seems to be affected by the levels of glutamate and glutamine, but not by ammonia concentrations. It also appears to be constitutively synthesised in some microorganisms (Miller & Stadtman, 1972; Buchanan, 1973a; Miller, 1973; Tempest *et al.*, 1973; Brown *et al.*, 1974; Tyler, 1978). Similarly, the enzyme is not as well regulated as GS. Glutamate, aspartate and

methionine are inhibitors (at least in *E. coli*), but L-cysteine, serine, glycine, homoserine, asparagine, alanine and histidine are less inhibitory (Meers *et al.*, 1970; Miller & Stadtman, 1972; Buchanan, 1973a; Bernlohr *et al.*, 1984).

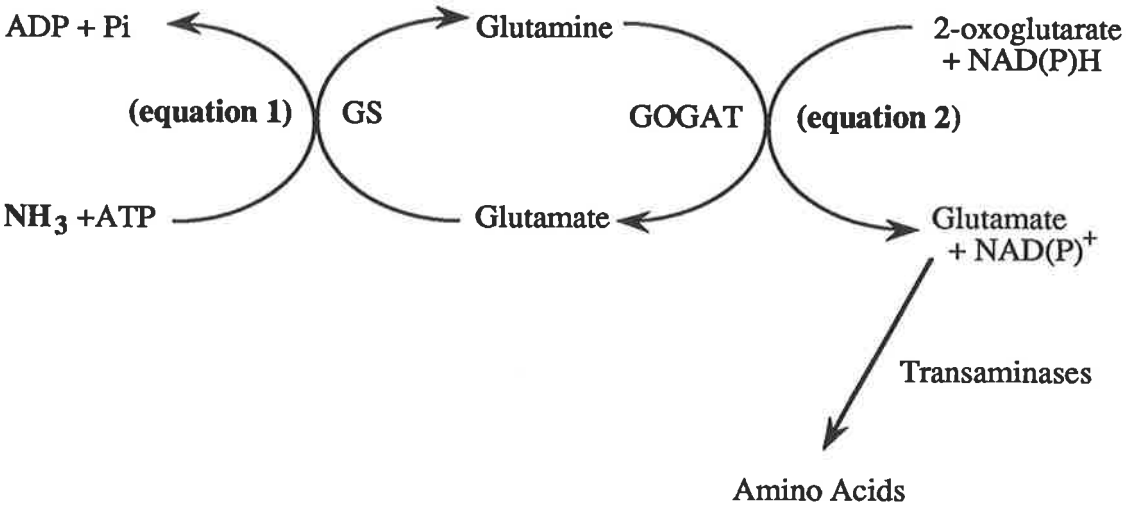
1.9.1.3 Glutamate Dehydrogenase

Prior to the discovery of GOGAT by Tempest, Meers and Brown in 1970, it was generally assumed that the principal mechanism for amino acid synthesis in most organisms involved the direct assimilation of ammonia via glutamate dehydrogenase (GDH) - i.e. the reductive amination of 2-oxoglutarate by ammonia in a reversible reaction utilizing NADH or NADPH and yielding glutamate (Equation 3; Figure 1.4 : Doherty, 1970; Stadtman, 1973; Brown *et al.*, 1974; Smith *et al.*, 1975; Miflin & Lea, 1976; Tyler, 1978; Brown, 1980; Marzluf, 1981; Stryer, 1981). This is the same overall reaction as catalysed by the GS/GOGAT couple (Equations 1 & 2, respectively; Figure 1.4), but is achieved at a lower energy cost to the cell, since NAD(P)H only is required, rather than NAD(P)H and one mole of ATP for the GS/GOGAT couple (Brown, 1980).

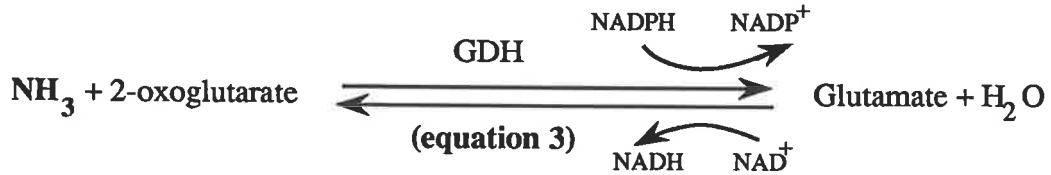
GDH's in general are widely distributed in microorganisms (Smith *et al.*, 1975; Miflin & Lea, 1976). As for GOGAT, GDH enzymes can utilise either NADH or NADPH as reductants, but the enzyme can utilise either cofactor in some organisms (Brown *et al.*, 1974; Smith *et al.*, 1975; Tyler, 1978). The NADH-dependent activity of this enzyme primarily operates catabolically (i.e. the oxidation of glutamate to ammonia and 2-oxoglutarate), whereas the NADPH-dependent activity operates biosynthetically. All three forms of the enzyme are, however, similar in gross structure (i.e. 6 identical subunits) although *Neurospora* has 4 subunits only (Smith *et al.*, 1975; Miflin & Lea, 1976; Tyler, 1978; Marzluf, 1981). The enzymes vary not only in coenzyme specificity, but also in regulatory properties such as enzyme synthesis, which is regulated by the levels of various metabolites, as well as by the concentrations of nitrate and aspartate in various microorganisms (Shapiro & Stadtman, 1970b; Brown *et al.*, 1974; Smith *et al.*, 1975; Brown, 1980; Marzluf, 1981; Stryer, 1981). In addition, there is allosteric control of enzyme activity (mainly in animals) through the actions of purine nucleoside di- and triphosphates (e.g. ADP, GDP, ATP, GTP), however there is apparently no feedback inhibition by amino acids (Shapiro & Stadtman, 1970b; Smith *et al.*,

Figure 1.4 : Major pathways of ammonia assimilation for the production of glutamate and glutamine (compiled from Tempest *et al.*, 1970; Stadtman, 1973; Brown *et al.*, 1974; Tyler, 1978).

GS / GOGAT PATHWAY



GDH PATHWAY



1975; Miflin & Lea, 1976; Tyler, 1978). Carbon catabolite repression may also control enzyme activity (Marzluf, 1981). Since few control mechanisms have been detected for bacterial GDH, it seems unlikely that this enzyme is a major control point for nitrogen metabolism in these organisms (Smith *et al.*, 1975). Nevertheless, Marzluf (1981) suggests the NADH- and NADPH-dependent forms of GDH may be subject to some form of concurrent regulation, since both types are often present in the same organism.

In contrast to the properties of GS, GDH has a relatively high K_m-value for ammonia (and 2-oxoglutarate; > 1 mM and usually about 4 mM). It is therefore likely that the major biosynthetic activity of GDH occurs when the environmental ammonia concentration is high (Brown *et al.*, 1974; Miflin & Lea, 1976; Tyler, 1978; Brown, 1980). The general conclusion regarding the two major ammonia-assimilatory pathways is that GS activity is high, and GDH activity is low, when the environmental ammonia level is low, resulting in the GS/GOGAT couple assimilating most of the ammonia. Conversely, when the environmental ammonia concentrations rise above about 1 mM, GDH is the dominant ammonia-assimilating enzyme (Stadtman, 1973; Brown *et al.*, 1974; Brown, 1980; Braña *et al.*, 1986).

1.9.1.4 Alanine Dehydrogenase

Although glutamate and glutamine are undoubtedly the key molecules in nitrogen metabolism, serving as nitrogen donors for biosynthetic reactions in most microorganisms, a number of other ammonia-assimilatory enzymes exist, but are not as important as either the GS/GOGAT, or GDH, pathways (Tyler, 1978; Umbarger, 1978; Brown, 1980; Magasanik, 1982). One of these enzymes is alanine dehydrogenase (AlaDH : E.C. 1.4.1.1), which catalyses the reductive amination of pyruvate by ammonia in a reversible reaction utilizing NADH or NADPH, and yielding alanine (Equation 4; Figure 1.5 : Wiame *et al.*, 1962; Yoshida & Freese, 1965; Brown *et al.*, 1974; Miflin & Lea, 1976).

Although AlaDH and similar amino acid dehydrogenases are not as important in ammonia assimilation as GS, GOGAT and GDH, the enzyme has been detected in plant tissues, as well as in many microorganisms. Its main physiological function is apparently the conversion of L-alanine to pyruvate, which can be readily used as a carbon source (Brown *et al.*, 1974; Brown, 1980). There are, however, a number of examples of the presence of AlaDH

when GDH activity is absent, indicating a possible role in ammonia assimilation. Such a role has been suggested in *Anabaena cylindrica, Cyanidium caldarium, Methanobacterium thermoautotrophicum, Rhodopseudomonas capsulata* and *Streptomyces clavuligerus* (Dunn & Klucas, 1973; Brown *et al.*, 1974; Kenealy *et al.*, 1982; Moreno-Vivián *et al.*, 1983; Braña *et al.*, 1986). AlaDH is also required for germination in *Bacillus subtilis* (Freese *et al.*, 1964).

Since AlaDH generally has a much higher K_m-value for ammonia than GDH (8 - 133 mM in algae, 38 - 360 mM in *Bacillus* species : Yoshida & Freese, 1965; Brown *et al.*, 1974; Stewart, 1980; Moreno-Vivián *et al.*, 1983), it has been suggested that it is an alternative ammonia-assimilatory pathway for organisms living in environments with high ammonia levels (by catalysing the incorporation of ammonia nitrogen into alanine, followed by glutamate production via transamination : Brown *et al.*, 1974; Moreno-Vivián *et al.*, 1983). Universal support for this view is lacking, however, since many workers consider that the enzyme does not have a primary role in ammonia assimilation (Brown *et al.*, 1974; Deuel & Prusiner, 1974; Meeks *et al.*, 1977; Brown, 1980; Kenealy *et al.*, 1982; Braña *et al.*, 1986). Braña *et al.* (1986) suggest that, although the enzyme may not have a role in ammonia assimilation, it may be involved in regulating the intracellular level of alanine, which acts as one of the fine controls on GS activity.

Dalziel (1975) indicated that the major regulatory mechanisms for dehydrogenase enzymes are repression/derepression of enzyme synthesis, and substrate inhibition/activation and product inhibition (allosteric controls), as well as cofactor competition at the active site (Brown *et al.*, 1974; Braña *et al.*, 1986). Thus, enzyme synthesis of AlaDH is induced by alanine, and also by high concentrations of ammonia (e.g. 20 mM), and repressed by catabolites (Brown *et al.*, 1974; Braña *et al.*, 1986). In general, dehydrogenase enzymes have similar active sites and cofactor binding sites, hence it is likely that AlaDH has similar control mechanisms (Rossman *et al.*, 1975). In algae the enzyme is composed of 6 identical subunits (Stewart, 1980).

1.9.1.5 Asparagine Synthetase

The other ammonia-assimilating enzyme is asparagine synthetase (AsS : E.C.

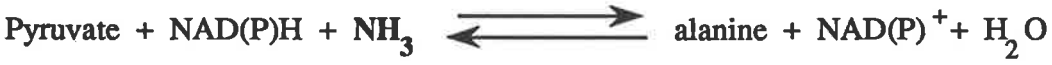
6.3.1.1 & 6.3.1.4), which catalyses the ATP-dependent production of L-asparagine from ammonia and aspartate, in a very similar fashion to the way in which GS catalyses the synthesis of glutamine. There is also a second version of the enzyme present in plants and animals which utilises L-glutamine rather than ammonia as the amide nitrogen donor (Equations 5 & 6, respectively; Figure 1.5 : Meister, 1974a; Miflin & Lea, 1976; Umbarger, 1978; Humbert & Simoni, 1980; Magasanik, 1982; Reitzer & Magasanik, 1982). Humbert & Simoni (1980) described a glutamine-dependent AsS in *E. coli* and Reitzer & Magasanik (1982) have now found both enzyme activities in *K. aerogenes*. Clearly the ammonia-dependent AsS is the only one potentially involved in primary ammonia assimilation.

Asparagine synthesised from aspartate can be either degraded by transamination, oxidation, or hydrolysis, or utilized for the synthesis of protein (i.e. similar to the reactions observed for glutamine). Thus, ammonia-dependent AsS is, in effect, part of a biosynthetic pathway (Meister, 1974a). It is interesting to note that the structure and free energy of asparagine are similar to that for glutamine. Evolution, however, has favoured glutamine as the amino-nitrogen donor for important steps in the synthesis of nucleic acids, proteins, and certain structural polysaccharides (see Figure 1.3), as well as its role in advanced life forms of providing a non-toxic vehicle for amino-nitrogen for synthesis and detoxification. Asparagine plays a similar role in certain higher plants (Wade, 1980). Nevertheless, AsS enzymes are important in some instances, especially in *E. coli* and *K. aerogenes* (Humbert & Simoni, 1980; Magasanik, 1982; Reitzer & Magasanik, 1982).

A possible reason for a minor role of AsS in ammonia assimilation in microorganisms is that this reaction requires more energy than that of glutamine formation via GS, since AsS utilises two equivalents of ATP (forming AMP and pyrophosphate, which would be hydrolysed to P_i) compared to only one for GS (Meister, 1974a; Umbarger, 1978; Humbert & Simoni, 1980; Magasanik, 1982). Similarly, the specific activity of AsS is usually considerably lower than that of GS, despite the fact that it has very similar catalytic properties to those of GS, but a different amino acid substrate and product, and a higher K_m-value for ammonia (about 4 mM). These similar properties even extend to possessing ß-aspartyl transferase activity, which is comparable to the γ-glutamyl transferase activity possessed by many GS's (Ravel, 1970).

Figure 1.5 : Possible minor pathways of ammonia assimilation.

AlaDH (equation 4)



Ammonia-dependent AsS (equation 5)

Glutamine-dependent AsS (equation 6)

The glutamine-dependent AsS, a tetramer, is controlled by asparagine through both repression of synthesis, and inhibition of activity, whereas the ammonia-dependent enzyme has more complex regulatory mechanisms (Meister, 1974a; Umbarger, 1978; Magasanik, 1982; Reitzer & Magasanik, 1982). As well as repression and inhibition of the enzyme by asparagine, the ammonia-dependent enzyme is also repressed by the *gln* G product, which is a regulatory protein of nitrogen assimilation found at high concentrations whenever GS is present at high activity levels (GS is the product of *gln* A, its structural gene) (Magasanik, 1982; Reitzer & Magasanik, 1982). Meister (1974a) and Umbarger (1978) also include AMP and aspartate as end product competitive inhibitors of the ammonia-dependent AsS. Thus, when cell growth is nitrogen-limited, AsS and GS do not compete for the available ammonia, which is clearly an advantage for the organism (Reitzer & Magasanik, 1982).

1.9.1.6 Other Ammonia-Assimilating Enzymes?

Some other enzymes are also described in the literature that catalyse the assimilation of ammonia under certain conditions, however these enzymes are not considered to be important in metabolism. One such group of enzymes, the glutamine amidotransferases, mediate the utilisation of the amide nitrogen of glutamine for particular synthetic reactions, however almost all of these enzymes utilise ammonia instead of glutamine, albeit at a lower affinity (Buchanan, 1973a; Brown *et al.*, 1974; Meister, 1984).

Glutamate synthase and the glutamine-dependent asparagine synthetase, which have been discussed earlier are, in fact, glutamine amidotransferases. Other examples include carbamoyl-phosphate synthase (glutamine-hydrolysing) (the first reaction in the synthesis of pyrimidines and arginine : E.C. 6.3.5.5), phosphoribosylformylglycinamidine synthase (an intermediate in purine synthesis : E.C. 6.3.5.3), amidophosphoribosyltransferase (the first step in purine synthesis : E.C. 2.4.2.14) and glutamine-fructose-6-phosphate aminotransferase (isomerising) (E.C. 5.3.1.19), all of which are shown in Table 1.1 (Buchanan, 1973a; Brown *et al.*, 1974; Hespell, 1984; Meister, 1984). In addition, carbamate kinase (E.C. 2.7.2.2 : see Table 1.1), which catalyses the synthesis of carbamoyl phosphate, but may play a greater role in the breakdown of carbamoyl phosphate than in its synthesis, is sometimes considered to be an ammonia-assimilating enzyme, as is aspartate ammonia-lyase (aspartase) (E.C. 4.3.1.1)

Table 1.1 : A selection of other potential ammonia-assimilating enzymes (Buchanan, 1973a; Brown *et al.*, 1974; Buttery, 1976; Hespell, 1984; Meister, 1984).

ENZYME	REACTION
Amidophosphoribosyltransferase (E.C. 2.4.2.14)	5-Phospho-ß-D-ribosylamine + pyrophosphate + L-glutamate → L-glutamine + 5-phospho-α-D-ribose 1-diphosphate + H_2O (also known as : phosphoribosyldiphosphate 5-amidotransferase)
Aspartate ammonia-lyase (E.C. 4.3.1.1)	L-aspartate → fumarate + NH_3 (also known as : aspartase)
Carbamate kinase (E.C. 2.7.2.2)	ATP + NH_3 + CO_2 → ADP + carbamoyl phosphate (also known as : carbamoyl phosphokinase)
Carbamoyl-phosphate synthase (glutamine-hydrolysing) (E.C. 6.3.5.5)	2ATP + L-glutamine + CO_2 + H_2O → 2ADP + orthophosphate + L-glutamate + carbamoyl phosphate (formerly E.C. 2.7.2.9; also known as carbomoyl phosphate synthetase (glutamine-hydrolysing))
Glutamine-fructose-6-phosphate aminotransferase (isomerising) (E.C. 2.6.1.16)	L-glutamine + D-Fructose-6-phosphate → L-glutamate + D-glucosamine 6-phosphate (also known as : glucosamine-6-phosphate synthetase & glucosamine phosphate isomerase (glutamine-forming); formerly E.C. 5.3.1.19)
Phosphoribosylformyl-glycinamidine synthase (E.C. 6.3.5.3)	ATP + 5'-phosphoribosylformylglycinamide + L-glutamine + H_2O → ADP + orthophosphate + L-glutamate + 5'-phosphoribosylformylglycinamidine (also known as : phosphoribosylformylglycinamide synthetase)

(Buchanan, 1973a; Buttery, 1976; Hespell, 1984). Nevertheless, it seems doubtful that any of these enzymes contribute greatly to ammonia assimilation.

1.9.2 COORDINATED ACTION OF AMMONIA-ASSIMILATING ENZYMES

It appears that when bacteria are grown on a poor nitrogen source, GS, with its low K_m-value for ammonia, is the main route for ammonia assimilation, with the glutamine produced used to synthesise glutamate by GOGAT and asparagine by the glutamine-dependent AsS. Loss of either GOGAT or AsS (by mutation) makes it impossible for these cells to grow in media containing low levels of ammonia unless glutamate or asparagine, respectively, are provided. When ammonia is in excess, GDH, with its high K_m-value for ammonia, replaces the GS/GOGAT pathway of assimilation, and the ammonia-dependent AsS replaces the glutamine-dependent version (Brenchley, 1973; Brown *et al.*, 1974; Miflin & Lea, 1976; Brown, 1980; Reitzer & Magasanik, 1982). These enzymes are able to provide glutamate and asparagine at a lower cost to the organism (in terms of ATP) than GS/GOGAT, however loss of either of these enzyme systems does not result in growth inhibition at high environmental ammonia levels, since other enzymes exist which can synthesise glutamate and asparagine from ammonia under these conditions (Magasanik, 1982; Reitzer & Magasanik, 1982). The amino acid dehydrogenases, in particular AlaDH, are examples of such enzymes, since they generally have high K_m-values for ammonia (Brown *et al.*, 1974; Brown, 1980; Braña *et al.*, 1986), as do carbamoyl-phosphate synthase and aspartate ammonia-lyase (Brown *et al.*, 1974; Buttery, 1976).

Thus, the classical view of ammonia assimilation is that the GDH pathway operates under conditions of high ammonia concentration, whereas the GS / GOGAT pathway is important at low ammonia concentrations to "scavenge" the available nitrogen. GS and GDH are therefore the major ammonia-assimilating enzymes and, due to this coordinated action, are rarely found at significant levels concurrently in bacteria (Stadtman, 1973; Brown *et al.*, 1974; Senior, 1975; Miflin & Lea, 1976; Tyler, 1978; Brown, 1980; Stewart, 1980; Stryer, 1981; Magasanik, 1982; Reitzer & Magasanik, 1982; Vargas & Strohl, 1985; Braña *et al.*, 1986). In addition, AsS, AlaDH and other amino acid dehydrogenases, carbamoyl-phosphate synthase (glutamine-hydrolysing), amidophosphoribosyltransferase, phosphoribosylformyl-glycinamidine synthase, glutamine-fructose-6-phosphate aminotransferase (isomerising),

aspartate ammonia-lyase and others are also present in many organisms, but their role in ammonia assimilation is not clear. Transaminases are responsible for the further metabolism of these amino acids to form all of the twenty amino acids necessary for growth of bacterial cells (Conn & Stumpf, 1976; Stryer, 1981).

1.9.3 THE CLASSICAL SITUATION IS NOT THE ONLY ONE

Although most bacteria modulate the synthesis of GS (and its activity), GOGAT and GDH to suit the availability of nitrogen in the environment, there are a number of variations of this classical concept (Brown, 1980). The most striking examples involve a deficiency of GDH activity accompanied by a concomitant increase in AlaDH activity at high ammonia levels, and the constitutive use of the GS / GOGAT pathway for ammonia assimilation (Dunn & Klucas, 1973; Deuel & Prusiner, 1974; Buttery, 1976; Miflin & Lea, 1976; Herbert *et al.*, 1978; Tachiki *et al.*, 1978; Brown, 1980; Kenealy *et al.*, 1982; Magasanik, 1982; Vairinhos *et al.*, 1983; Moreno-Vivián *et al.*, 1983; Rachim & Nicholas, 1985; Bhatnagar *et al.*, 1986a; Braña *et al.*, 1986).

Similarly, a number of examples can be found of organisms lacking GOGAT activity, and therefore relying on GDH to assimilate ammonia irrespective of the ammonia level in the environment (Miflin & Lea, 1976). This either involves a modified GDH enzyme with lower than the usual K_m-value, which operates effectively at low ammonia concentrations, or the synthesis of increased amounts of the biosynthetic form of this enzyme (Brown *et al.*, 1974; Brown, 1980; Kumar & Nicholas, 1982)

GS is also not universally present, but more particularly is not always subject to the same extensive set of controls as outlined earlier. Thus, the GS's of numerous organisms do not have adenylylation/deadenylylation control of their activity, but usually have some form of feedback inhibition of enzyme activity (Smith *et al.*, 1980; Stewart, 1980; Fisher *et al.*, 1984; Kustu *et al.*, 1984; Bodasing *et al.*, 1985; Bhatnagar *et al.*, 1986b). The lack of adenylylation/ deadenylylation control of GS in *Bacillus subtilis in vivo* does not mean that the enzyme operates uncontrolled, since GS in this bacterium can be regulated by changes in mRNA levels of the enzyme. Moreover, expression of the structural gene accounts for some of the specific activity regulation in *E. coli, K. aerogenes, Rhizobium* sp. and *Salmonella typhimurium* .

These bacteria, however, cannot be fully controlled by gene expression, as is the case for *Bacillus subtilis* (Magasanik, 1982; Fisher *et al.*, 1984).

A number of organisms have also been studied which have "classical" GS activity and control mechanisms, but which do not possess the ability to catalyse the γ-glutamyl transferase reaction often used to measure GS activity (Hubbard & Stadtman, 1967; Smith *et al.*, 1980; Yamamoto *et al.*, 1984).

1.9.4 AMMONIA ASSIMILATION IN RUMEN BACTERIA

Although it has long been recognised that ammonia is the major nitrogen source in the rumen (Blackburn, 1965; Chalupa, 1972; Hobson & Wallace, 1982b; Owens & Bergen, 1983; Hume, 1984; Leng & Nolan, 1984), and that it is converted to protein by the microorganisms ultimately for use by the animal, relatively little work has been done to determine how the ammonia is assimilated in the rumen, and especially by rumen bacteria.

1.9.4.1 The Early Studies

It has been assumed that ammonia assimilation by rumen microorganisms follows the same pathways as those determined for enteric bacteria. Early reviews by Allison (1969) and Chalupa (1972) considered that the major ammonia-assimilating mechanism involved GDH, since this enzyme(s) had been detected in extracts of *Streptococcus bovis* grown with ammonia (Burchall *et al.*, 1964a; Joyner & Baldwin, 1966), as well as those of *B. ruminicola, Butyrivibrio fibrisolvens, F. succinogenes, Peptostreptococcus elsdenii, Ruminobacter (Bacteroides) amylophilus, Ruminococcus flavefaciens, R. albus* and *S. ruminantium* (Joyner & Baldwin, 1966). Burchall *et al.* (1964b), however did not detect GS activity in *Streptococcus bovis* , but did find AsS activity which they purified. Griffith & Carlsson (1974) suggested that GDH was the major ammonia-assimilatory pathway in *Streptococci*, including *Streptococcus bovis*. Similarly, Hoshino *et al.* (1966), Palmquist & Baldwin (1966), Chalupa *et al.* (1970) and Salem *et al.* (1973) detected GDH activity in mixed rumen samples as well as in the rumen mucosa (Hoshino *et al.*, 1966; Salem *et al.*, 1973). Chalupa *et al.* (1970) also detected GS and carbamate kinase activities, and Salem *et al.* (1973) found GS, carbamate kinase and carbamoyl-phosphate synthase activities in mixed rumen samples.

Interestingly, either NADH- or NADPH-dependent activities of GDH (but not both) were detected in the species grown in pure culture (Burchall *et al.*, 1964a; Joyner & Baldwin, 1966), but Palmquist & Baldwin (1966) and Chalupa *et al.* (1970) found both activities in mixed rumen bacterial samples, with the NADH-dependent activity exceeding the NADPH-dependent one. Palmquist & Baldwin (1966) suggest that the activities of enzymes in various species probably do not accurately reflect those which occur in the rumen. The rumen environment, they suggest, might be expected to modify enzyme activities, however such effects could not entirely account for the gross differences in NADH to NADPH ratios for GDH between single species and in rumen fluid samples. A number of the species not studied must contain active NADH-linked GDH's (Palmquist & Baldwin, 1966).

1.9.4.2 A Landmark *In Vitro* Study

Although early workers in the field of rumen ammonia assimilation reported that GDH was the key enzyme for ammonia assimilation in rumen bacteria, they qualified this statement by stating that other amino acid dehydrogenases, transaminases and synthetases were probably also important (Allison, 1969; Chalupa *et al.*, 1970; Chalupa, 1972). Salem *et al.* (1973) recorded GS activity in rumen microorganisms, thus confirming the observations of Chalupa *et al.* (1970). As a consequence of this early work, others showed the induction of GS in non-rumen microorganisms. The discovery of GOGAT in non-rumen bacteria (Tempest *et al.*, 1970) led to an investigation of the effects of ammonia on the activities of GS, GOGAT, GDH, AsS, and aspartate and alanine aminotransferase in continuous cultures of mixed rumen bacteria (Erfle *et al.*, 1977).

This work showed that the activity of GS was markedly induced at low ammonia concentrations, and that both NADH- and NADPH-dependent GDH activities were also present in the rumen. This work also demonstrated the presence of GOGAT activity in rumen microorganisms utilising both NADH and NADPH. Griffith & Carlsson (1974) had detected GOGAT in *Streptococcus bovis* . In addition, Erfle *et al.* (1977) detected AsS and aspartate aminotransferase activities and high levels of free alanine in the cultures. They concluded that rumen bacteria synthesise glutamate via pathways other than GDH, such as GS/GOGAT and AsS. They also speculated that these enzymes could assimilate ammonia at low concentrations

(2 - 4 mM), whereas the high K_m-value measured for GDH indicated that this enzyme probably operates at high ammonia concentrations.

A catabolic role for the NADH-dependent GDH supported by the work of Palmquist & Baldwin (1966) and Chalupa *et al.* (1970) helps to explain the shift in the ratio of the NADH- to the NADPH-dependent activity with ammonia as the nitrogen source (i.e. as the ammonia concentration decreased, the level of NADH-dependent activity also decreased). Interestingly, GOGAT activity showed a lack of specificity for amide donors. Thus, AsS and GS in these samples provide a glutamate synthesising system even more efficient than that in some non-rumen microorganisms, and certainly more effective than GDH at low levels of ammonia (Erfle *et al.*, 1977). Erfle *et al.* (1977) also speculated that the high concentration of free alanine in rumen bacteria may provide a means of trapping pyruvate and ammonia for subsequent utilization by the host animal. This reaction is mediated by an alanine aminotransferase which favours alanine accumulation, and also by AlaDH (Erfle *et al.*, 1977).

1.9.4.3 Some Pure Culture Studies

Jenkinson *et al.* (1979) studied ammonia assimilation in the rumen bacterium *Ruminobacter (Bacteroides) amylophilus* and discovered that this species does not strictly follow the classical route. Instead, GS activity is induced at ammonia levels below about 0.5 mM and is inactivated at levels above about 1 mM (i.e. "classical" action), but GDH is a constitutive enzyme with a relatively low K_m-value for ammonia (1-2 mM), and utilises either NADH or NADPH as an electron donor (the NADPH-dependent activity is usually more pronounced) (Jenkinson *et al.*, 1979; Hespell, 1984). AsS was also detected, however Hespell (1984) considers it to only contribute to ammonia assimilation when high energy levels are available. AlaDH and GOGAT activities were not recorded, although Hespell (1984) suggests that this may be more due to an inability to find an appropriate electron donor for GOGAT than due to a lack of activity *per se* (Jenkinson *et al.*, 1979; Hespell, 1984).

Similarly, Smith *et al.* (1980, 1981) concluded that the "classical" routes via GS/ GOGAT and GDH appear to be responsible for ammonia assimilation in *S. ruminantium*, based on studies of the properties and regulation of the major ammonia-assimilating enzymes. These two pathways accounted for all of the ammonia assimilated by *S. ruminantium* (Hespell,

1984). Thus, a GS without adenylylation control and γ-glutamyl transferase activity is the most active enzyme under ammonia-limiting or other growth-limiting conditions, and is coupled to a GOGAT with unusual electron donor requirements (i.e. maximum activity with dithionite-reduced methyl viologen *in vitro*, as well as with NADH or NADPH). In addition, GS in *S. ruminantium* is not subject to the same feedback inhibition as for enteric bacteria and low ammonia concentrations *per se* do not significantly increase GS activity except when values are less than 1 mM. High levels of GS activity may therefore be necessary in this species for adequate ammonia assimilation and glutamate formation under ammonia-limited conditions. In contrast, NADH- and NADPH-dependent GDH activities were highest when the levels of ammonia were not rate limiting and in glucose-deficient cultures. Although this enzyme is less regulated by ammonia level than GS, it does appear to be important when the energy supply is limited. Although it is a non-ATP requiring pathway the catalysis does require an electron donor/oxidation step which may place a competitive constraint on the reaction. Nevertheless, GDH can still synthesise glutamate when the ammonia concentration is very low, albeit less efficiently than the GS/ GOGAT pathway, thus it may have an important role under a variety of external conditions (Smith *et al.*, 1980, 1981; Hespell, 1984).

Succinivibrio dextrinosolvens is the only other ruminal bacterium whose pattern of ammonia assimilation has been studied in any detail. A GS which is subject to adenylylation control and inhibition by alanine, glycine and serine has been detected in this species, however GOGAT and AlaDH activities have not been reliably shown (Hespell, 1984; Patterson & Hespell, 1985). In addition, GDH and possibly AsS, have been detected (Patterson & Hespell, 1985). Pettipher & Latham (1979) have shown GDH activity in *Ruminococcus flavefaciens* which is three-fold greater in cells grown under ammonia-limitation than cellobiose limitation.

1.9.4.4 Recent *In Vivo* Studies

The most recent *in vivo* studies of rumen ammonia assimilation have been carried out by Bhatia *et al.* (1978), Salter *et al.* (1979), Wallace (1979), Bhatia *et al.* (1980), Blake *et al.* (1983) and Lenártová *et al.* (1985), who all indicate that GDH is probably the major pathway. Wallace (1979) and Lenártová *et al.* (1985), however, were the only ones to measure the activity of these enzymes. Wallace (1979) found that GDH was the major pathway of ammonia assimilation by rumen bacteria, since the activity of this enzyme was relatively high at

the two rumen ammonia concentrations studied. In contrast, the activities of GS and GOGAT were both low and NADH-dependent, but not NADPH-dependent, AlaDH activities were detected. These are hardly surprising results, since the enzyme activities were measured in extracts of cells grown *in vivo* at ammonia concentrations of 6.1 or 13.4 mM, which are above those usually associated with GS activity.

An interesting, and as yet unsolved, feature of much of this *in vivo* work is that nitrogen was most rapidly and effectively incorporated into glutamine, aspartate and alanine from ammonia *in vivo* (Salter *et al.*, 1979; Wallace, 1979; Blake *et al.*, 1983). This feature was also observed by Erfle *et al.* (1977) with their *in vitro* continuous cultures. These compounds may be the initial recipients of amino groups from ammonia for subsequent transfer to other amino acids by transamination reactions (Tsubota & Hoshino, 1969; Bhatia *et al.*, 1979, 1980; Lenártová *et al.*, 1985), but the high level of alanine is a feature for which an adequate explanation is yet to be provided (Hobson & Wallace, 1982b). Wallace (1979) and Blake *et al.* (1983) suggested that this initial synthesis of alanine may be due to high activities of AlaDH, since Blake *et al.* (1983) did not detect adequate transaminase activities that could explain the alanine levels. Smith (1979) considers that the results for $^{15}NH_3$ incorporation into glutamic acid, aspartic acid and alanine were consistent with the labelling of the amide groups of glutamine and/or asparagine followed by synthesis of alanine, aspartate and glutamate.

It is interesting to speculate about the reasons for the apparently futile synthesis of alanine by rumen bacteria. Blake *et al.* (1983) suggest three possible reasons : (1) as a short-term storage mechanism for ammonia, hence alanine released into the rumen fluid would be rapidly deaminated to ammonia and pyruvate; (2) as a control mechanism within the bacteria to prevent excess levels of ammonia accumulating; and (3) as a means of removing excess pyruvate from bacteria when readily available energy is supplied and bacterial metabolism is rapid.

1.9.4.5 Summary

It has been shown that rumen bacteria in continuous cultures of mixed populations, *in vivo*, and in pure cultures of several species, possess the type of ammonia-assimilating enzymes found in other environments, with the GS / GOGAT and GDH pathways predominating (Buttery, 1976; Baldwin & Denham, 1979; Harrison & McAllan, 1980; Russell

& Hespell, 1981; Hobson & Wallace, 1982b; Hespell, 1984). Russell & Hespell (1981), however point out that ". . . *an interplay of ammonia assimilation pathways probably takes place at various times after feeding and with various dietary changes, but the nature of these is not known,* ". Thus, it is probable that other ammonia-assimilating enzymes, such as AsS, AlaDH and other amino acid dehydrogenases, aspartate ammonia-lyase, carbamoyl-phosphate synthase, carbamate kinase, and transaminases, may also play roles in ammonia assimilation in the mixed flora of the rumen (Chalupa *et al.*, 1970; Chalupa, 1972; Buttery, 1976; Hobson & Wallace, 1982b; Hespell, 1984).

By assuming that GS is the major enzymic contributor at low concentrations of ammonia, with an increasing dependence upon GDH at high ammonia levels, Baldwin & Denham (1979) found that the GS/GDH pathways would account for the experimental estimates of microbial growth rates throughout the day in an animal fed alfalfa hay once daily. GS alone over-estimated the growth rate, whilst for GDH the reverse was true (Baldwin & Denham, 1979). Hespell (1984) suggests that both pathways may be used simultaneously in different microorganisms in the rumen, and that no one pathway is likely to predominate in overall ammonia assimilation in the rumen. Nevertheless, the point made by Miflin & Lea (1976) that the ". . . *presence of GS and GOGAT does not prove that they are used in vivo as the major route of nitrogen assimilation* ", is relevant to rumen microbial studies, since, although these enzymes may not be important under the prevailing experimental conditions, they may be important under other conditions, or in some microenvironments.

1.10 USE OF AMINO ACID ANALOGUES TO STUDY AMMONIA ASSIMILATION

Four major methods, namely enzymological studies, ^{15}N labelling, mutagenesis, and the action of amino acid analogues, are routinely used to determine the major routes of ammonia assimilation in organisms, and to confirm that the main pathway is via the GS and GOGAT enzymes rather than GDH (Lea & Norris, 1976; Brown, 1980).

Growth inhibition following the addition of an amino acid analogue to a bacterial culture may affect the exponential phase of growth, resulting in immediate linearity, or linearity may result after a lag period of about one generation (Fowden *et al.*, 1967; Norris & Lea, 1976).

Nevertheless, the capacity of analogues to bind to individual enzymes varies with the type of organism concerned (Lea & Norris, 1976).

To be a potent inhibitor of growth, a compound must not only be similar in structure to the amino acid, but must also exist in a similarly charged form (Fowden *et al.*, 1967). This may occur when the analogue is an isomer of the substrate (i.e. isomeric analogue), or as a result of a replacement of one or more chemical groups on the substrate (i.e. substitution analogue) (Webb, 1966; Norris & Lea, 1976). The two analogues used in this thesis to investigate the roles of GS and GOGAT in cell growth are both substitution analogues (Webb, 1966; Ronzio & Meister, 1968; Buchanan, 1973b; Lea & Norris, 1976; Norris & Lea, 1976; Pinkus, 1977).

Thus, L-methionine DL-sulphoximine (MSX or L-methionine-S-sulphoximine) and azaserine (O-diazoacetyl-L-serine) were chosen as analogues of glutamate and glutamine, respectively, for inhibitor studies of a number of rumen bacteria.

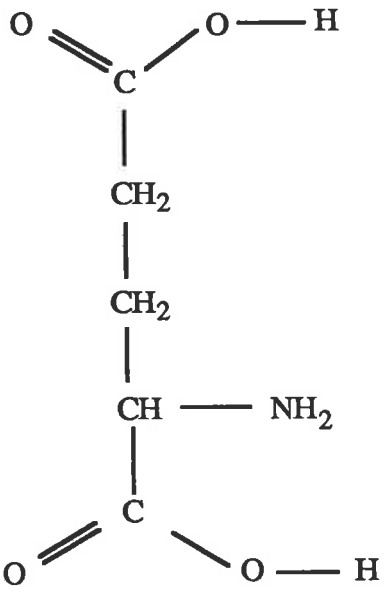
1.10.1 L-METHIONINE DL-SULPHOXIMINE

This compound, which was used extensively in active-site mapping studies of GS (Tate & Meister, 1973; Meister, 1980, 1984), has more recently been employed as an inhibitor of cell growth in a number of organisms. Inhibition in this situation indicates that the particular organism relies on the GS/GOGAT pathway for ammonia assimilation. It has also been shown to inhibit GS activity in many microorganisms (Heathcote & Pace, 1950; Brenchley, 1973; Stewart & Rhodes, 1976; Herbert *et al.*, 1978; Stewart *et al.*, 1980; Moreno-Vivián *et al.*, 1983; Vairinhos *et al.*, 1983; Yamamoto *et al.*, 1984; Rachim & Nicholas, 1985; Vargas & Strohl, 1985; Bhatnagar *et al.*, 1986b).

Thus, MSX is an analogue of glutamate (Figure 1.6) which can act as an effective inhibitor of GS (Ronzio & Meister, 1968; Ronzio *et al.*, 1969; Brenchley, 1973; Brown, 1980). MSX is more accurately defined as an analogue of the γ-glutamyl phosphate enzyme complex of GS (i.e. the enzyme-bound intermediate of the GS catalytic reaction). When MSX binds to GS, its sulphoximide nitrogen occupies the same site as that of the oxygen of glutamate which is phosphorylated by GS, and the methyl group of MSX binds to the ammonia binding site of the enzyme (Figure 1.7) (Ronzio & Meister, 1968; Ronzio *et al.*, 1969; Tate & Meister, 1973; Miflin & Lea, 1976; Meister, 1980, 1984). In both cases (i.e. analogue and

Figure 1.6 : Comparative structures of glutamate and L-methionine DL-sulphoximine (MSX) (compiled from Lea and Norris, 1976; Norris and Lea, 1976).

Glutamate



MSX

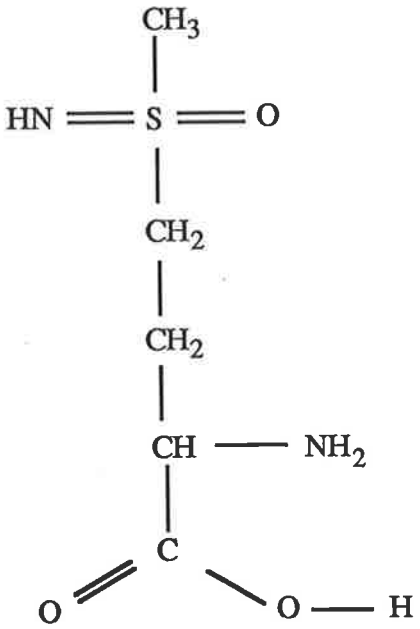
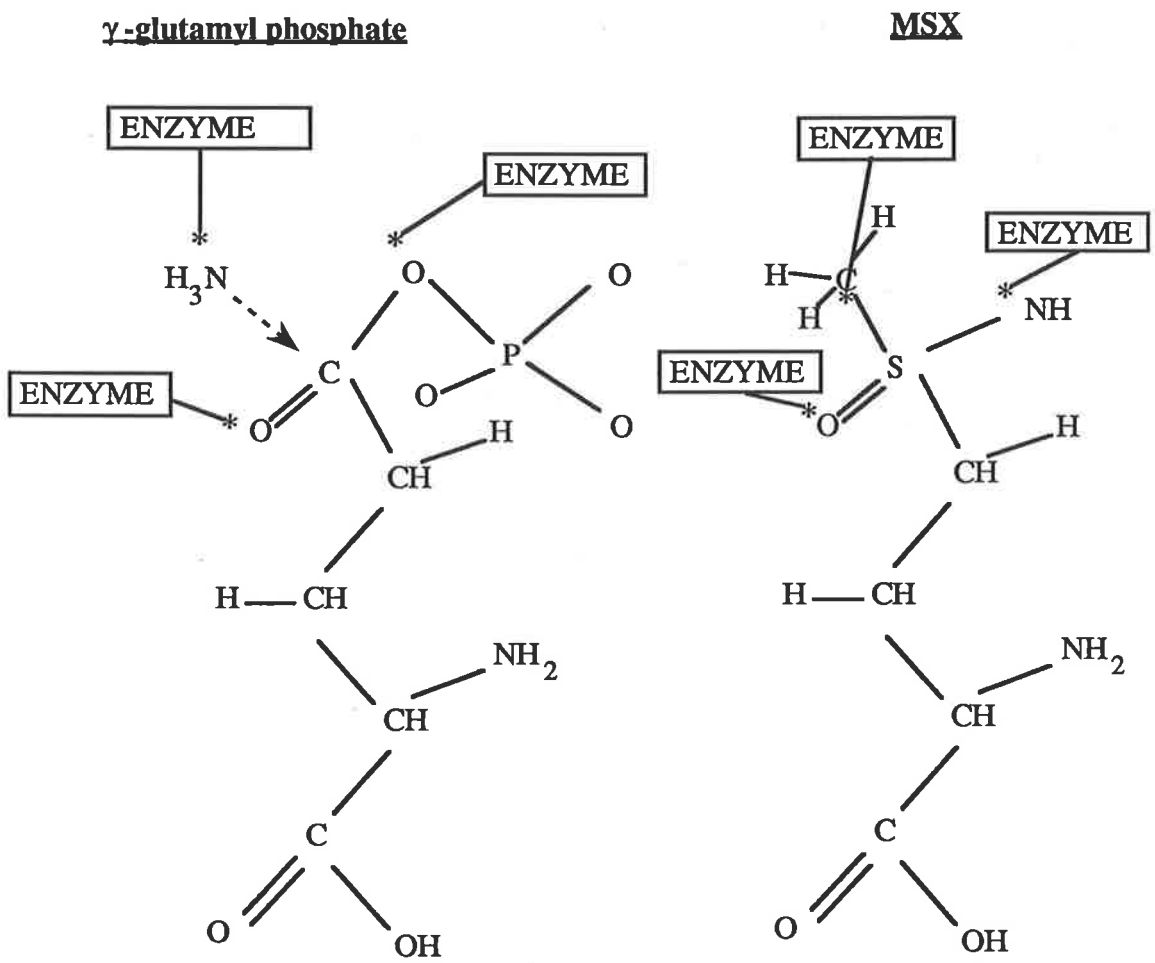


Figure 1.7 : Comparison of γ-glutamyl phosphate (with ammonia) and MSX at the GS active site (compiled from Ronzio and Meister, 1968; Meister, 1980, 1984).



normal substrate), ATP and a divalent cation are required for the reaction (Tate & Meister, 1973; Meister, 1980).

It is interesting to note that only the L, S-isomer of MSX (i.e. L-methionine-S-sulphoximine) undergoes this reaction, whereas the three other isomers do not inhibit the enzyme (Manning *et al.*, 1969; Meister, 1980, 1984). Although GS is therefore susceptible to this chemical arrangement, MSX is not completely specific for GS (Brenchley, 1973; Miflin & Lea, 1975; Miflin & Lea, 1976; Brown, 1980). Thus, Brenchley (1973) has shown that the activity of GS is 70 % inhibited by only 0.01 mM MSX, however GOGAT is also completely inhibited at much higher concentrations (10 mM). In contrast, GDH activity is not affected by MSX (Brenchley, 1973; Lea & Norris, 1976; Miflin & Lea, 1976), hence MSX is primarily an inhibitor of GS, and to a lesser extent of GOGAT, therefore interfering with the normal growth of cells by preventing glutamine production (Brenchley, 1973).

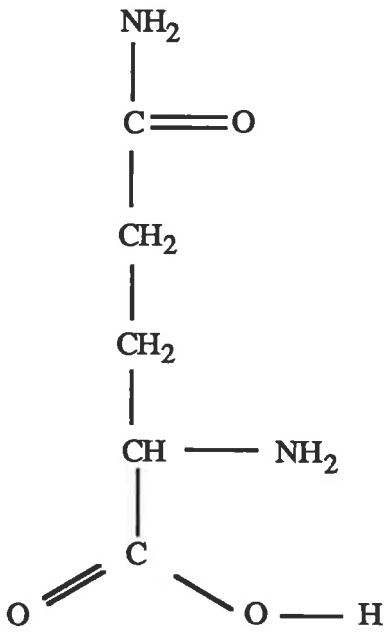
1.10.2 AZASERINE

Azaserine is one of a number of analogues of glutamine (two other common ones are albizziine and 6-diazo-5-oxo-L-norleucine (DON)), which inhibit all glutamine amide transfer reactions, including that catalysed by GOGAT (Webb, 1966; Lea & Norris, 1976; Miflin & Lea, 1976; Norris & Lea, 1976; Pinkus, 1977; Fentem *et al.*, 1983). The steric properties of the diazo-group (i.e. the two nitrogen atoms on the fifth carbon atom), as well as the chain length of azaserine, are important features of this glutamine analogue (Figure 1.8). Its structural similarity to glutamine enables it to react at the glutamine active site of enzymes catalysing glutamine amide transfer (Buchanan, 1973b). The chemically active diazoacetyl group attaches the inhibitor to the enzyme irreversibly via a covalent bond despite the fact that this inhibitor is one atom longer than the normal substrate, glutamine, and the alignment of atoms in the enzyme active site only approximates those of glutamine (Webb, 1966; Buchanan, 1973b).

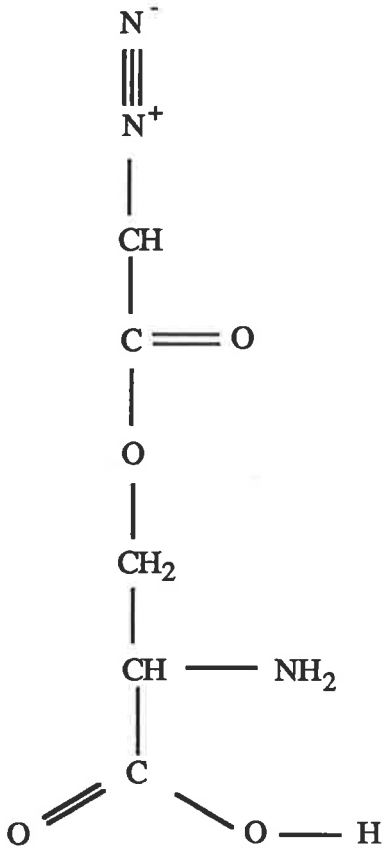
This inhibitory mechanism of azaserine is based on its effect on phosphoribosyl-formylglycinamidine synthase (E.C. 6.3.5.3), an enzyme involved in the synthesis of purine nucleotides (see Table 1.1 : Webb, 1966; Buchanan, 1973b; Norris & Lea, 1976; Pinkus, 1977). Presumably, the same type of mechanism operates when azaserine inhibits GOGAT.

Figure 1.8 : Comparative structures of glutamine and azaserine (compiled from Webb, 1966; Buchanan, 1973b; Lea and Norris, 1976; Norris and Lea, 1976; Pinkus, 1977).

Glutamine



Azaserine.



When the GS/GOGAT pathway is operating azaserine usually results in a rapid increase in the concentrations of glutamine and 2-oxoglutarate, and a decrease in glutamate, as a result of inhibition of GOGAT activity (Miflin & Lea, 1976). Consequently, azaserine has been used extensively to confirm the presence of GOGAT (Pace & McDermott, 1952; Nagatani *et al.*, 1971; Dunn & Klucas, 1973; Miflin & Lea, 1975; Stewart & Rhodes, 1976; Stewart *et al.*, 1980; Smith *et al.*, 1981; Moreno-Vivián *et al.*, 1983; Vairinhos *et al.*, 1983; Paress & Streicher, 1985; Rachim & Nicholas, 1985; Vargas & Strohl, 1985). GS and GDH are not affected by azaserine (Lea & Norris, 1976; Miflin & Lea, 1976), although Dunn & Klucas (1973) report that GDH was inhibited by only 13% by this analogue. Azaserine therefore appears to be a relatively specific inhibitor of glutamine-dependent reactions.

1.10.3 PROBLEMS WITH INHIBITOR STUDIES

Although inhibition of growth by MSX and azaserine is a good indication that an organism is dependent on the GS/GOGAT system for ammonia assimilation under the prevailing environmental conditions, there are limitations to this method.

Webb (1966) points out that the actions of analogues which are attributed to competition at the enzyme level may actually affect the uptake of substrates. This has not been shown for azaserine inhibition of GOGAT, but MSX has been reported to inhibit methylammonium and/or NH_4^+ transport in *Klebsiella pneumoniae, Azotobacter vinelandii, Azospirillum brasilense, Rhodospirillum rubrum, Rhodopseudomonas sphaeroides, Anabaena flos-aquae* and *Anacystis nidulans* (Kleiner, 1985). Nevertheless, MSX did not affect methylammonium transport in *Anabaena variabilis* (Kleiner, 1985). Although some of these observations argue for an effect of MSX on the NH_4^+ carrier, possibly via the glutamine-binding site of the carrier (e.g. in cyanobacteria), another interpretation is that transport is inhibited by MSX because of the build-up of an intracellular NH_4^+ pool as a result of inhibition of GS activity. It is not possible to decide which hypothesis is correct (Kleiner, 1985). In barley plants (*Hordeum vulgare* L. cv Golden Promise) there is also a suggestion that MSX is poorly transported by some tissues (Fentem *et al.*, 1983).

1.11 AIMS OF THIS RESEARCH

A new approach to solving the problems of nitrogen utilisation in the ruminant, and which has the potential for very great positive effects on the efficient utilisation of feed in ruminants (Leng, 1985), involves genetic manipulation of the rumen ecosystem. Thus, work is underway in a number of laboratories to manipulate cellulase genes in various species of rumen bacteria so that the rate and extent of cellulose degradation can be enhanced. In this way it may be possible to increase the utilisation of fibrous diets by ruminants (Smith & Hespell, 1983; Leng, 1985).

Another potential application of the techniques of molecular biology to the rumen is the transfer of genes coding for enzymes involved in various degradative processes in rumen bacteria, or the modification of bacterial fermentation pathways in these bacteria (Smith & Hespell, 1983). The project reported in this thesis is part of a larger gene manipulation program, aimed at modifying nitrogen metabolism in ruminants. The specific aim of the work to be described is to provide basic information about the processes of ammonia assimilation and metabolism, and their regulation, in rumen bacteria. If successful, this project would enable rumen microorganisms to utilize the ammonia available in their environment more effectively, thus reducing the amount to be excreted and detoxified by the animal in high production, or non-protein nitrogen-supplemented, conditions.

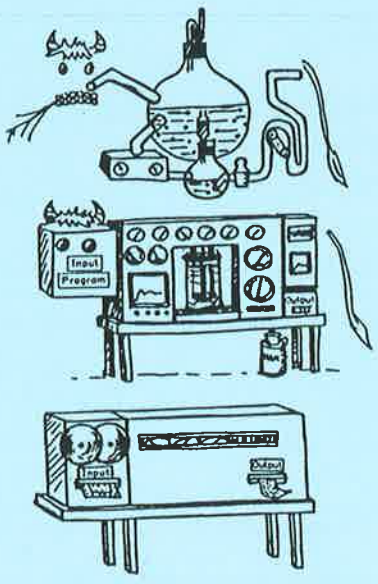
Thus, the ultimate aim of this thesis is to understand more fully the processes of nitrogen metabolism and assimilation in the rumen, and in particular those associated with ammonia. The enzymes, interactions and fluxes of metabolites through the pathways of ammonia assimilation are considered, since ammonia is the major nitrogen source for microbial growth in the rumen.

CHAPTER 2

Activity of Ammonia-Assimilating Enzymes in Rumen Bacteria *in vivo*.

"Ruminants have complex stomachs to compensate for their deficient teeth "

(Aristotle)



Development of the rumen (3)

Czerkawski (1986)

Chapter 2

Activity of Ammonia-Assimilating Enzymes in Rumen Bacteria *in vivo*.

2.1 INTRODUCTION

The ammonia-assimilating enzymes discussed in the Literature Review (section 1.9 : i.e. GS, GOGAT, GDH, AlaDH and AsS) have been detected in bacterial cell-free extracts prepared from a number of pure culture studies of rumen species (Burchall *et al.*, 1964b; Joyner & Baldwin, 1966; Allison, 1969; Griffith & Carlsson, 1974; Buttery, 1976; Jenkinson *et al.*, 1979 ; Smith *et al.*, 1980, 1981; Hobson & Wallace, 1982b; Hespell, 1984; Patterson & Hespell, 1985), however very few comprehensive studies of ammonia assimilation have been performed using *in vivo* rumen bacterial samples or *in vitro* cultures of mixed rumen bacteria. For example, Hoshino *et al.* (1966) and Bhatia *et al.* (1978, 1980) only measured the activities of GDH in mixed bacterial samples from various ruminants, whilst this was also the only ammonia-assimilating enzyme whose activity was measured in a study of carbohydrate utilization in the rumen by Palmquist & Baldwin (1966).

In addition to measuring both NADH- and NADPH-dependent GDH activities in rumen bacterial samples when investigating potential pathways of ammonia metabolism in sheep fed urea or soy protein diets, Chalupa *et al.* (1970) also measured GS, carbamate kinase and two transaminase activities. Similarly, Salem *et al.* (1973) measured GDH, GS, carbamoyl-phosphate synthase and carbamate kinase activities in samples from iso-nitrogenous semi-purified diets containing 0, 50, or 100 % added nitrogen as urea. Salter *et al.* (1979) and Blake *et al.* (1983), in contrast, studied nitrogen assimilation *in vivo* by following the incorporation of nitrogen into intracellular amino acids in rumen bacteria. Nevertheless, it was concluded from all of these studies that GDH is the major nitrogen-assimilatory pathway in the rumen.

Although GOGAT, AsS and AlaDH activities were not measured in the previous *in vivo* studies, Erfle *et al.* (1977) included the first two enzymes in their investigation of nitrogen assimilation in cultures of rumen bacteria taken from a cow fed a diet supplemented with non-protein nitrogen. They concluded that the GS/GOGAT pathway and/or AsS were responsible

for ammonia assimilation when less than 2 mM ammonia was present, and that at higher ammonia concentrations the GDH pathway was responsible for this process, while Lenártová *et al.* (1985) came to similar conclusions from a more limited study of ammonia assimilation *in vivo*. In addition, Erfle *et al.* (1977) showed that GS activity was induced at low ammonia concentrations while no relationship was evident between GDH activity and ammonia concentration in the cultures. Although Wallace (1979) concluded that GDH was the major pathway in rumen bacteria (above), at least at the two rumen ammonia concentrations studied, this conclusion is clearly a consequence of the moderate rumen ammonia levels studied (6.1 and 13.4 mM for diets of whole barley and whole barley supplemented with urea, respectively). Such concentrations apparently favour GDH activity over GS activity.

Thus, the importance and presence of each of the enzymes of ammonia assimilation in rumen bacteria appear to vary from diet-to-diet, from one microorganism to another, and within microorganisms depending upon growth conditions. Despite this trend, the evidence suggests that the primary routes of assimilation are via the GS/GOGAT and GDH pathways (Buttery, 1976; Baldwin & Denham, 1979; Harrison & McAllan, 1980; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Hespell, 1984). Nevertheless, it is probable that other ammonia-assimilating enzymes, such as AsS, AlaDH and other amino acid dehydrogenases, aspartate ammonia-lyase, carbamoyl-phosphate synthase, carbamate kinase and transaminases, also play roles in ammonia assimilation in the rumen, especially considering the diversity of both growth conditions and microbial species in this environment (Chalupa *et al.*, 1970; Chalupa, 1972; Buttery, 1976; Hobson & Wallace, 1982b; Hespell, 1984).

Further detailed determinations of the occurrence of these enzymes *in vivo* are clearly unnecessary. A comprehensive study of the presence and activity of the various enzymes in bacterial samples taken from the rumen of animals fed a number of diets is, nevertheless, a logical starting point for investigating the mechanisms of ammonia assimilation in rumen bacteria, since it will provide comparative data for the single species culture work presented in Chapter 3. These data should also add to the observations of the workers mentioned above regarding the presence of the various ammonia-assimilating enzymes and pathways.

It is evident that a deficiency in the above literature is a comprehensive study of the factors which influence the presence and activities of ammonia-assimilating enzymes *in vivo*. As already mentioned, Erfle *et al.* (1977) showed that GS activity was induced in mixed cultures of

rumen bacteria *in vitro* at low ammonia concentrations (< 2 mM), while similar relationships were not evident for GDH, GOGAT or AsS activities. Paradoxically, Erfle *et al.* (1977) also showed that the biosynthesis and utilisation of amino acids appear to be regulated by ammonia concentration in the rumen despite GS being the only assimilatory enzyme similarly affected. Salem *et al.* (1973) also showed some effects of rumen ammonia levels on GS and GDH activities *in vivo*, however Wallace (1979) did not find similar effects, probably due to the moderate ammonia levels of the diets tested. Wallace (1979) was able to show increased alanine synthesis when the rumen ammonia level was increased from 6.1 mM to 13.4 mM by supplementation with urea. These studies indicate that the activities of ammonia-assimilating enzymes may be influenced by the levels of rumen parameters such as ammonia, but this is clearly not the only important parameter, since ammonia level alone could not fully explain the effects measured by Salem *et al.* (1973), Erfle *et al.* (1977) and Wallace (1979).

Thus, the first series of experiments for this thesis studies the effects of rumen ammonia and free protein levels on the activity of the ammonia-assimilating enzymes in mixed bacterial samples from the rumen using animals fed lucerne chaff plus wheat grain, lucerne chaff only, or sheep pellets plus oaten chaff. These diets were chosen so that the resulting rumen conditions would differ in ammonia level to test the hypothesis that these rumen fluid parameters are important determinants of the activity of the ammonia-assimilating enzymes *in vivo* from day-to-day, a suggestion made by Hespell (1984), but which is also intuitively possible based on the literature presented above.

In their study of the effects of ammonia concentration on the activity of the ammonia-assimilatory enzymes, Erfle *et al.* (1977) took single samples from mixed rumen bacterial cultures immediately prior to "feeding" the continuous fermenter. At this time a stable ammonia concentration clearly should have been established for most of the previous 24 hr, with the probable exception of day 2 of the experiment when the NH_4Cl infusion was stopped for 6 hr prior to sampling. Their conclusion that GS activity is induced in rumen bacteria at low ammonia concentrations, and conversely decreases at high concentrations does not address the question of how rapidly the activity can be induced or decreased. Consequently, the second series of experiments described in this chapter study the regulation of ammonia-assimilating enzymes in rumen bacteria *in vivo* to test the hypothesis that the activities are influenced in the short-term (0 - 4 hr) by increases in rumen ammonia, and/or energy (glucose) levels.

In order to adequately investigate this hypothesis it was necessary to ensure that the results were not confounded by normal daily changes in the microbial population, and in rumen parameters. Thus, the diets fed to the two animals for these experiments were such that the digestion of ingested feed materials was spread as evenly as possible throughout the day. The first of these diets consisted solely of poor quality wheaten straw (very low digestibility and nitrogen content) fed twice daily. This diet should result in a fairly constant fermentation pattern in the rumen throughout the day, resembling that from a low energy/low nitrogen feedstuff. The second diet also consisted of this low grade straw, but a continuous infusion of mixed carbohydrates and ammonia was superimposed on it to maintain a fairly constant fermentation pattern appropriate for a rumen supplied with a high energy/high nitrogen feedstuff. Thus, although the rumen bacteria studied were to be from *in vivo* samples, they were also intended to be from carefully-controlled rumen fermentations more commonly expected from *in vitro* mixed ruminal cultures.

The aims of these experiments were therefore to determine which, if any, rumen fluid parameters affect the activity of the ammonia-assimilating enzymes from day-to-day; to generate data on the activity of the ammonia-assimilatory enzymes under a variety of *in vivo* conditions for comparison with later pure culture activity data and to enable a picture to be built up of ammonia assimilation in the rumen; and to investigate the short-term regulation of these enzymes *in vivo* under conditions in which the variability within the diet, such as the day-to-day variation in rumen conditions, was minimised.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

2.2.1.1 Dietary Components

Wheat grain (approx. 2.0 % Nitrogen (N) on a dry matter basis and 87 % Dry Matter Digestibility (DMD)†), lucerne chaff (approx. 2.5 % N (dry matter basis) and 65 %

† Ostrowski-Meissner (1987).

DMD†) and oaten chaff (approx. 0.67 % N (dry matter basis) and 52 % DMD†) were obtained from stocks at the Waite Agricultural Research Institute. Chopped wheaten straw (91.7 % Dry Matter ; 22 % DMD (determined by the method of Tilley & Terry, 1963) ; 0.48 % N (determined by the Kjeldahl method with titration from a Markham still)) was selected from poor quality hay stocks at the Waite Institute. Sheep pellets were standard shed issue pellets (Milling Industries Ltd, Adelaide, Australia).

The minerals mixture added to diets D and E (experiment 1.2) was taken from a stockpile previously prepared to the specifications of Moir & Harris (1962).

Cornflour was purchased from Fielders Starches, Tamworth, Australia. Ammonium chloride (NH_4Cl) was purchased from ACE Chemical Company, Adelaide, Australia. D(+)-xylose was obtained from B.D.H. Chemicals (Australia Pty. Ltd.), Kilsyth, Australia. *Iso*-butyric acid, *n*-valeric acid and *iso*-valeric acid were purchased from B.D.H. Chemicals Ltd., Poole, England. 2-methylbutyric acid was obtained from Aldrich Chemical Company, Milwaukee, U.S.A. Gum arabic was purchased from Analytical & Research Chemical Company, Adelaide, Australia. D(+)-cellobiose was purchased from Sigma Chemical Company, St. Louis, U.S.A.

Components of "synthetic saliva", the best purity available, were obtained from Ajax Chemicals (Sydney, Australia) and ACE Chemical Company (Adelaide, Australia).

2.2.1.2 General Chemicals and Biochemicals

Adenosine 5'-diphosphate (sodium salt [Na-ADP]), adenosine 5'-triphosphate (sodium salt [Na-ATP]), L-aspartic acid, DL-aspartic acid β-monohydroxamate (β-aspartyl hydroxamate), bovine albumin (Fraction V [BSA]), casein, D(+)-cellobiose, α-cellulose, Coomassie Brilliant Blue G250, L-cysteine hydrochloride, L-glutamic acid (mono-sodium salt), L-glutamic acid γ-monohydroxamate (γ-glutamyl hydroxamate), L-glutamine, hydroxylamine monohydrochloride, 2-oxoglutarate, maltose (hydrate, grade I), 2-mercaptoethanol, β-nicotinamide adenine dinucleotide (β-NADH: reduced form), β-nicotinamide adenine dinucleotide phosphate (β-NADPH: reduced form), pectin, pyruvic acid (sodium salt), sodium arsenate, xylan, D(+)-xylose, vitamins {thiamine-hydrochloride (vit. B_1), Ca-D-pantothenate, nicotinic acid, riboflavin (vit. B_2), pyridoxine-monohydrochloride (vit. B_6), *p*-aminobenzoic acid,

d-biotin (vit. H), folic acid and cyanocobalamin (vit. B_{12})}, HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid] - biological buffer) and Tris(hydroxymethyl)aminomethane (Tris-HCl buffer) were obtained from Sigma Chemical Company, St. Louis, U.S.A.

Bacto-Agar and Proteose Peptone were purchased from DIFCO Laboratories, Detroit, U.S.A. Resazurin and hemin-1,4-napthoquinone (bovine hemin) were purchased from Aldrich Chemical Company, Milwaukee, U.S.A. Dithiothreitol was obtained from Boehringer Mannheim Australia Pty Ltd, Sydney, Australia.

All other chemicals, the best quality available, were obtained from the following sources : Ajax Chemical (Sydney, Australia); B.D.H. Chemicals Ltd. (Poole, England); B.D.H. Chemicals, Australia Ltd. (Kilsyth, Australia).

2.2.1.3 Buffers

5 mM HEPES buffer and 1.0 M stocks of Tris-HCl buffer solutions were prepared according to the standard methods of Dawson (1986).

All solutions, including buffers, were made using water purified by Reverse Osmosis (R.O. water).

2.2.2 METHODS

2.2.2.1 Preparation of Samples for Enzyme Assay

Rumen bacterial pellets, prepared as outlined in section 2.2.2.5 were used in a preliminary experiment to determine the most effective and efficient way of preparing cell-free extracts for assay of enzyme activity. Seven cell pellets were prepared from crude rumen fluid and each was resuspended in TKD buffer (10 ml) (50 mM Tris-HCl; 1 % [w/v] KCl; 1 mM dithiothreitol, [pH 7.4]). One of the following methods was then used to prepare the cells for enzyme assay :

(i) No treatment other than resuspension in TKD buffer;
(ii) Passed through the French Pressure Cell twice (10,000 p.s.i.; 10°C);
(iii) Passed through the French Pressure Cell four times (10,000 p.s.i.; 10°C);
(iii) Sonicated (5 min.; medium probe; 350 W [Labsonic 1510 sonicator; B.Braun Melsungen AG]);
(iv) Sonicated (10 min.; medium probe; 350 W);

(v) Sonicated (10 min.; medium probe; 350 W; snap frozen in liquid nitrogen; thawed; 5 min.; 350 W)

(vi) Resuspended in 10 ml of 5 % [w/v] cetyltrimethylammonium bromide [CTAB] in 0.5 N HCl; snap frozen and thawed (modified from Helenius *et al.*, 1979; Schnaitman, 1981).

Following their respective treatments, samples were centrifuged (18,000 x *g* ; 30 min.; 4°C in a Sorvall® RC-5B Centrifuge (DuPont Instruments, Wilmington, U.S.A.)) and the supernatants retained. The amount of free protein in each supernatant, determined by the method of Sedmak & Grossberg (1977) using Coomassie Brilliant Blue G250, with BSA as standard (see section 2.2.2.5), was used to assess the effectiveness of each disruption method.

2.2.2.2 Experiment 1 - Animals, Diets and Design

Experiment 1 was designed to study the ammonia-assimilating enzymes in mixed rumen bacterial samples from the rumen of animals on various diets. Three Merino wethers fitted with permanent rumen fistulae were housed in individual pens with unrestricted access to water and were fed their respective diet at 0845 hours daily. Diet A consisted of 500 g lucerne chaff plus 400 g wheat grain; diet B was 1 kg lucerne chaff only; and diet C was 800 g sheep pellets plus 100 g oaten chaff, with one diet allocated to each animal.

Five sampling days were spread over a 22 day period. For each of the experimental animals a 125 ml aliquot of rumen fluid was sampled into two 100 ml glass centrifuge tubes ("*in vitro*" tubes) and stoppered at 1145 hours on each of the five days. The pH was determined immediately (section 2.2.2.5) and the samples placed on ice, transported to the laboratory and each subdivided into a 25 ml and two 50 ml aliquots. This protocol is summarised in Figure 2.1.

Each 25 ml aliquot was strained through four layers of cheesecloth and centrifuged (22,000 x *g* ; 20 min.; 4°C). Twenty ml of the resulting supernatant [clarified rumen fluid] from each sample was stored at -20°C for subsequent determination of ammonia concentration (section 2.2.2.6) and 2 ml was base-hydrolysed (0.1 M NaOH; 70°C; 10 min.) according to the method of Leedle *et al.* (1982). This was also stored at -20°C for subsequent protein determination (section 2.2.2.6).

The 50 ml aliquots of each sample were processed as outlined in section 2.2.2.5 to produce pellets of bacterial cells. One of these pellets was used to measure the amino acid

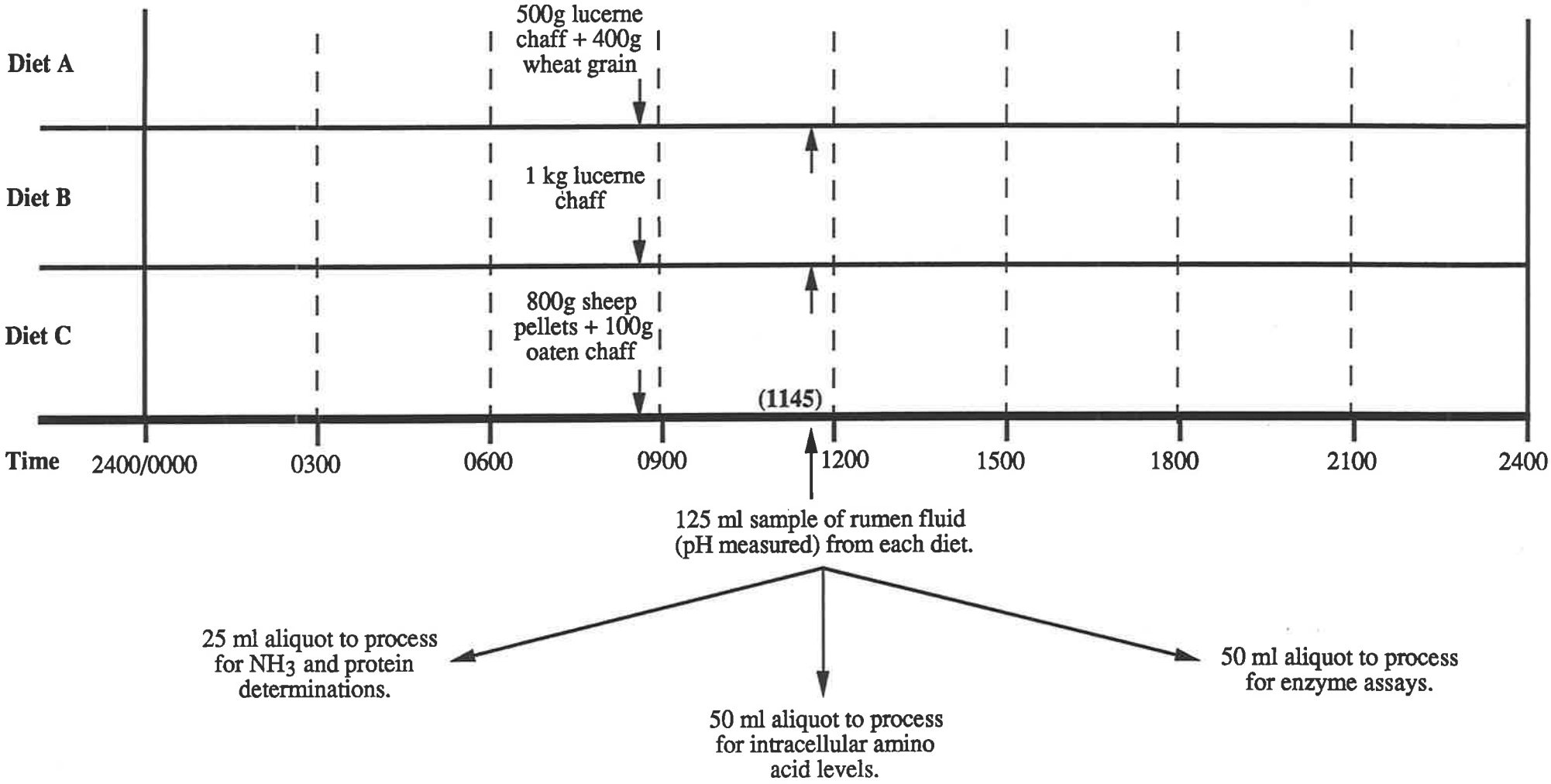


Figure 2.1: Summary of the experimental protocol on a sampling day for Experiment 1.

profile of the intracellular fluid of the rumen bacteria present (section 2.2.2.8). The other pellet from each sample was resuspended in TKD buffer (5 ml) and passed through a French Pressure Cell (twice x 10,000 p.s.i.; 10°C) to disrupt the cells. The resulting homogenates were centrifuged (18,000 x *g* ; 20 min.; 4°C) and the supernatants were stored at 4°C until assayed for GS, GOGAT, GDH and AlaDH activities (Patterson & Hespell, 1985). No attempt was made to keep samples anaerobic after separating the bacteria from protozoa, fungi and feed particles, since it was considered that this would have little effect on enzyme activities. Processing to this stage (i.e. preparation of cell-free extracts) was carried out on the day of sampling and samples were always kept on ice.

2.2.2.3 Experiment 2 - Animals, Diets and Design

Experiment 2 was designed to study the regulation of ammonia-assimilating enzymes in rumen bacteria *in vivo*. Two Merino wethers (sheep D & E) fitted with permanent rumen fistulae were individually housed in metabolism pens with unrestricted access to water. In addition to the basal diet of chopped wheaten straw *ad libitum* plus 7.5 g minerals mixture (Moir & Harris, 1962) fed at 0845 and 1645 hours daily (1 kg offered in two equal portions daily), 1.8 - 2 litres/day of "synthetic saliva" as a buffer (made according to the specifications of McDougall, 1948) was continuously infused into the rumen of each animal. Sheep D only received the "synthetic saliva" infusate, resulting in a low nitrogen/low energy diet, whereas the daily infusate for sheep E was supplemented with a suspension of mixed carbohydrates and NH_4Cl (i.e. an artificial high nitrogen/high energy diet). This design is summarised in Figure 2.2. Of the daily ration of chopped wheaten straw offered, sheep D consumed approximately 400 g per day compared to 700 - 800 g per day by sheep E.

The daily mixed carbohydrates suspension consisted of 170 g cornflour, 15 g D(+)-xylose, 10 g gum arabic, 1 g D(+)-cellobiose {to provide a variety of carbon sources to the microbial population}, and 12.5 ml of volatile fatty acids mixture (1.8 ml *iso* -butyric acid; 2.0 ml *n* -valeric acid; 2.0 ml 2-methylbutyric acid; 2.0 ml *iso* -valeric acid per 100 ml; pH adjusted to 7.0 with NaOH (modified from Papas *et al.*, 1984)).

Sheep E was gradually introduced to the final suspension of mixed carbohydrates

and NH_4Cl (equivalent to approx. 200 g starch plus 54 g NH_4Cl per day) over seven days†. The final diets of chopped wheaten straw (plus minerals mixture) and appropriate infusion, were offered and infused, respectively, to the animals for three weeks prior to experimentation.

INFUSION TECHNIQUE : A two litre solution of "synthetic saliva", and another of "synthetic saliva" plus mixed carbohydrates and NH_4Cl, was prepared daily. The latter was stirred continuously to maintain a suspension of carbohydrates and to prevent the 2 mm diameter tubing running to the animal from becoming blocked. A Gilson "Minipuls 2" variable speed infusion pump (Gilson Medical Electronics, Villiers-le-Bel, France) and blue coded (1.60 c.c./min.) Elkay infusion pump tubes (Elkay Products Inc., Shrewsbury, U.S.A.) were previously calibrated to deliver 1.8 - 2 litres of infusate per day to the rumen of each animal via a tube inserted through the rumen fistula. A 30 cm length of tubing with random outlet holes and a 10 g weight on the end was used to discharge the infusate directly into the rumen to facilitate faster and more even mixing with rumen contents. This experimental setup was determined during a nine day preliminary infusion.

EXPERIMENTAL PROCEDURE : On the day of an experiment both animals were fed at 0845 hours. At 1145 hours 250 - 300 ml of "synthetic saliva" plus treatment# (treatment "pulse") was added through the rumen fistula of the animal being tested and 150 ml rumen fluid samples were taken at 0, 30, 60, 120, and 240 min. "post-pulse", as summarised in Figure 2.2. The pH of each sample was determined immediately and the sample was subdivided into three 50 ml aliquots.

One aliquot was treated identically to rumen fluid samples used to determine the diurnal levels of ammonia, free protein and carbohydrate (section 2.2.2.6). The remaining two aliquots were processed as outlined for the 50 ml aliquots in experiment 1 (section 2.2.2.2).

The effects of various bolus additions ("pulses") of NH_4Cl in "synthetic saliva" (SS) were investigated during the preliminary infusion mentioned above. Three boluses were tested : (i) 1 mole NH_4Cl in 200 ml SS; (ii) 0.01 moles NH_4Cl in 200 ml SS; (iii) 200 ml SS

† During a preliminary experiment, infusions of 1 (54 g) and 2 (108 g) moles NH_4Cl per day were tested. The former was decided upon because it resulted in a rumen ammonia concentration of between 35 and 50 mM, whereas the latter resulted in ammonia toxicity, with rumen ammonia concentration of about 125 mM.

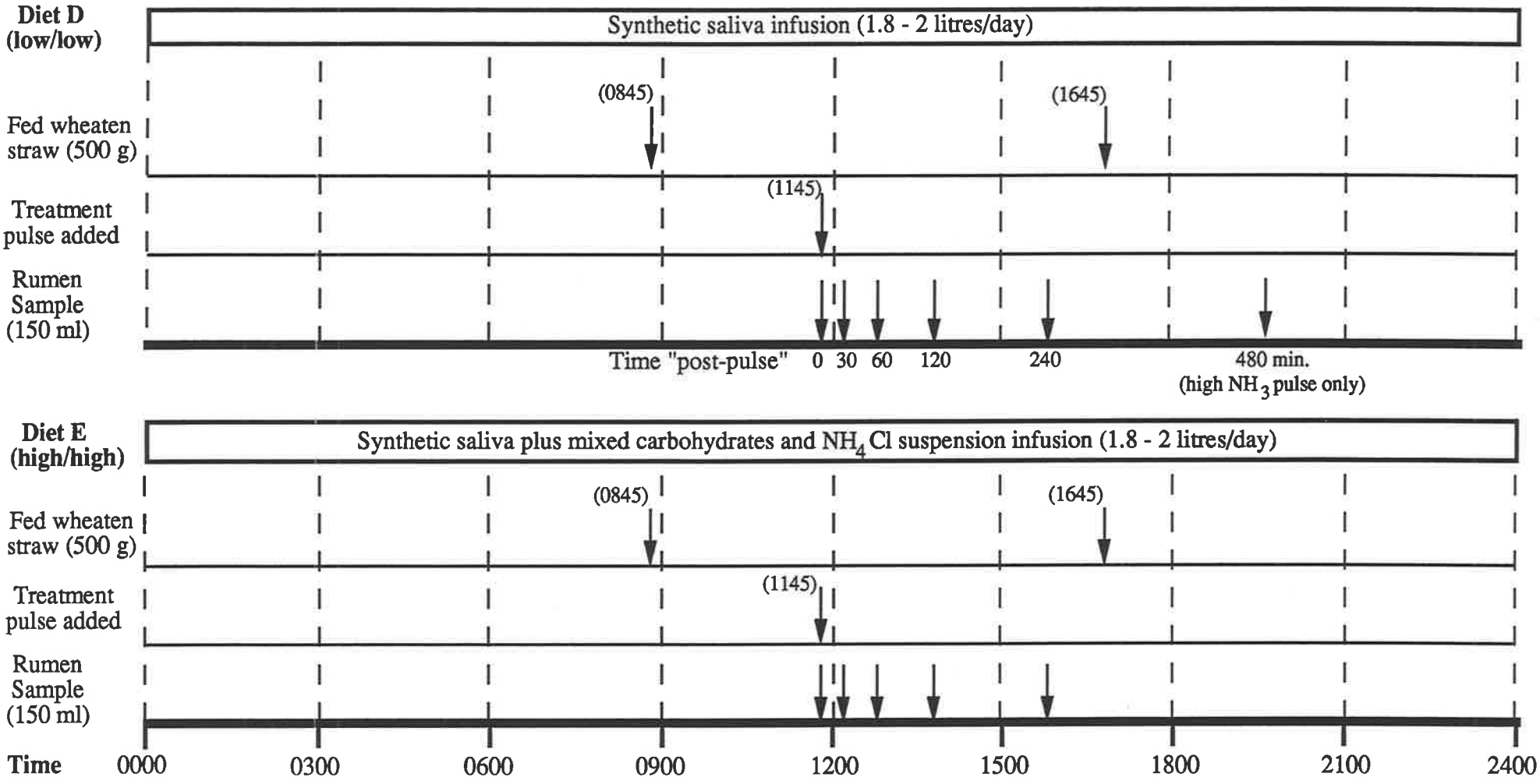


Figure 2.2: Summary of the experimental protocol for Experiment 2.

only. Rumen fluid samples, taken at 30 min. intervals up to 4 hrs, and again at 6 hrs "post-pulse", were used for pH and ammonia determinations. As a consequence, the bolus doses chosen for the "pulse" experiment were 0.005 and 0.5 moles NH_4Cl in 250-300 ml SS.

Sheep D received the following "pulses" on separate days of the experiment : (i) SS only; (ii) 0.005 moles NH_4Cl in SS; (iii) 0.5 moles NH_4Cl in SS; (iv) 0.5 moles glucose in SS; and (v) 0.5 moles NH_4Cl + 0.5 moles glucose in SS. Sheep E received the following "pulses", also on separate days : (i) SS only; (ii) 0.5 moles NH_4Cl in SS; (iii) 0.5 moles glucose in SS; (iv) 0.5 moles NH_4Cl + 0.5 moles glucose in SS. All "pulse" experiments were separated by at least four days to allow the rumen to return to its "pre-pulse" state.

2.2.2.4 Justification of Experimental Design

In vivo studies of rumen bacteria were chosen for the initial experiments in this thesis so that the processes of nitrogen metabolism and assimilation could be studied, and the results interpreted, without the added difficulty of having to extrapolate from *in vitro* experiments (Barnett & Reid, 1961; Chalupa, 1975; Russell & Hespell, 1981). The problems which exist with this type of work, such as mixed microbial populations, animal factors, etc., are acknowledged, but were considered to be an unavoidable consequence of an investigation of the processes of nitrogen metabolism and assimilation in the rumen.

Rumen-fistulated animals were used in these experiments to enable samples to be taken which were as representative as possible of the total microbial population (see section 1.6.1 of the Literature Review for a discussion of this topic).

2.2.2.5 Rumen Fluid Sampling and Initial Sample Processing

Rumen contents for assay of enzyme activity and measurement of rumen fluid parameters were sampled into stoppered 100 ml "*in vitro*" tubes using a short piece of plastic tubing (1.5 cm diameter) . The pH of each sample was determined immediately using a Solstat® FET pH/mV meter (model EPM-300 : Solstat Scientific Instrumentation, Laboratory Supply Pty. Ltd., Adelaide, Australia) with a Ross® pH electrode (Orion Research Inc., Cambridge, U.S.A.) and the samples placed on ice for transport to the laboratory.

Fifty ml of each crude rumen fluid sample was blended using a Janke & Kunkel KG (Staufen i. Breisgau, West Germany) Ultra-Turrax homogenizer (20,000 rpm; 1 min.) to release bacteria attached to feed particles (Mackie *et al.*, 1983). The blended mixtures were then squeezed through four layers of cheesecloth and centrifuged (100 x *g* ; 1 min.; 4°C) to remove protozoa and dense food particles. The supernatant of each sample was decanted into 40 ml screw-capped polyallomer centrifuge tubes (Beckman Instruments Inc., Palo Alto, U.S.A.) and recentrifuged (22,000 x *g* ; 10 min.; 4°C) to produce a pellet of bacteria, as described by Hutton *et al.* (1971). The pellet was resuspended twice in TKD buffer and centrifuged (22,000 x *g* ; 10 min.; 4°C) each time, resulting in thoroughly washed bacterial cells. This pellet, snap frozen by immersing the 40 ml centrifuge tube in liquid nitrogen for one min., was stored at -20°C (modified from Patterson & Hespell, 1985).

The above protocol was designed to obtain bacterial samples representative of all of the bacteria present in samples, rather than ones consisting predominantly of bacteria present only in the rumen fluid.

2.2.2.6 Measurement of Rumen Fluid Parameters

Experiment 1 - Clarified rumen fluid samples prepared during experiment 1 and stored at -20°C were subsequently used to measure ammonia and free protein levels.

AMMONIA MEASUREMENT : Stored samples were thawed and ammonia measured by the Ion Specific Electrode method using an Orion® Ammonia Gas Sensing Electrode (model 95-12; Orion Research Incorporated, Boston, U.S.A.) linked to a model EA940 Orion® Expandable Ion Analyser. Triplicate measurements were made on each sample.

PROTEIN MEASUREMENT : Stored, base-hydrolysed samples were thawed and protein measured by the method of Sedmak & Grossberg (1977), with BSA as a standard (0 - 75 μg). Triplicate measurements were made on each sample.

Experiment 2 - The low quality straw diet plus a continuous infusion of "synthetic saliva" as buffer, or carbohydrates and NH_4Cl suspended in this buffer, was planned so that feed materials would be fermented at a constant rate throughout the day. The following

procedures were used to determine whether this aim was achieved, and to characterise the fermentation patterns of the diets.

(i) Diurnal Levels of Rumen Parameters

Rumen fluid (50 ml) was sampled from each animal at two hr intervals beginning at 0830 hours and continuing until 0830 hours the following day (total of 13 samples). The pH of these samples was measured immediately and the samples subsequently subdivided as shown in Figure 2.3. This sampling and measurement protocol was performed three times and the data pooled to produce the mean ± the standard error of the mean from triplicate data. The data from each day were from triplicate assays for each parameter except the pH, which was from a single measurement.

AMMONIA MEASUREMENT : Clarified rumen fluid (20 ml) was stored at -20°C until all measurements could be made together. Ammonia concentration was measured by the method outlined for experiment 1 (above). Each sample was measured in triplicate.

PROTEIN MEASUREMENT : Clarified rumen fluid (2 ml) was base-hydrolysed (0.1 M NaOH; 10 min.; 70°C) and stored at -20°C until all samples could be assayed together. Protein was measured by the method of Sedmak & Grossberg (1977) with BSA as a standard. Triplicate measurements were made on each sample.

CARBOHYDRATE CONCENTRATION : Clarified rumen fluid (5 ml) was acid-hydrolysed (5 ml concentrated HCl; 100°C; 4 hr) and neutralised (10 N NaOH) according to the method of Leedle *et al.* (1982). Samples were stored at -20°C until all could be assayed together. Thawed hydrolysates were centrifuged (22000 x *g* ; 20 min.; 4°C) to remove debris and 2 ml of the resulting supernatant was added to a wide-mouthed test tube (16 - 20 mm diameter). Phenol Reagent† (0.05 ml) was then added, followed by rapid addition of 5 ml of concentrated H_2SO_4. Colour was developed by allowing the sample to stand at room temperature for 30 min. and the optical density was measured at 487.5 nm (glucose absorbs light between 485 and 490 nm) in a Lambda 5 UV/VIS Spectrophotometer (Bodenseewerk Perkin-Elmer & Co. GmbH, Überlingen, West Germany) according to the method of Ashwell (1966). Glucose (0 - 200 µg) was arbitrarily chosen as the standard with the knowledge that clarified rumen fluid samples contain a mixture of sugars. Triplicate measurements were made on each sample.

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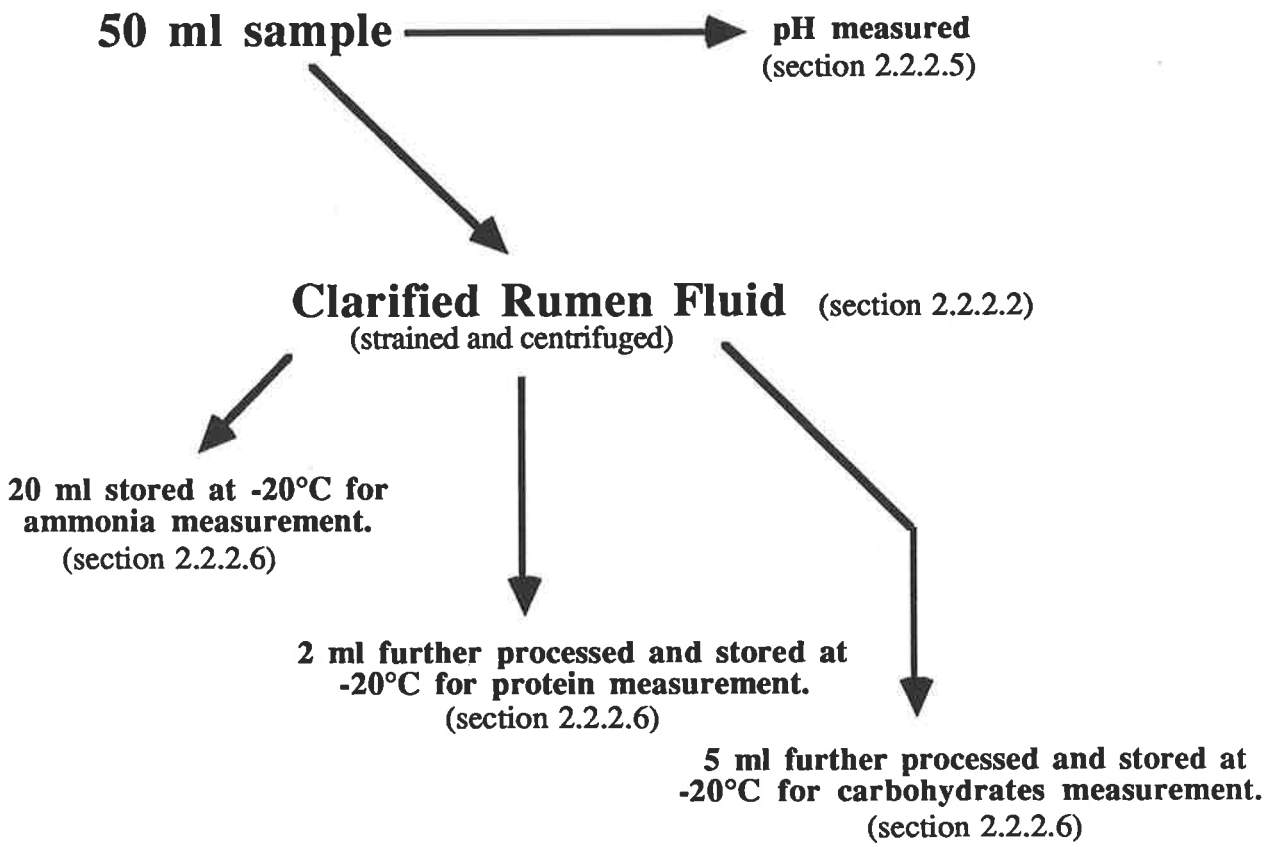
† Phenol Reagent : Phenol, 80 % [w/v]. Prepared by adding 20 g glass distilled water to 80 g redistilled, reagent grade phenol.

Figure 2.3 : Subdivision of rumen fluid sampled for measurement of diurnal pH, free protein, ammonia and carbohydrate levels in Experiment 2.



(ii) Feed Degradation Rate

The nylon bag technique of Mehrez & Orskov (1977) was used to determine the rate of degradation of the basal diet (chopped wheaten straw) fed to the animals. The fermentative activity developed with the two diets was calculated from this information. Results are from regression analysis of triplicate measurements.

(iii) Culture Counts

The rumen microbial population of both animals was characterised by counts of total viable bacteria and the number of cellulolytic, amylolytic and proteolytic bacteria using a modified version of Medium 5-4, originally developed by Baker & Moir (1983).

PROTOCOL : Bacterial cell pellets were prepared from 40 ml of crude rumen fluid sampled from both animals (sections 2.2.2.3 and 2.2.2.5), except that every effort was made to keep the samples anaerobic. Also, instead of washing in TKD buffer, they were

washed in anaerobic "S" buffer (Table 2.1), centrifuged (3000 x *g* ; 10 min.; 4°C) and the final pellets were resuspended in 40 ml of "S" buffer (i.e. equivalent to original sample volume). These cell suspensions were serially diluted in 10-fold amounts with "S" buffer to produce dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} for inoculation onto the appropriate test plates.

Agar plates (15-20 ml media in 60 x 15 mm plastic petri dishes) were prepared for each media and allowed to equilibrate in an anaerobic glove box (90 % CO_2 : 10 % H_2 atmosphere) for at least 48 hrs before use. Plates were inoculated in triplicate with 50 µl portions of the appropriate serial dilutions and the inoculum dispersed with a sterile glass spreading rod.

Plates were stacked into BBL® "GasPak 100™ Anaerobic System" anaerobic jars (BBL Microbiology Systems, Cockysville, U.S.A.) which were sealed and removed from the anaerobic glove box to a 39°C incubator. At 48 and 72 hrs the anaerobic jars were returned to the glove box for counts of growing colonies. Only plates containing between 10 and 100 colonies were used to calculate the number of viable bacteria on each media (Leedle & Hespell, 1980).

MEDIA : Components of the basal medium, modified from Baker & Moir (1983), are listed in Table 2.2. Complete carbohydrates Medium 5-4, used for counts of total viable bacteria, contained 0.05 g each of α-cellulose, D(+)-cellobiose, glucose, maltose, pectin, soluble starch, xylan and D(+)-xylose per 100 ml media in addition to the listed components. Cellulolytic bacteria were enumerated in Medium 5-4 containing 0.4 g α-cellulose (as substrate) per 100 ml media and amylolytic bacteria in this medium containing 0.05 g soluble starch per 100 ml media. Proteolytic bacteria were enumerated in this medium containing 0.5 g casein and 0.1 g glucose per 100 ml media. Proteose Peptone was not included in proteolytic Medium 5-4.

2.2.2.7 Enzyme Assays

Glutamine synthetase (GS : forward and γ-glutamyl transferase activities), glutamate synthase (GOGAT), glutamate dehydrogenase (GDH), alanine dehydrogenase (AlaDH) and asparagine synthetase (AsS) activities were assayed in bacterial cell-free extracts prepared as outlined in sections 2.2.2.2 (experiment 1) and 2.2.2.3 (experiment 2). Optimal

Table 2.1 : Composition of "S" buffer [a] (modified from Leedle & Hespell, 1980).

COMPONENT	AMOUNT per 100 ml
$CaCl_2.H_2O$	0.02 g
$MgSO_4$	0.025 g
KCl	0.06 g
NaCl	0.06 g
0.1 M Tris-HCl buffer, pH 7.0	5.0 ml
Resazurin (0.1 % solution)	0.10 ml
R_1 salts solution [b]	0.10 ml
R.O. water	91.3 ml
Dithiothreitol (0.1 M)	*1.5 ml*
NaH_2PO_4 (0.5 M; pH 7.0)	*2.0 ml*

[a] Buffer was prepared without the components in italics (heat labile), the pH adjusted to 7.0 with KOH and equilibrated with CO_2 before autoclaving; heat labile components were filter sterilised and added to cool, autoclaved buffer. The solution was stored in the anaerobic glove box to keep it anaerobic.

[b] As described by Hespell & Canale-Parola (1970) {25 ml modified Pfennig's trace element solution (0.5 g $AlCl_3$, 0.25 g KI, 0.25 g KBr, 0.25 g LiCl, 3.5 g $MnCl_2.4H_2O$, 5.5 g H_3BO_3 (heat to dissolve), 0.5 g $ZnCl_2$, 0.5 g $CuCl_2.2H_2O$, 0.5 g $NiCl_2.6H_2O$, 0.5 g $CoCl_2.6H_2O$, 0.15 g $SnCl_2.2H_2O$ (white precipitate forms when added), 0.15 g $BaCl_2.2H_2O$, 0.25 g $NaMoO_4.2H_2O$*, 0.05 g $NaVO_3$* (heat to dissolve). Each salt was dissolved separately in R.O. water; the pH of asterisked (*) metals is adjusted to 7.0 prior to the solutions being added in order, starting with $AlCl_3$; solution is brought to final volume of 1800 ml with R.O. water and pH adjusted to 3.0 - 4.0); 1 g tetrasodium ethylenediaminetetraacetic acid (EDTA), 3.75 g $CaCl_2.2H_2O$, 12.5 g $MgCl_2.6H_2O$, 0.5 g $FeSO_4.7H_2O$; made up to 100 ml with R.O. water}.

enzyme assay procedures outlined below were determined in preliminary assays with cell-free extracts of mixed rumen bacterial samples from animals on diets A, B and C (experiment 1 - section 2.2.2.2). Cell-free extracts stored at 4°C during experiments 1 and 2 were assayed for enzyme activity within 64 hrs of initial rumen sampling (GS was assayed within 36 hrs). The level of protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard. This value was used to calculate the specific activity of each enzyme, expressed per mg of protein in the cell-free extract.

1) Glutamine Synthetase

Glutamine synthetase (GS) activity was measured by the forward and γ-glutamyl transferase assays.

Table 2.2 : Composition of modified Medium 5-4 [a] (Baker & Moir, 1983).

COMPONENT	AMOUNT PER 100 ml
Proteose Peptone	0.2 g
Bacto-Agar	1.2 g
L-Cysteine hydrochloride	0.05 g
SUBSTRATE	Refer to text
Minerals A [b]	15.0 ml
Volatile fatty acids solution [c]	0.31 ml
Vitamins Solution [d]	0.1 ml
Hemin (0.1 % solution [w/v])	0.1 ml
Resazurin (0.1 % solution [w/v])	0.1 ml
R.O. water	59.39 ml
Minerals B [e]	*15.0 ml*
$NaHCO_3$ (4 % solution)	*10.0 ml*

[a] pH of media adjusted to 6.8 and equilibrated with CO_2 before autoclaving; components in italics were filter sterilised and added to autoclaved, cooled media.

[b] Minerals A : 0.24 %[w/v] KH_2PO_4; 0.60 %[w/v] NaCl; 0.60 %[w/v] KCl; 0.24 %[w/v] $MgSO_4$; 0.53 mg% $CoCl_2.6H_2O$; 0.20 mg% $ZnCl_2.2H_20$; 0.19 mg% $FeCl_3$; 0.18 mg% $CuCl_2.2H_20$.

[c] V.F.A. solution : 1 ml *n*-valeric acid; 1 ml *iso*-valeric acid; 1 ml 2-methyl butyric acid; 1 ml *iso*-butyric acid per 100 ml; adjusted to pH 7.0 with NaOH.

[d] Vitamins solution : 20 mg thiamine-hydrochloride; 20 mg Ca-D-pantothenate; 20 mg nicotinic acid; 20 mg riboflavin; 20 mg pyridoxine-monohydrochloride; 1 mg para-aminobenzoic acid; 0.5 mg d-biotin; 0.125 mg folic acid; 0.2 mg vitamin B_{12} ; made up to 1000 ml in 5 mM HEPES buffer ; adjusted to pH 7.3 and autoclaved for 5 min. (after Bryant & Robinson, 1962).

[e] Minerals B : 0.3 %[w/v] $CaC_{12}.6H_2O$; 0.09 mg% $(NH_4)_6Mo_{24}.4H_2O$.

(a) FORWARD ASSAY : This assay, modified from Patterson & Hespell (1985), is a measure of glutamine formation by GS (Bender *et al.*, 1977). Components of the assay (Table 2.3) were added individually to 10 ml plastic screw-capped tubes, mixed and pre-incubated at 37°C for 10 min. Triplicate assays, initiated by adding 60 µl of 0.2 M Na-ATP, were terminated and colour developed after 60 min. with the addition of 1 ml of "stop solution" (made with : 400 ml 10 % [w/v] $FeCl_3$, 100 ml 24 % [w/v] trichloroacetic acid, 50 ml 6 N HCl and R.O. water to 1200 ml). The final solution was mixed to dissolve the precipitate that forms transiently upon the addition of stop solution. Samples were centrifuged (3,000 x *g* ; 10 min.; 25°C) to remove any residual precipitate and the absorbances were measured at 540 nm in a Lambda 5 UV/VIS Spectrophotometer. The amount of γ-glutamyl hydroxamate formed in the

reaction was determined by reference to a standard curve for γ-glutamyl hydroxamate. Controls without cell-free extract and with boiled cell-free extract were run with each set of assays to check for background levels of γ-glutamyl hydroxamate. Other controls (minus Na-ATP; minus L-glutamic acid) were determined during preliminary experiments with only minor colour development evident. Specific activity is expressed as nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$. Results are the mean ± the standard error of the mean for the triplicate assays.

(b) γ-GLUTAMYL TRANSFERASE ASSAY : The γ-glutamyl transferase (γ-GT) assay, modified from that described by Patterson & Hespell (1985), was used to measure the total amount of glutamine synthetase enzyme, since both the adenylylated and deadenylylated forms of the enzyme are detected by this assay (Bender *et al.*, 1977). Components for all assays (Table 2.3) were added individually to 10 ml plastic screw-capped tubes, mixed and pre-incubated at 37°C for 10 min. A modified assay containing 60 mM $MgCl_2$, in addition to all other components, was used to determine the proportion of adenylylated enzyme present. Adenylylation percentage of the enzyme was calculated as shown in Figure 2.4 (Shapiro & Stadtman, 1970a). Triplicate assays were initiated with the addition of cell-free extract. Assays were terminated and colour was developed after 20 min. by the addition and mixing of 2 ml of stop solution. Samples were centrifuged (3,000 x *g* ; 10 min.; 25°C) to remove turbidity and the absorbance was measured at 540 nm. The amount of γ-glutamyl hydroxamate formed was determined by reference to a standard curve obtained with γ-glutamyl hydroxamate. Controls (minus cell-free extract; boiled cell-free extract) were assayed with each set of samples to detect any γ-glutamyl hydroxamate present in cell-free extracts. Other controls (minus L-glutamine; minus Na-ADP; minus sodium arsenate) were assayed during preliminary experiments with only minor colour development evident. Specific activity was expressed as nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$. Results are the mean ± the standard error of the mean for the triplicate assays.

2) Glutamate Synthase

Glutamate synthase (GOGAT) activity was determined spectrophotometrically (Lambda 5 UV/VIS Spectrophotometer with printer/plotter) from the initial rate of oxidation of

Table 2.3 : Composition of glutamine synthetase (GS) forward and γ-glutamyl transferase assay mixtures (modified from Patterson & Hespell, 1985).

Assay	Volume (µl)	Stock Solution [a]	Final Concⁿ
Forward reaction assay mixture	50	1.0 M Tris-HCl buffer, pH 6.7	50 mM
	45	1.0 M Hydroxylamine solution [b]	45 mM
	30	2.0 M $MgCl_2$	60 mM
	160	1.0 M L-Glutamic acid	160 mM
	x	Cell-free enzyme extract (in TKD buffer)	
	155 - *x*	"Extraction" buffer (50 mM Tris-HCl; 2.5 mM $MgCl_2$; 2 mM 2-mercaptoethanol; pH 6.7)	
γ-GT assay mixture	20	1.0 M Tris-HCl buffer, pH 6.5	20 mM
	60	1.0 M Hydroxylamine solution [b]	60 mM
	1.5	0.1 M $MnCl_2.4H_2O$; pH 7.0	0.15 mM
	300	0.1 M L-Glutamine, pH 7.0	30 mM
	20	1.0 M Sodium arsenate, pH 7.0	20 mM
	40	0.01 M Na-ADP, pH 7.0 (prepared fresh as required)	0.4 mM
	58.5	R.O. water (reduced volume for modified assay)	
	x	Cell-free enzyme extract (in TKD buffer)	
	500 - *x*	"Extraction" buffer (40 mM Tris-HCl; 0.3 mM $MnCl_2$; 2 mM 2-mercaptoethanol; pH 6.5)	

[a] All components were prepared as stock solutions prior to the experiment, unless stated otherwise.
[b] 2.0 M Hydroxylamine-monohydrochloride neutralised with 2.0 M NaOH.

NADH or NADPH measured at 340 nm in a 1 cm path length cuvette (modified from the method of Kumar & Nicholas, 1982).

Two cuvettes, both containing all the assay components listed in Table 2.4, were prepared for each assay. One of these cuvettes was placed in the reference beam of the spectrophotometer and the other in the sample beam. After "zeroing" to this reaction mixture the sample cuvette was removed and 5 µl of 10 mM NADH or NADPH was added (final concentration of 0.05 mM) with mixing. The cuvette was immediately returned to the sample beam of the spectrophotometer and the change in absorbance at 340 nm was plotted on continuous feed chart paper. Controls (minus NH_4Cl and minus 2-oxoglutarate) were assayed with each set of samples to detect any non-specific oxidation of NADH or NADPH. Initial reaction rate was calculated from the initial slope of the plotted curve and used to calculate GOGAT specific activity, expressed as µmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$ (Figure 2.5). Results are the mean ± the standard error of the mean for the triplicate assays.

3) Glutamate Dehydrogenase

Glutamate dehydrogenase (GDH) activity was determined spectrophotometrically using the assay mixture listed in Table 2.5 (modified from Fahien & Cohen, 1970) by the method outlined for GOGAT activity. Initial reaction rate was calculated from the initial slope of the plotted curve and used to calculate GDH specific activity, expressed as µmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$. Calculations were the same as those outlined above for GOGAT activity. Results are the mean ± the standard error of the mean for the triplicate assays.

4) Alanine Dehydrogenase

Alanine dehydrogenase (AlaDH) activity was determined spectrophotometrically using the assay mixture listed in Table 2.6 (modified from Yoshida & Freese, 1970) by the method outlined for GOGAT activity (above). Initial reaction rate, calculated as for GOGAT, was used to calculate AlaDH specific activity, expressed as µmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$. Results are the mean ± the standard error of the mean for the triplicate assays.

5) Asparagine Synthetase

Asparagine synthetase (AsS) activity was measured by the transferase assay of Burchall *et al.* (1964a) and Ravel (1970). In this assay the activity was determined by

Figure 2.4 : Calculation of adenylylation percentage from glutamine synthetase γ-GT activities (from Shapiro & Stadtman, 1970a).

$$\text{Number of enzyme subunits adenylylated } (n) = 12 - 12 \times \frac{\text{Activity in modified assay}}{\text{Activity in standard assay}}$$

$$\text{Percent Adenylylation} = \frac{n}{12} \times 100$$

Figure 2.5 : Calculation of GOGAT activity from initial slope of spectrophotometer plots.

Initial Slope of Plot = Change in optical density per min. {units $min.^{-1}$}

= Rate of NAD(P)H oxidation.

$$\text{Change in NAD(P)H level } min.^{-1} = \frac{\text{Change in optical density } min.^{-1}}{\sum_{340}^{mM}\ ^{\dagger}}$$

$= ???$ mM $min.^{-1}$

$= ???$ µmoles $ml.^{-1}$ $min.^{-1}$

$$\text{SPECIFIC ACTIVITY} = \frac{??? \text{ µmoles NAD(P)H oxidised } ml.^{-1}\ min.^{-1}}{\text{mg protein in cell-free extract}}$$

$= ??$ µmoles NAD(P)H oxidised $min.^{-1}$ $(\text{mg protein})^{-1}$

---

† $\sum_{340}^{mM} = 6.2$ for NADH and NADPH.

Table 2.4 : Components of glutamate synthase (GOGAT) assay mixture (modified from Kumar & Nicholas, 1982).

Volume (µl)	Stock Solution [a]	Final Conc[n]
25	0.2 M 2-oxoglutarate, pH 8.6 [b]	5 mM
50	0.1 M L-Glutamine, pH 7.0	5 mM
x	Cell-free extract (in TKD buffer)	
920-*x*	"Extraction" buffer (50 mM Tris-HCl; 0.1 mM EDTA; 2 mM 2-mercaptoethanol; pH 8.6 [b])	

[a] All components were prepared as stock solutions prior to the experiment, unless stated otherwise.
[b] pH 8.6 for NADH; pH 9.0 for NADPH.

Table 2.5 : Components of glutamate dehydrogenase (GDH) assay mixture (modified from Fahien & Cohen, 1970).

Volume (µl)	Stock Solution [a]	Final Conc[n]
50	0.2 M 2-oxoglutarate, pH 7.5 [b]	10 mM
50	1.0 M NH_4Cl	50 mM
x	Cell-free extract (in TKD buffer)	
895-*x*	"Extraction" buffer (50 mM Tris-HCl; 0.1 mM EDTA; 2 mM 2-mercaptoethanol; pH 7.5 [b])	

[a] All components were prepared as stock solutions prior to the experiment, unless stated otherwise.
[b] pH 7.5 for NADH; pH 7.7 for NADPH.

measuring the amount of ß-aspartyl hydroxamate produced. The assay procedure was identical to the γ-glutamyl transferase assay for GS, except for the assay mixture, which is shown in Table 2.7. Specific activity is expressed as nmoles ß-aspartyl hydroxamate formed $min.^{-1}$ (mg $protein)^{-1}$. Results are the mean ± the standard error of the mean for the triplicate assays.

2.2.2.8 Amino Acid Analysis

The remaining bacterial cell pellet prepared from each sample taken during experiments 1 and 2 (section 2.2.2.2 and 2.2.2.3, respectively) was resuspended in 2 ml TKD buffer and passed through the French Pressure Cell (twice x 10,000 p.s.i.). The suspensions were centrifuged (18,000 x *g* ; 20 min.; 4°C) and the supernatants retained. Samples were deproteinized by adding 0.5 ml 30% [v/v] sulpho-salicylic acid (SSA) to 1 ml of cell-free extract (duplicate samples were treated this way from each initial sample). After mixing, samples were left to stand for 10 min. and then SSA and precipitates were removed by centrifugation (18,000 x *g* ; 20 min.; 4°C). The supernatant was stored at -80°C.

Levels of aspartic acid, glutamic acid and alanine were measured using ion exchange chromatography with lithium citrate buffers, and post column fluorometric detection with *o*-phthalaldehyde on a Dionex D-300 Amino Acid Analyser. These amino acids were chosen since ammonia is first incorporated into them (Salter *et al.*, 1979). Glutamine, which is also important, but unstable, proved difficult to detect because the levels were very low and near the lower limits of detection. Results are the means of duplicates which were within 10 % of each other.

Results were not adjusted to reflect the number of cells per sample, unlike the specific activity data for the enzyme assays. It was assumed that the same number of cells were present in each sample used for amino acid measurements for each animal, since the same volume of rumen fluid was used for each sample. This may be a reasonable assumption within diets for experiment 2, since the rumen fermentations for each animal were relatively constant. For the less controlled diets of experiment 1 such an assumption may not be valid, nor may it be for comparisons between diets in experiment 2.

Table 2.6 : Components of alanine dehydrogenase (AlaDH) assay mixture (modified from Yoshida & Freese, 1970).

Volume (µl)	Stock Solution [a]	Final Concⁿ
20	0.1 M Pyruvic acid, pH 6.4 [b]	2 mM
100	1.0 M NH_4Cl	100 mM
x	Cell-free extract (in TKD buffer)	
875-*x*	"Extraction" buffer (50 mM Tris-HCl; 0.1 mM EDTA; 2 mM 2-mercaptoethanol; pH 6.4 [b])	

[a] All components were prepared as stock solutions prior to the experiment, unless stated otherwise.
[b] pH 6.4 for NADH; pH 6.5 for NADPH.

Table 2.7 : Components of asparagine synthetase (AsS) assay mixture (modified from Burchall *et al.*, 1964a; Ravel, 1970).

Volume (µl)	Stock Solution [a]	Final Concⁿ
50	Tris-$MnCl_2$ solution, pH 5.8 [b]	
80	1.0 M Hydroxylamine solution [c]	80 mM
200	0.1 M L-Aspartate, pH 6.4	20 mM
100	0.1 M Na-ATP (prepared daily and neutralised)	10 mM
70	R.O. water	
x	Cell-free extract (in TKD buffer)	
500-*x*	"Extraction" buffer (40 mM Tris-HCl; 5 mM $MnCl_2$; 2 mM 2-mercaptoethanol; pH 5.8)	

[a] All components were prepared as stock solutions prior to the experiment, unless stated otherwise.
[b] Made in the proportions : 40 ml 1.0 M Tris-HCl, pH 5.8; 5 ml 1.0 M $MnCl_2.4H_2O$; 5 ml H_2O.
[c] 2.0 M Hydroxylamine-monohydrochloride neutralised with 2.0 M NaOH.

2.2.2.9 Statistical Analyses

Statistical analyses were confined to Student's T-tests performed with the StatWorks™ computer package (version 1.2; Cricket Software, Philadelphia, U.S.A.) for the Apple Macintosh. These were used to compare between diets or treatments within particular parameters (e.g. activity of GS between diets A, B and C).

The mean ± the standard error of the mean were routinely calculated for triplicate assay data.

2.3 RESULTS

2.3.1 PREPARATION OF SAMPLES FOR ENZYME ASSAY

The level of protein in cell-free extracts prepared in the preliminary experiment to determine the most effective and efficient method of disrupting rumen bacteria are shown in Table 2.8. Clearly, four passages through the French Pressure Cell was the superior method in terms of protein released. If efficiency of time and effort were also considered, two passages through the French Pressure Cell was a more appropriate procedure. The French Pressure Cell methods disrupted about 90 % of cells, whereas after sonication a large number of cells were still intact, and very few cells were disrupted by simply freezing and thawing cell suspensions. CTAB appeared to interfere with the protein assay.

Comparisons of protein contents of extracts from the seven cell disruption methods give an indication of the efficiency of extraction. Two passages through the French Pressure Cell was the best method and it was therefore used routinely to disrupt the bacteria. This method, however, may not result in a cell-free extract truly representative of the bacterial sample, since the larger bacteria which are present may be more readily disrupted than the smaller cocci.

Table 2.8 : Comparison of cell disruption methods for preparing cell-free bacterial extracts for assays of enzyme activities.

Seven rumen bacterial cell pellets, prepared as in section 2.2.2.5, were resuspended in TKD buffer (10 ml) and one of the methods listed in section 2.2.2.1 was used to disrupt each cell suspension. Cell debris was removed by centrifugation and the level of protein was measured by the method of Sedmak & Grossberg (1977) with B.S.A. as a standard. See section 2.2.2.1 for full details.

METHOD OF CELL DISRUPTION	PROTEIN LEVEL †	VISUAL ASSESSMENT OF CELL EXTRACT
i) No treatment	0.13	Very few cells disrupted
ii) French Pressure Cell x 2	0.70	Approx. 90 % disrupted
iii) French Pressure Cell x 4	1.02	Approx. 90 % disrupted
iv) Sonication 1	0.25	> 50 % disrupted
v) Sonication 2	0.23	> 50 % disrupted
vi) Sonication 3	0.36	> 50 % disrupted
vii) CTAB treatment	ND£	< 10 % disrupted

† μg protein. (μl cell-free extract)$^{-1}$; values are the mean from duplicate assays.
£ Not Detected.

2.3.2 EFFECT OF RUMEN FLUID PARAMETERS ON THE AMMONIA-ASSIMILATING ENZYMES IN RUMEN BACTERIA *in vivo* (Experiment 1)

2.3.2.1 Levels of Rumen Fluid Parameters

pH, ammonia and free protein levels in rumen fluid samples varied markedly from day-to-day for each animal in experiment 1 despite consistent diets being offered at the same time daily. Mean pH values, shown in Table 2.9, were not different between the three diets ($P > 0.05$), however diets A and B had much higher ammonia concentrations than diet C, but were not different from each other (5 % level of significance). The mean level of protein was higher for diet A than either diet B or diet C ($P < 0.05$).

2.3.2.2 Assay of Ammonia-Assimilating Enzyme Activity

Ideally the cell-free extracts for GS, GOGAT, GDH and AlaDH assays (and AsS assay in experiment 2 only) should be prepared with different "extraction" buffers tailored for each enzyme, since they have different optimal pH, etc. requirements. This is especially the case for GS, because this enzyme requires a metal cofactor (e.g. Mn^{++} or Mg^{++}) (Shapiro & Stadtman, 1970a,b), which is not required by the other enzymes. In this experiment, as well as in experiment 2, it was impractical to prepare separate extracts, hence a single buffer (TKD) was used to prepare cell-free extracts for all of the enzymes. Activity was detected for each enzyme in preliminary experiments with TKD as a "universal" buffer.

(1) Glutamine Synthetase Activities

GS activities on each of the five days for this experiment, measured using both the γ-GT and forward reactions, were very low or undetectable in cell-free extracts for the three diets (data not shown). These very low activity levels were largely due to very high activity values for the control assays (boiled enzyme) run with each assay set. The activity of these controls is normally subtracted from "active" assay activities, however the control and active assay values were similar in magnitude in this experiment. This was caused by the incomplete removal of the protein which precipitated during preparation of the boiled enzyme controls prior to optical density measurements.

Table 2.9 : pH, ammonia and protein levels measured in aliquots of rumen fluid sampled from the three animals in experiment 1 (see Figure A1.1, Appendix 1 for the values for each day).

Rumen fluid samples (125 ml) from each animal were taken at 1130 hours on 5 days spread over a 22 day period. The pH was determined immediately, and the sample divided into one 25 ml and two 50 ml aliquots. The 25 ml aliquot of each sample was processed for storage and subsequent measurement of ammonia and free protein levels (see section 2.2.2.6 for details). One of the 50 ml aliquots of each sample was processed into a cell-free bacterial extract for assays of the activity of GS, GOGAT, GDH and AlaDH. The other 50 ml aliquot was processed into a cell-free bacterial extract for measurement of the intracellular levels of glutamic acid, glutamine, aspartic acid and alanine.

All values are the mean ± standard error of the mean from combined data from individual days. Measurements within each day were in triplicate, except pH for which only single measurements were recorded.

Dissimilar superscripts within rumen fluid parameters are significantly different ($P < 0.05$), according to Student's T-tests, with $n = 5$.

DIET	RUMEN FLUID PARAMETERS		
	pH	Ammonia†	Free Protein††
A	5.80 ± 0.099 [a]	26.9 ± 8.03 [a]	460.0 ± 61.77 [a]
B	5.77 ± 0.194 [a]	32.6 ± 2.50 [a]	156.6 ± 16.31 [b]
C	5.41 ± 0.160 [a]	1.72 ± 0.040 [b]	209.2 ± 28.75 [b]

† mM ammonia
†† µg protein (ml rumen fluid)$^{-1}$

Attempts to determine the extent of adenylylation of GS using a modified γ-GT assay, containing 60 mM $MgCl_2$ in addition to the normal assay constituents, were inconclusive for this experiment. Adenylylation percentages calculated from these assays ranged from -43 % to + 65 %.

(2) Glutamate Synthase Activities

NADH- and NADPH-dependent GOGAT activities (expressed as μmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$) were detected in cell-free extracts from the three diets of experiment 1 (Table 2.10). A consistent correlation was not evident between the day-to-day levels of rumen fluid ammonia, free protein or pH, and the large variation in GOGAT activities between days within any of these diets (compare Figures A1.1 and A1.2, Appendix 1). Comparisons between diets showed that the mean NADH-dependent activity for diet C was much lower than for diets A and B ($P < 0.05$), which were similar to each other ($P > 0.05$). In contrast, the NADPH-dependent activities were not significantly different for the three diets ($P > 0.05$). The ratios of NADH to NADPH dependent activities were therefore similar for diets A and B (7.1 and 6.6, respectively), but much lower for diet C (1.6). Nevertheless, the NADH-dependent activities were always greater than those with NADPH.

(3) Glutamate Dehydrogenase Activities

As for GOGAT, NADH- and NADPH-dependent GDH activities (expressed as μmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$) were detected in cell-free extracts from the three diets of experiment 1 (Table 2.10). A consistent correlation between the levels of rumen fluid ammonia, free protein or pH from day-to-day for each diet, and the GDH activities, was not evident (compare Figures A1.1 and A1.2, Appendix 1). Diet comparisons showed that the mean NADH-dependent GDH activity was highest for diet B, followed by diet A and much lower for diet C ($P < 0.05$). In contrast, the mean NADPH-dependent GDH activities for the three diets were not significantly different from each other ($P > 0.05$). Diet B therefore had the highest NADH to NADPH enzyme activity ratio (12.6), followed by diet A (9.8) and finally diet C (3.1) (i.e. NADH-dependent activities were greater than those of NADPH : Table 2.10).

(4) Alanine Dehydrogenase Activities

NADH- and NADPH-dependent activities (expressed as μmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$) were also detected for AlaDH, however these activities were much lower than those for GOGAT and GDH measured in the same samples (Table 2.10). AlaDH activities were also very variable between days, however they did not correlate with the data for rumen fluid pH, ammonia and free protein levels (compare Figures A1.1 and A1.2, Appendix 1). Neither the NADH-dependent nor the NADPH-dependent activities were significantly different between the diets ($P > 0.05$). This was associated with the very large variation in activity evident between days. The NADH to NADPH ratio for enzyme activity varied from 2 to 12 (Table 2.10). Thus, the NADH-dependent activities were always greater than those specific for NADPH, as was the case for GOGAT and GDH.

(5) Asparagine Synthetase Activity

Assays to detect the activity of this enzyme were not performed in experiment 1.

2.3.2.3 Intracellular Levels of Amino Acids

Consistent trends were not apparent in the intracellular levels of glutamic acid, glutamine, aspartic acid and alanine between days for each of the diets, nor was there any correlation with rumen fluid pH, ammonia or free protein levels (compare Figures A1.1 and A1.2, Appendix 1).

Comparisons of the concentrations of these amino acids between the diets were not possible, since the values were not expressed on a per mg protein (or similar) basis. Comparisons between days also may not be valid, since the number of bacteria was not necessarily constant for each sample.

2.3.3 REGULATION OF THE AMMONIA-ASSIMILATING ENZYMES IN RUMEN BACTERIA *in vivo* (Experiment 2)

2.3.3.1 Diurnal Levels of Rumen Fluid Parameters

In contrast to experiment 1, where diets A, B and C were not closely controlled, an attempt was made in experiment 2 to reduce differences in fermentation pattern from day-to-

Table 2.10 : Specific activities of GOGAT, GDH and AlaDH, and intracellular levels of amino acids, in cell-free extracts prepared from rumen bacterial samples from diets A, B and C (experiment 1).

The day-to-day variation can be seen in Figure A1.2 (Appendix 1).
Rumen fluid sample and sampling details are as for Table 2.9.
Full enzyme assay details are in section 2.2.2.7.
Amino acid measurement details are in section 2.2.2.8.

All values are the mean ± standard error of the mean from combined data from individual days. Enzyme activity measurements within each day were from triplicate assays; amino acid values within each day were means of duplicates which were within 10 % of each other.

Dissimilar superscripts within enzymes are significantly different ($P < 0.05$), according to Student's T-tests ($n = 5$).

ENZYME	DIET A	DIET B	DIET C
GOGAT Activity §			
NADH-dependent	444.4 ± 111.14 [a]	451.0 ± 31.60 [a]	159.5 ± 19.79 [b]
NADPH-dependent	62.9 ± 14.13	68.8 ± 5.86	102.0 ± 19.98
NADH / NADPH ratio	*7.1*	*6.6*	*1.6*
GDH Activity §			
NADH-dependent	979.0 ± 116.19 [a]	1524.2 ± 155.21 [b]	469.4 ± 52.94 [c]
NADPH-dependent	100.2 ± 13.77	120.7 ± 11.78	149.9 ± 24.80
NADH / NADPH ratio	*9.8*	*12.6*	*3.1*
AlaDH Activity §			
NADH-dependent	72.1 ± 26.88	35.4 ± 11.63	19.3 ± 9.94
NADPH-dependent	6.0 ± 2.45	8.2 ± 2.55	9.0 ± 1.62
NADH / NADPH ratio	*12.0*	*4.3*	*2.1*
Intracellular Amino Acids ¥			
Glutamic Acid	2.63 ± 0.789	1.65 ± 0.132	1.93 ± 0.251
Glutamine	0.38 ± 0.100	0.16 ± 0.071	0.44 ± 0.090
Aspartic Acid	1.25 ± 0.359	1.28 ± 0.152	0.84 ± 0.105
Alanine	2.01 ± 0.236	1.51 ± 0.264	1.52 ± 0.046

§ Specific activity expressed as μmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$
¥ Level expressed as nmoles amino acid $(ml\ rumen\ fluid)^{-1}$

day (and even from hour-to-hour) within the diets, thus controlling rumen fluid conditions. The diurnal levels of rumen fluid pH, ammonia, free protein and carbohydrates (as glucose), measured on three days for the animals fed diets D and E of this experiment, show that pH, free protein and ammonia levels remained fairly constant throughout each day, and between days, for the animals fed diet D (Table 2.11). The very low concentration of ammonia (maximum value of 0.718 mM) in the rumen fluid of this diet is a result of the low nitrogen status of the chopped wheaten straw fed *ad libitum*.

The pH also varied in a consistent manner throughout each day for samples taken from animal E, however the level of ammonia in the rumen fluid increased slightly following feeding (Figure A1.3, Appendix 1) and was not constant throughout each day (Table 2.11). The pH of this diet was significantly lower than for diet D ($P < 0.01$), and the mean ammonia level was more than 10 times greater than for diet D ($P < 0.01$). The concentration of free protein in rumen fluid samples similarly was significantly higher for the animal fed diet E than diet D ($P < 0.01$), and was more variable for diet E (Table 2.11). The amount of carbohydrates (as glucose) was significantly higher in rumen samples from the animal fed diet D than diet E ($P < 0.01$), although both varied markedly throughout the day, and between days, with no obvious relationship to feeding time or other daily activity (Figure A1.3, Appendix 1).

2.3.3.2 Feed Degradation Rate

An index of the fermentative activity of diets D and E was determined by the nylon bag technique of Mehrez & Orskov (1977). The $T_{0.5}$ (time taken for half the ground wheaten straw in a nylon bag to be degraded while in the rumen) was 5.7 for diet D and 3.9 for diet E, indicating greater fermentative activity in diet E than diet D.

2.3.3.3 Culture Counts

The total number of viable bacteria, and the number of viable cellulolytic, proteolytic and amylolytic bacteria, from diets D and E of experiment 2 are presented in Table 2.12. The number of viable bacteria in diet D is extremely low. Counts of viable bacteria in the rumen are usually only about 25 % of the total number present, with the lowest counts usually coming from animals on fibrous diets (Hobson & Wallace, 1982a). The present data, however, represents only about 0.01 % of the normal rumen population (Czerkawski, 1986). Such low

Table 2.11 : Mean diurnal levels of rumen fluid pH, ammonia, free protein and carbohydrates (as glucose) for the low nitrogen/low energy (D) and high nitrogen/high energy (E) diets of experiment 2 (see Figure A1.3, Appendix 1 for the diurnal trends).

Rumen fluid (50 ml) was sampled at intervals of 2 hrs. The pH was measured immediately and the sample subdivided and processed for subsequent measurement of ammonia, free protein and carbohydrates levels according to the scheme in Figure 2.3, and as outlined in section 2.2.2.6.

Sampling and measurement protocols were repeated and the data pooled. Data are therefore the mean ± the standard error of the mean of data from three individual days of measurements (n = 39, except for the asterisked (*) points where n = 38). Measurements within each day were in triplicate, except pH values for which only single measurements were recorded.

Dissimilar superscripts between diets for a parameter are significantly different ($P < 0.05$), according to Student's T-tests.

DIET	pH	Ammonia #	Free Protein †	Carbohydrates §
D	7.06 ± 0.019 ^{d}	0.176 ± 0.0280 ^{*d}	189.6 ± 2.14 ^{*d}	113.7 ± 9.54 ^{d}
E	6.61 ± 0.022 ^{e}	2.12 ± 0.185 ^{e}	292.1 ± 7.90 ^{e}	69.5 ± 5.39 ^{e}

mM ammonia.
† µg protein (ml rumen fluid)$^{-1}$.
§ µg glucose (ml rumen fluid)$^{-1}$.

Table 2.12 : Counts of total viable bacteria and viable cellulolytic, proteolytic and amylolytic bacteria cultured from rumen bacterial samples from diets D and E (experiment 2) taken at the end of the experiment.

Bacterial cell pellets prepared as in section 2.2.2.6 (iii) from diets D and E were serially diluted in 10-fold amounts with anaerobic "S" buffer. Triplicate anaerobic agar plates of complete carbohydrates Medium 5-4, α-cellulose Medium 5-4, soluble starch Medium 5-4, and casein Medium 5-4 (section 2.2.2.6 (iii)), were inoculated with the appropriate serial dilutions (50 µl) and the inoculum spread with a sterile glass rod. Plates were incubated anaerobically at 39°C and colonies counted after 48 and 72 hrs.

Results are the mean of plates with 10 - 100 growing colonies (Leedle & Hespell, 1980). Values in brackets ( ) are the percentage of the total viable bacteria which grew on the respective media.

Means with dissimilar superscripts within a media type are significantly different ($P < 0.05$).

Diet	Total Viable Bacteria †	Viable Cellulolytic Bacteria †	Viable Proteolytic Bacteria †	Viable Amylolytic Bacteria †
D	3.2×10^6 a	4.4×10^4 a (1.4 %)	3.6×10^6 a (113 %)	2.1×10^6 a (65 %)
E	1.1×10^8 a	4.3×10^7 b (39 %)	2.3×10^5 a (0.2 %)	1.3×10^7 a (12 %)

† expressed as cells (ml rumen fluid sample)$^{-1}$

viabilities may be a real effect or may be an artifact due to deficiencies in media or methods (Hobson & Wallace, 1982a). Nevertheless, there is an obvious trend towards a greater number of viable bacteria in the sheep fed diet E than diet D, indicating a more active bacterial population in the rumen of E, although the differences are not significant ($P > 0.05$). The lack of statistical significance is probably due to the large amount of variation between plates even though only plates with 10 - 100 colonies were included in the calculations. As a consequence of this restriction the number of plates included in calculations varied for each medium.

The number of viable cellulolytic bacteria was greater for sheep fed diet E than diet D ($P < 0.05$) and the proportion of total viable bacteria which were cellulolytic was also greater for sheep fed diet E than for diet D. In contrast, the number of viable proteolytic and amylolytic bacteria were not significantly different between diets ($P > 0.05$), however when these data were expressed as a proportion of the total viable bacteria present the values were greater for the sheep fed diet D than diet E. Each set of counts was determined only at the end of the experiment, thus information is not available about variation between days. These differences do not strictly relate to dietary effects due to sheep x diet effects (i.e. intake level, fluid and particulate pool sizes, flow rates, dilution rates, etc). Thus, it is actually the whole ruminal system which has resulted in this pattern of viable counts.

2.3.3.4 Day-to-Day Levels of Rumen Fluid Parameters

As well as the diurnal measurements of rumen fluid, similar measurements were made as part of the study of the regulation of the ammonia-assimilating enzymes in rumen bacteria *in vivo* (experiment 2). These measurements, made on rumen fluid samples taken prior to the bolus addition of ammonia, glucose, or ammonia plus glucose (treatment "pulse"), show that levels of pH, ammonia and free protein were not as variable between days for this experiment (Table 2.13) as was the case for experiment 1 (Table 2.9). The mean pH, ammonia and carbohydrates (as glucose) levels, however were different between diets D and E for experiment 2 ($P < 0.05$: Table 2.13). The mean level of free protein did not change between these diets (Table 2.13).

These data show very similar mean pH values to those measured during the diurnal rumen samplings, with diet E still having significantly different values for each parameter than did diet D (Table 2.11). In contrast, the rumen fluid carbohydrates levels were higher for diet D than diet E in the diurnal data of Table 2.11, but the reverse is the case for the day-to-day values in Table 2.13. Comparisons between diets from experiments 1 and 2 were

Table 2.13 : Mean values for rumen fluid pH, ammonia, free protein and carbohydrates for diets D and E, measured in aliquots of rumen fluid samples taken prior to the addition of treatment "pulses" in experiment 2 (the day-to-day variation can be seen in Figure A1.4, Appendix 1).

Rumen fluid samples (150 ml) were taken immediately prior to bolus additions of ammonia, glucose, or ammonia plus glucose, to the rumen of each animal. Fifty ml of each sample was processed as outlined for diurnal samples (section 2.2.2.6 (i)), stored and ultimately used to measure ammonia, free protein and carbohydrate levels. The pH was determined immediately following sampling.

Values are the mean ± the standard error of the mean from combined data from individual days. Measurements within each day were in triplicate, except the pH, for which only single measurements were recorded.

Dissimilar superscripts within rumen fluid parameters are significantly different ($P < 0.05$) according to Student's T-tests. n = 5 for diet D; n = 4 for diet E.

DIET	pH	Ammonia #	Free Protein †	Carbohydrates §
D	7.11 ± 0.056 ^{d}	0.294 ± 0.1172 ^{d}	246.7 ± 20.18	115.3 ± 7.42 ^{d}
E	6.61 ± 0.031 ^{e}	4.15 ± 0.996 ^{e}	282.7 ± 13.93	219.1 ± 44.36 ^{e}

mM ammonia.
† µg protein (ml rumen fluid)$^{-1}$
§ µg glucose (ml rumen fluid)$^{-1}$

not analysed statistically, however clear differences are evident in pH, ammonia and free protein levels, illustrating the extent of differences between the diets from these experiments (see Tables 2.9 and 2.13).

2.3.3.5 Day-to-Day Enzyme Activities

(1) Glutamine Synthetase Activities

Unlike diets A, B and C of experiment 1, GS γ-GT and forward activities were detected in all rumen bacterial samples from the two diets of experiment 2 (Table 2.14). High control activities, which confounded the GS activity data from experiment 1, were not recorded for experiment 2 due to more exacting assay procedures being employed in the latter experiment. The data from experiment 2 is/are therefore probably a more reliable indication of GS activity in rumen bacteria *in vivo* than the data from experiment 1.

The GS activity values measured in experiment 2 show a large variation between days for diets D and E. This variation does not consistently follow the same fluctuations evident for the rumen fluid parameters (compare Figures A1.4 and A1.5, Appendix 1),suggesting the activity of this enzyme is independent of the levels of these parameters. Nevertheless, Table 2.14 indicates that GS activity in the low nitrogen/low energy diet (D) was invariably higher than that for the high nitrogen/high energy diet (E) ($P < 0.005$ for γ-GT activity; $P < 0.01$ for forward activity).

As was the case for experiment 1, attempts to determine the extent of adenylylation of GS were inconclusive for this experiment. Adenylylation percentages calculated from these assays ranged from 0.03 % to 74 %.

(2) Asparagine Synthetase Activities

AsS activity was not detected in samples assayed from diet D, however low activities were detected in samples taken on three of the four days in diet E (Table 2.14).

(3) Glutamate Synthase Activities

NADH- and NADPH-dependent GOGAT activities were detected in cell-free extracts of bacteria from both diets, as was the case for the three diets of experiment 1 (Table

2.14). These activities varied greatly from day-to-day, despite the defined nature of diets D and E. As in experiment 1, the variation in activity did not correspond to that of rumen fluid ammonia, free protein or pH levels measured concurrently (compare Figures A1.4 and A1.5, Appendix 1). Mean NADH-dependent GOGAT activities for diets D and E (experiment 2) were similar (Table 2.14), and were not significantly higher than those for diet C in experiment 1 ($P > 0.05$; Table 2.11). The mean NADH-dependent GOGAT activities were also similar between diets A and E ($P > 0.05$), however the activity for diet A was higher than for diet D, and diet B was higher than both diets D and E ($P < 0.05$; Tables 2.11 & 2.14). NADPH-dependent GOGAT activities, although variable, were fairly similar between diets within experiment 2, as well as between experiments 1 and 2 ($P > 0.05$). The NADH to NADPH ratio for diet D was 1.5 and for diet E was 1.6, thus the NADH-dependent activities were greater than the NADPH-dependent activities in this experiment, as was the case in experiment 1.

(4) Glutamate Dehydrogenase Activities

A great deal of variation of NADH- and NADPH-dependent GDH activities was also evident between days in this experiment (Table 2.14). This variation could not be attributed to the variable values for rumen fluid pH, ammonia, free protein or carbohydrates (compare Figures A1.4 & A1.5, Appendix 1). Despite different levels of pH, ammonia and carbohydrates in diets D and E ($P < 0.05$), the mean activity of NADH- and NADPH-dependent GDH was similar for both diets ($P > 0.05$; Tables 2.13 & 2.14). NADH-dependent activities were consistently about twice that of NADPH-dependent activities, with NADH to NADPH ratio values of 3.4 and 2.6 for diets D and E, respectively. The mean values for NADH-dependent GDH activity were similar for diets C (experiment 1), D and E (experiment 2) ($P > 0.05$), however samples from the sheep on diets A and B (experiment 1) had higher activities than from diets D and E ($P < 0.05$: Tables 2.11 & 2.14). The NADPH-dependent activity was higher for diet E than diets A and B ($P < 0.05$), but no other differences were evident between the diets. Similarly, the NADPH-dependent activities for diets D and E were not significantly different ($P > 0.05$).

(5) Alanine Dehydrogenase Activities

The NADH- and NADPH-dependent AlaDH activities for diets D and E were

also very variable from day-to-day (Table 2.14) and showed no correlation with rumen fluid ammonia, free protein or pH levels (compare Figures A1.4 & A1.5, Appendix 1). As for experiment 1, the NADH-dependent activity was 2 - 10 times greater than the NADPH-dependent activity of the enzyme in experiment 2 (average NADH to NADPH ratio was 3.3 for diet D and 11.1 for E), however both activities were low compared with GOGAT and GDH activities. NADPH-dependent activities were similar for both diets ($P > 0.05$), however the NADH-dependent value for diet E was greater than for diet D ($P < 0.05$) (Table 2.14). Higher NADH-dependent AlaDH activities in diet E (experiment 2) than diets B and C (experiment 1) were the only significant differences between the two experiments ($P < 0.05$: Tables 2.11 & 2.14).

2.3.3.6 Day-to-Day Levels of Intracellular Amino Acids

These data are similar to those of experiment 1 in that no trends were evident within either of the diets between days, nor was there a correlation with rumen fluid parameters (Table 2.14 & compare Figures A1.4 & A1.5, Appendix 1). Comparisons between the two experiments are not possible, since data were not corrected for possible differences in the numbers of cells per sample.

Glutamine was detected in most samples taken from diets A, B and C, but was not detected in those from diet D, nor in these initial samples from diet E. It was, however, above detectable limits in three other samples from diet E.

2.3.3.7 Effects of Ammonia, Glucose, and Ammonia plus Glucose, Boluses on Enzyme Activities

As well as studying the variation of GS, GOGAT, GDH, AlaDH and AsS activities, and intracellular amino acid levels, between days in two artificially-controlled rumen fermentations (diets D and E), experiment 2 was also designed to study the effects of bolus additions to the rumen of ammonia, glucose (energy), or ammonia plus glucose, on the activity of these enzymes and the amounts of amino acids produced. Samples, taken from the rumen at 0, 30, 60, 120 and 240 min. after the addition of each bolus, were processed for the assays of enzyme activities in cell-free extracts (Figures 2.6 & 2.7 and Tables 2.15 & 2.16). Figures 2.6 and 2.7 also show the concentrations of ammonia and carbohydrates (as glucose) in clarified

Table 2.14 : Mean specific activities of GS, AsS, GOGAT, GDH and AlaDH, and intracellular amounts of amino acids, in cell-free extracts prepared from rumen bacterial samples from diets D and E immediately prior to the addition of treatment "pulses" in experiment 2 (the day-to-day variation can be seen in Figure A1.5, Appendix 1).

Rumen fluid sample and sampling details are as for Table 2.13.
Full enzyme assay details are in section 2.2.2.7.
Amino acid measurement details are in section 2.2.2.8.

All values are the mean ± the standard error of the mean from the combined data from individual days. Enzyme activity measurements within each day were from triplicate assays; amino acid values within each day were means of duplicates which were within 10 % of each other.

Dissimilar superscripts within enzymes or amino acids are significantly different ($P < 0.05$), according to Student's T-tests, with $n = 5$ for diet D and $n = 4$ for diet E.

ENZYME	DIET D (low nitrogen/low energy)	DIET E (high nitrogen/high energy)
GS Activity #		
γ-GT Assay	337.1 ± 57.86 d	66.0 ± 17.35 e
Forward Assay	33.5 ± 6.92 d	5.5 ± 1.40 e
AsS Activity £		
	Not Detected	11.5 ± 5.44
GOGAT Activity §		
NADH-dependent	146.6 ± 46.12	175.2 ± 34.33
NADPH-dependent	95.1 ± 19.38	110.4 ± 21.59
NADH / NADPH ratio	*1.5*	*1.6*
GDH Activity §		
NADH-dependent	465.5 ± 40.53	478.6 ± 28.74
NADPH-dependent	137.6 ± 17.63	185.8 ± 26.59
NADH / NADPH ratio	*3.4*	*2.6*
AlaDH Activity §		
NADH-dependent	37.3 ± 14.31	101.0 ± 19.23
NADPH-dependent	11.2 ± 1.19	9.1 ± 1.70
NADH / NADPH ratio	*3.3*	*11.1*
Intracellular Amino Acids ¥		
Glutamic Acid	1.54 ± 0.134 d	2.91 ± 0.106 e
Glutamine	Not Detected	Not Detected
Aspartic Acid	0.48 ± 0.102 d	1.68 ± 0.126 e
Alanine	0.46 ± 0.063 d	1.35 ± 0.163 e

Specific activity expressed as nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$
£ Specific activity expressed as nmoles ß-aspartyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$
§ Specific activity expressed as µmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$
¥ Level expressed as nmoles amino acid $(ml\ rumen\ fluid)^{-1}$

rumen fluid samples taken concurrently for diet D, and Figure 2.8 presents data for diet E. A "control" bolus of "synthetic saliva" only was also administered to each rumen followed by the same sampling, enzyme assay and rumen fluid measurement protocols (Figures 2.6 & 2.8 and Tables 2.15 & 2.16).

Figure 2.6 (c & e), Figure 2.7 (a & c) and Figure 2.8 show that bolus additions of ammonia, glucose, or ammonia plus glucose, raised the level of ammonia, glucose, or both, in the rumen for the duration of each day's sampling. The pH and free protein values were also measured, however these parameters remained fairly stable for the control bolus additions, but declined in response to treatment boluses (Tables 2.15 & 2.16).

(1) Glutamine Synthetase Activities

The "control" boluses of "synthetic saliva" only did not have any clear effect on GS activities (γ-GT and forward) in the rumen bacterial populations of the animals on diets D and E over the sampling period (Figure 2.6 (b) & Table 2.16 for diets D & E, respectively). In contrast, the low ammonia pulse (0.005 moles NH_4Cl) administered to diet D resulted in a 17 and 30 % decrease in GS γ-GT and forward activities, respectively, 30 min. after addition. This effect lasted only while the ammonia concentration was elevated, despite the fact that it only increased from 0.12 to 0.73 mM (Figure 2.6 (c-ii & d)). The high ammonia (0.5 moles NH_4Cl) bolus added to diet D similarly resulted in about a 10 % decrease in GS activities within 30 min. of addition, at which time the ammonia concentration of the rumen fluid was about 96 mM (cf. 0.34 mM before addition of the bolus). The enzyme activities decreased to about 40 % of their pre-treatment levels, respectively, after four hrs (the ammonia concentration was still 54 mM : Figure 2.6 (e & f)).

The ammonia plus glucose bolus did not have a significant effect on the forward activity one hr after the addition to the rumen population in this animal, whereas the γ-GT activity increased by 20 %. The level of both of these activities decreased to about 40 % of their pre-treatment values four hrs after addition to the rumen (ammonia concentration was about 35 mM compared to 0.73 mM before the addition : Figure 2.7 (c & d)). Following this bolus addition of ammonia plus glucose to diet D, rumen samples were taken over a period of eight hrs (compared to four hrs for the other pulses), by which time the GS activities were only 20 % of their pre-treatment values. In fact,the activities were similar to those of diet E (Table

2.16). A consistent effect was not observed for GS activities in diet D in response to the glucose bolus (Figure 2.7 (a & b)).

Figure 2.6 (c-i & c-ii) shows that the low ammonia bolus (0.005 moles of NH_4Cl) added to diet D only resulted in a small increase in rumen ammonia (from 0.12 to 0.73 mM), hence this treatment was not included for diet E, which had a higher basal ammonia concentration than diet D (Table 2.11).

The basal GS activities of bacterial cell-free extracts from diet E (Table 2.16) were about one-quarter of those in diet D (Figures 2.6 & 2.7). Positive effects of the four bolus additions ("control", ammonia [0.5 moles only], glucose, and ammonia plus glucose) were not evident under this dietary regime.

(2) Asparagine Synthetase Activities

AsS activity was not detected in samples assayed from diet D (Table 2.15), however low activities were found in samples taken after the "control", glucose, and ammonia plus glucose, boluses administered to diet E (Table 2.16). Despite the relatively low initial AsS activities in diet E, these were decreased by 100 % four hrs after the glucose bolus and by 83 % four hrs after the ammonia plus glucose bolus. No activity was detected on the day when the ammonia bolus was administered.

(3) Glutamate Synthase Activities

As was observed for GS activity, the "control" boluses of "synthetic saliva" given to diets D and E had no immediate effects on NADH- or NADPH-dependent GOGAT activities in rumen bacterial samples (Tables 2.15 & 2.16), although the NADH-dependent activity fluctuated from 20 % above, to 35 % below, the initial activity over the sampling period for diet D. The ammonia, glucose, and ammonia plus glucose, boluses added to diets D and E also did not have any consistent effects on NADH- or NADPH-dependent GOGAT activities (Table 2.15 & 2.16), despite the fact that these boluses raised ammonia and/or glucose concentrations of the rumen (Figures 2.6, 2.7 & 2.8).

Figure 2.6 : Effects of "control", low ammonia and high ammonia boluses on rumen fluid ammonia and carbohydrates (as glucose) levels, and GS specific activities, in rumen bacterial samples from diet D (see Table 2.15 for the enzyme activities, and the levels of other rumen fluid parameters and intracellular amino acids).

Rumen fluid samples (150 ml) were taken before (0), and at 30, 60, 120 and 240 min. after, the bolus addition of "synthetic saliva" only ("control" bolus), or "synthetic saliva" plus 0.005 moles of NH_4Cl (low ammonia bolus) or 0.5 moles of NH_4Cl (high ammonia bolus).

The pH was measured immediately and the samples divided into three 50 ml aliquots, two of which were processed into cell-free bacterial extracts for assay of GS, AsS, GOGAT, GDH and AlaDH activity and measurement of glutamic acid, glutamine, aspartic acid and alanine levels, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.
Full details of rumen fluid sampling, and ammonia and carbohydrates measurements, are in sections 2.2.2.3 and 2.2.2.6, respectively.

Ammonia, carbohydrates and enzyme activity values are the mean ± the standard error of the mean from triplicate assays.

(a) Ammonia (•) and carbohydrates [as glucose] (◦) levels for the control bolus added.

(b) GS γ-GT (♦) and forward (◇) specific activities for the control bolus.

(c-i) Ammonia concentrations (•) for the low ammonia bolus.

(c-ii) Ammonia (•) and carbohydrates (◦) levels for the low ammonia bolus.

(d) GS γ-GT (♦) and forward (◇) specific activities for the low ammonia bolus.

(e) Ammonia (•) and carbohydrates (◦) concentrations for the high ammonia bolus.

(f) GS γ-GT (♦) and forward (◇) specific activities for the high ammonia bolus.

(a)
(c)
(i)
(ii)
(e)
Ammonia (mM)
Carbohydrates (µg glucose (ml rumen fluid)$^{-1}$)
(b)
(d)
(f)
γ-GT Specific Activity (nmoles γ-glutamyl hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$)
Forward Specific Activity (nmoles γ-glutamyl hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$)
Time after addition of bolus (mins)

Figure 2.7 : Effects of glucose, and ammonia plus glucose, boluses on rumen fluid ammonia and carbohydrates (as glucose) levels, and GS specific activities, in rumen bacterial samples from diet D (see Table 2.15 for the levels of other rumen fluid parameters, enzyme activities and intracellular amino acid levels).

Rumen fluid samples (150 ml) were taken before (0), and at 30, 60, 120 and 240 min. after, the bolus addition of "synthetic saliva" plus 0.5 moles of glucose (glucose bolus) or 0.5 moles of NH_4Cl plus 0.5 moles of glucose (ammonia plus glucose bolus).

The pH was measured immediately and the samples divided into three 50 ml aliquots, two of which were processed into cell-free bacterial extracts for assay of GS, AsS, GOGAT, GDH and AlaDH activity and measurement of glutamic acid, glutamine, aspartic acid and alanine levels, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.
Full details of rumen fluid sampling, and ammonia and carbohydrates measurements, are in sections 2.2.2.3 and 2.2.2.6, respectively.

Ammonia, carbohydrates and enzyme activity values are the mean ± the standard error of the mean from triplicate assays.

(a) Ammonia (•) and carbohydrates (◦) concentrations for the glucose bolus.

(b) GS γ-GT (♦) and forward (◇) specific activities for the glucose bolus.

(c) Ammonia (•) and carbohydrates (◦) levels for the ammonia plus glucose bolus.

(d) GS γ-GT (♦) and forward (◇) specific activities for the glucose bolus.

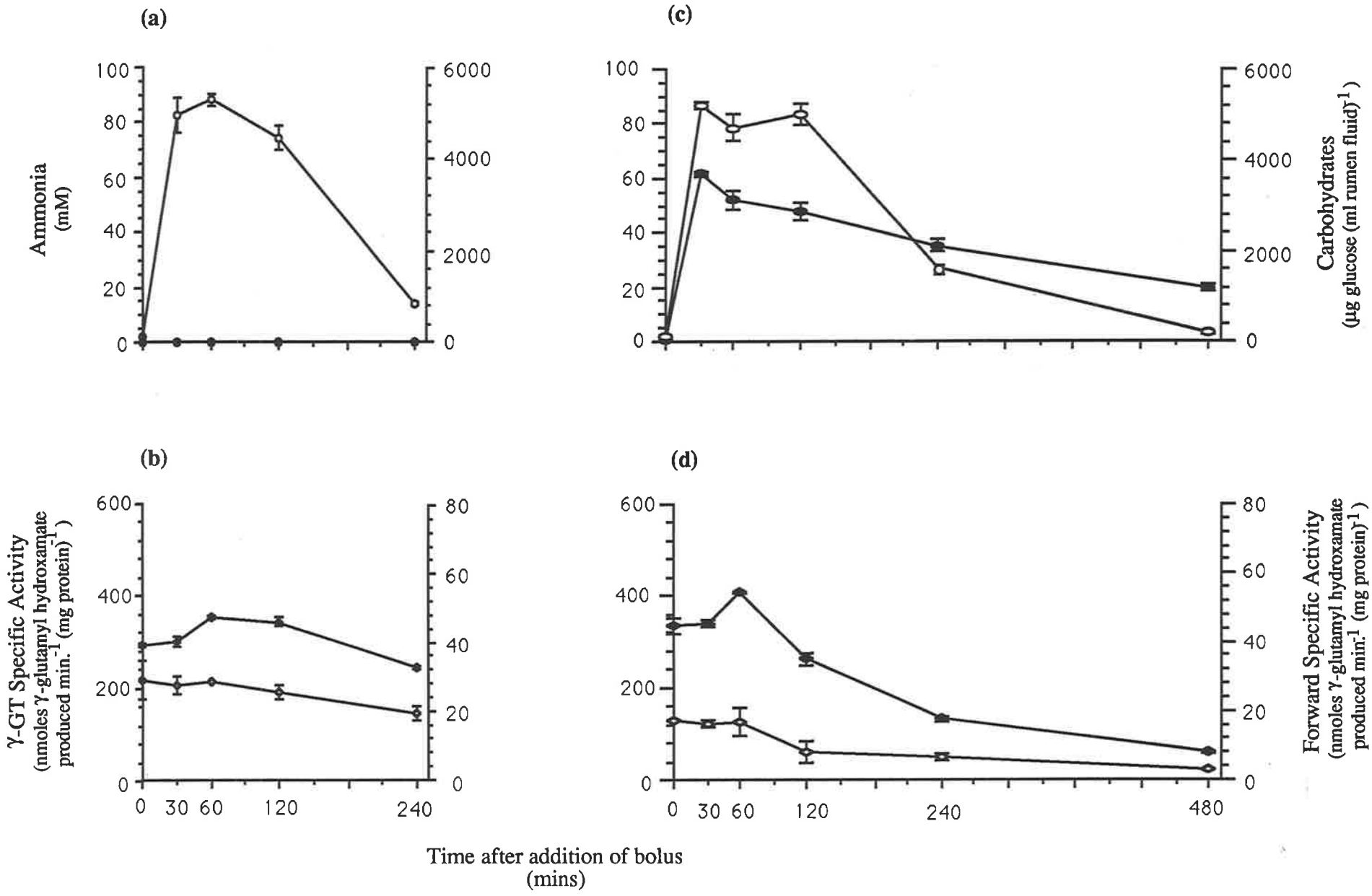

(a)
(b)
(c)
(d)
Ammonia (mM)
γ-GT Specific Activity (nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
Carbohydrates (µg glucose (ml rumen fluid)-1)
Forward Specific Activity (nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
Time after addition of bolus (mins)

Figure 2.8 : Effects of "control", ammonia, glucose, and ammonia plus glucose, boluses on rumen fluid ammonia and carbohydrates (as glucose) levels in rumen bacterial samples from diet E (see Table 2.16 for the levels of other rumen fluid parameters, enzyme activities and intracellular amino acid levels).

Rumen fluid samples (150 ml) were taken before (0), and at 30, 60, 120 and 240 min. after, the bolus addition of "synthetic saliva" only ("control" bolus) or "synthetic saliva" plus 0.5 moles of NH_4Cl (ammonia bolus), 0.5 moles of glucose (glucose bolus), or 0.5 moles of NH_4Cl plus 0.5 moles of glucose (ammonia plus glucose bolus).

The pH was measured immediately and the samples divided into three 50 ml aliquots, two of which were processed into cell-free bacterial extracts for assay of GS, AsS, GOGAT, GDH and AlaDH activity and measurement of glutamic acid, glutamine, aspartic acid and alanine levels, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.
Full details of rumen fluid sampling, and ammonia and carbohydrate measurements, are in sections 2.2.2.3 and 2.2.2.6, respectively.

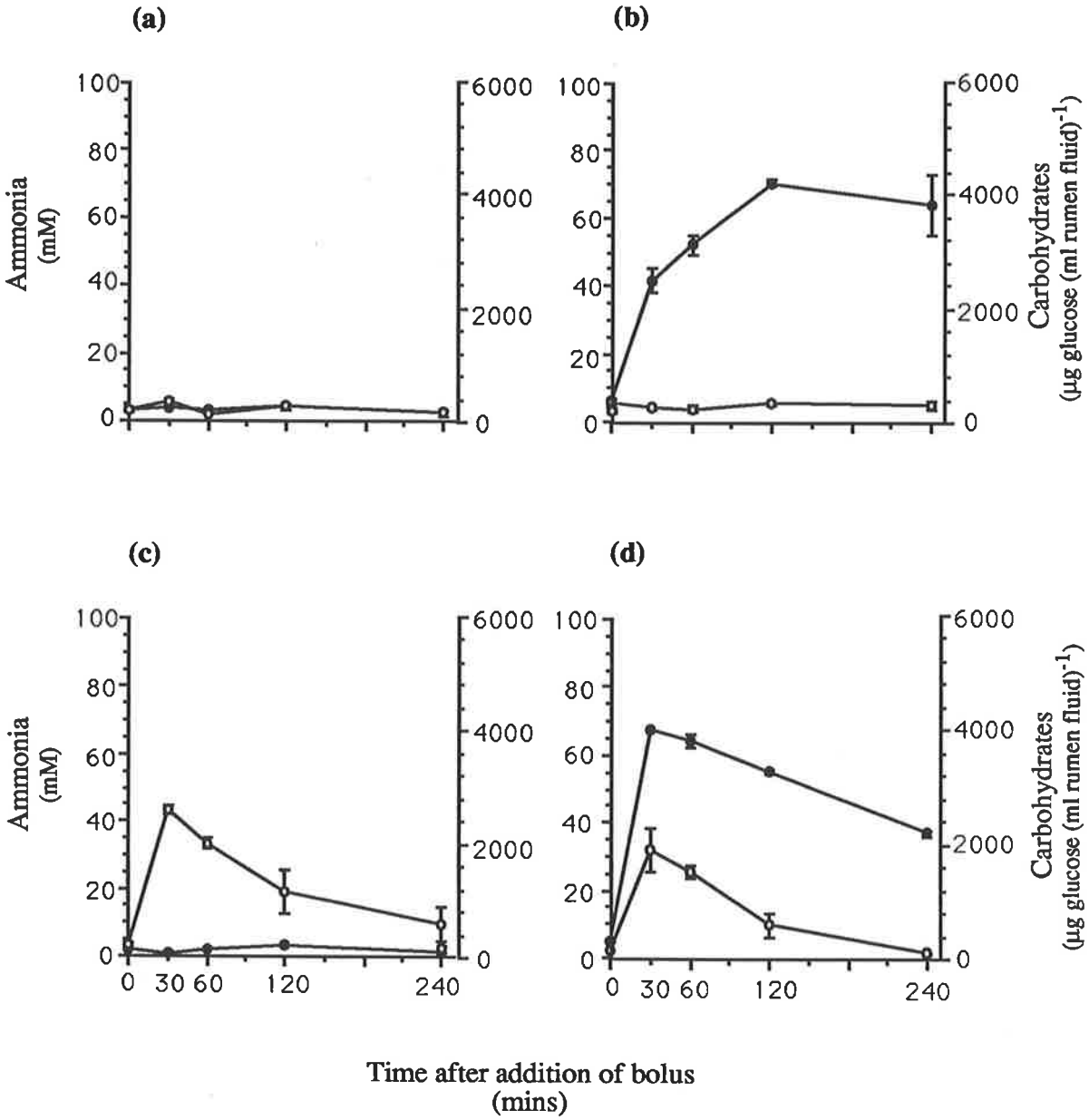
Ammonia and carbohydrates values are the mean ± the standard error of the mean from triplicate assays.

(a) Ammonia (•) and carbohydrates (◦) values for the control bolus.

(b) Ammonia (•) and carbohydrates (◦) concentrations for the ammonia bolus.

(c) Ammonia (•) and carbohydrates (◦) values for the glucose bolus.

(d) Ammonia (•) and carbohydrates (◦) concentrations for the ammonia plus glucose bolus.


(a)
(b)
(c)
(d)
Ammonia (mM)
Carbohydrates (µg glucose (ml rumen fluid)$^{-1}$)
100
80
60
40
20
0
6000
4000
2000
0
0 30 60 120 240
Time after addition of bolus (mins)

(4) Glutamate Dehydrogenase Activities

Similarly, GDH activities (NADH- and NADPH-dependent) in the rumen bacterial samples were not affected by the "control" bolus of "synthetic saliva" given to diet D (Table 2.15), however the NADPH-dependent GDH activity for diet E increased by 90 % compared to the initial activity (Table 2.16). These activities were not affected in a consistent way by the bolus additions of ammonia, glucose, or ammonia plus glucose, in either diet (Tables 2.15 & 2.16).

(5) Alanine Dehydrogenase Activities

There were also no consistent effects of "control", ammonia, glucose, or ammonia plus glucose, boluses on AlaDH activities in samples from sheep on either diet (Tables 2.15 & 2.16).

2.3.3.8 Effects of Ammonia, Glucose, and Ammonia plus Glucose, Boluses on Intracellular Levels of Amino Acids

The effects of the bolus additions of ammonia, glucose, or ammonia plus glucose, on the intracellular levels of glutamic acid, aspartic acid and alanine in rumen bacteria from diets D and E were variable, however there is a suggestion that the ammonia, and the ammonia plus glucose, boluses altered the proportions of the various amino acids (Table 2.15). Further work is needed to clarify these, as well as similar possible, trends from diet E (Table 2.16).

2.4 DISCUSSION

2.4.1 GENERAL FEATURES OF THE ENZYME ACTIVITY DATA

2.4.1.1 Sampling Considerations

A number of differences were evident in the pH, and the ammonia, free protein and carbohydrates (as glucose) levels, in rumen fluid samples within and between experiments 1 and 2 (Tables 2.9 & 2.11), indicating the extent of the dietary differences between the animals in these experiments. Different feeding regimes are known to change the species

Table 2.15 : Effects of "control", low ammonia, high ammonia, glucose, and ammonia plus glucose, boluses on rumen fluid pH and protein levels, AsS, GOGAT, GDH and AlaDH specific activities, and intracellular values for glutamic acid, aspartic acid and alanine, in rumen bacterial samples from diet D (the levels at each harvest time can be seen in Figures A1.6, A1.7 & A1.8, Appendix 1).

Rumen fluid samples (150 ml) were taken before (0), and at 30, 60, 120 and 240 min. after, the bolus addition of "synthetic saliva" only ("control" bolus) or "synthetic saliva" plus 0.005 moles of NH_4Cl (low ammonia bolus), 0.5 moles of NH_4Cl (high ammonia bolus), 0.5 moles of glucose (glucose bolus), or 0.5 moles of NH_4Cl plus 0.5 moles of glucose (ammonia plus glucose bolus).

The pH was measured immediately and the samples divided into three 50 ml aliquots, two of which were processed into cell-free bacterial extracts for assay of GS, AsS, GOGAT, GDH and AlaDH activity and measurement of glutamic acid, glutamine, aspartic acid and alanine levels, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.
Full details of rumen fluid sampling, and ammonia and carbohydrate measurements, are in sections 2.2.2.3 and 2.2.2.6, respectively.

Values are the mean ± the standard error of the mean from the five sampling times (n = 5) (six for the ammonia plus glucose treatment), except where indicated by an asterisk (*), where n = 4. Individual ammonia, carbohydrates, free protein and enzyme activity values were the result of triplicate assays; pH values were the result of single measurements; amino acid values were the mean of duplicates which were within 10 % of each other.

Amino acid values in brackets ( ) are the percentages of that amino acid relative to the total of the three acids measured.

PARAMETER / ENZYME	TREATMENT				
	Control	Low Ammonia	High Ammonia	Glucose	Ammonia plus Glucose
pH	7.18 ± 0.050	7.28 ± 0.015	6.81 ± 0.065	7.04 ± 0.065	6.75 ± 0.127
Free Protein †	213.6 ± 5.55	185.8 ± 7.77	365.4 ± 21.78	222.2 ± 13.01	246.1 ± 15.54
GS Activity #					
γ-GT Assay	See Figure 2.6	See Figure 2.6	See Figure 2.6	See Figure 2.7	See Figure 2.7
Forward Assay	See Figure 2.6	See Figure 2.6	See Figure 2.6	See Figure 2.7	See Figure 2.7
AsS Activity £	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
GOGAT Activity §					
NADH-dependent	176.2 ± 17.44	215.8 ± 23.55	82.4 ± 14.34	74.3 ± 6.88	61.7 ± 5.51
NADPH-dependent	122.8 ± 5.31	139.8 ± 3.14 *	53.1 ± 5.87	63.7 ± 1.75	59.4 ± 2.55
GDH Activity §					
NADH-dependent	462.3 ± 21.43	467.3 ± 29.39	435.9 ± 13.37	310.8 ± 9.09	504.7 ± 20.65
NADPH-dependent	167.9 ± 5.64	157.6 ± 19.08	112.5 ± 4.74	100.1 ± 3.54	164.8 ± 22.91
AlaDH Activity §					
NADH-dependent	32.5 ± 7.04	52.3 ± 13.47	32.6 ± 3.60	42.6 ± 3.42	19.1 ± 3.70 *
NADPH-dependent	12.8 ± 2.71	13.0 ± 1.52 *	12.2 ± 1.46 *	9.0 ± 1.80	12.1 ± 2.45
Amino Acids ¥					
Glutamic Acid	1.56 ± 0.133 (61 %)	1.41 ± 0.128 (68 %)	1.74 ± 0.276 (52 %)	1.70 ± 0.103 (62 %)	1.98 ± 0.111 (53 %)
Aspartic Acid	0.50 ± 0.068 (20 %)	0.32 ± 0.047 (15.5 %)	1.05 ± 0.284 (31 %)	0.59 ± 0.073 (22 %)	1.12 ± 0.129 (30 %)
Alanine	0.49 ± 0.038 (19 %)	0.34 ± 0.058 (16.5 %)	0.56 ± 0.084 (17 %)	0.45 ± 0.071 (16 %)	0.66 ± 0.032 (17 %)

† μg protein (ml rumen fluid)$^{-1}$
# Specific activity expressed as nmoles γ-glutamyl hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$
£ Specific activity expressed as nmoles ß-aspartyl hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$
§ Specific activity expressed as μmoles NAD(P)H oxidised min.$^{-1}$ (mg protein)$^{-1}$
¥ Level expressed as nmoles amino acid (ml rumen fluid)$^{-1}$

Table 2.16 : Effects of "control", ammonia, glucose, and ammonia plus glucose, boluses on rumen fluid pH and free protein levels, GS, AsS, GOGAT, GDH and AlaDH specific activities, and intracellular levels of glutamic acid, aspartic acid and alanine, in rumen bacterial samples from diet E (the levels at each harvest time can be seen in Figures A1.9, A1.10 & A1.11, Appendix 1).

Rumen fluid samples (150 ml) were taken before (0), and at 30, 60, 120 and 240 min. after, the bolus addition of "synthetic saliva" only ("control" bolus) or "synthetic saliva" plus 0.5 moles of NH_4Cl (ammonia bolus), 0.5 moles of glucose (glucose bolus), or 0.5 moles of NH_4Cl plus 0.5 moles of glucose (ammonia plus glucose bolus).

The pH was measured immediately and the samples divided into three 50 ml aliquots, two of which were processed into cell-free bacterial extracts for assay of GS, AsS, GOGAT, GDH and AlaDH activity and measurement of glutamic acid, glutamine, aspartic acid and alanine levels, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.
Full details of rumen fluid sampling, and ammonia and carbohydrates measurements, are in sections 2.2.2.3 and 2.2.2.6, respectively.

Values are the mean ± the standard error of the mean from the five sampling times (n = 5) (six for the ammonia plus glucose treatment), except where indicated by an asterisk (*), where n = 4. Individual ammonia, carbohydrates, free protein and enzyme activity values were the result of triplicate assays; pH values were the result of single measurements; amino acid values were the mean of duplicates which were within 10 % of each other.

Amino acid values in brackets ( ) are the percentages of that amino acid relative to the total of the three acids measured.

PARAMETER / ENZYME	TREATMENT			
	Control	Ammonia	Glucose	Ammonia plus Glucose
pH	6.65 ± 0.033	6.40 ± 0.114	6.48 ± 0.072	6.13 ± 0.149
Free Protein †	309.0 ± 8.13	252.5 ± 2.75	281.3 ± 4.30	241.3 ± 24.57
GS Activity #				
γ-GT Assay	68.1 ± 4.92	36.6 ± 9.07	135.6 ± 6.56	24.2 ± 9.78
Forward Assay	7.2 ± 1.06	1.3 ± 0.69	8.7 ± 0.32	8.1 ± 0.56
AsS Activity £	8.7 ± 1.39	Not Detected	8.6 ± 4.02	15.5 ± 3.66
GOGAT Activity §				
NADH-dependent	97.2 ± 5.48	165.7 ± 11.00	162.9 ± 11.34	229.9 ± 10.61
NADPH-dependent	75.5 ± 3.61	118.5 ± 5.08	97.2 ± 2.53	159.9 ± 4.61
GDH Activity §				
NADH-dependent	553.4 ± 28.56	428.2 ± 65.71	420.1 ± 17.53	497.1 ± 30.89
NADPH-dependent	229.6 ± 30.07	163.7 ± 21.64	178.3 ± 29.55	211.5 ± 11.29
AlaDH Activity §				
NADH-dependent	67.5 ± 6.07	59.1 ± 7.56	75.9 ± 19.10	105.6 ± 7.55
NADPH-dependent	9.6 ± 2.51	13.3 ± 1.38	8.5 ± 1.37	10.1 ± 1.12
Amino Acids ¥				
Glutamic Acid	2.82 ± 0.120 (52 %)	3.12 ± 0.057 (45 %)	2.91 ± 0.106 (51 %)	2.64 ± 0.246 (46 %)
Aspartic Acid	1.46 ± 0.039 (27 %)	2.00 ± 0.046 (29 %)	1.42 ± 0.103 (25 %)	1.59 ± 0.207 (28 %)
Alanine	1.12 ± 0.051 (21 %)	1.83 ± 0.069 (26 %)	1.35 ± 0.130 (24 %)	1.49 ± 0.161 (26 %)

† µg protein (ml rumen fluid)$^{-1}$
# Specific activity expressed as nmoles γ-glutamyl hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$
£ Specific activity expressed as nmoles ß-aspartyl hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$
§ Specific activity expressed as µmoles NAD(P)H oxidised min.$^{-1}$ (mg protein)$^{-1}$
¥ Level expressed as nmoles amino acid (ml rumen fluid)$^{-1}$

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composition of rumen microbial communities (Moir & Somers, 1957; Warner, 1962; Bryant & Robinson, 1968; Thorley *et al.*, 1968; Latham *et al.*, 1971; Mackie *et al.*, 1978; Leedle *et al.*, 1982; Mink *et al.*, 1982; Yokoyama & Johnson, 1988). The animals in experiments 1 and 2 are therefore likely to have different rumen microbial populations, each reflecting the nutritional characteristics of the particular diet, as well as the feed intake levels and feeding frequencies peculiar to the animals used and the diets offered (Warner, 1962, 1966b, c; Cecava *et al.*, 1990).

Samples of the microbial population of the rumen are also influenced by the post-prandial times at which the samples are taken from an animal, since the population changes throughout the day (Warner, 1962, 1966a, b, c; Bryant & Robinson, 1968; Wohlt *et al.*, 1976; Leedle *et al.*, 1982; Mink *et al.*, 1982; Yokoyama & Johnson, 1988). This is unlikely to have influenced population differences within each of the present experiments, since sampling was at the same time after feeding for each animal. Nevertheless, population differences between the experiments may have been influenced by the "pulse" and subsequent sampling performed as part of experiment 2, in addition to dietary factors. Similarly, although each rumen sample for these experiments was taken using the same procedures it is unlikely that representative bacterial samples were prepared, despite samples being processed to release plant-bound cells and separate the bacteria from the other constituents. This clearly is one of the drawbacks of all *in vivo* rumen studies.

2.4.1.2 Enzyme Considerations

A consistent feature of the GS activity data presented for experiment 2 is the much higher activities (3 - 25 times) from the γ-GT assays than from the corresponding forward assays. This difference is expected, since the standard γ-GT assay measures the total amount of enzyme present (both adenylylated and unadenylylated forms of the enzyme are active), whereas the forward assay measures the biosynthetic activity only (unadenylylated, unrepressed, uninhibited enzyme : Ebner *et al.*, 1970; Stadtman *et al.*, 1970; Bender *et al.*, 1977; Tyler, 1978). Nevertheless, the difference between the γ-GT activities and the corresponding forward activities appears to be too great to be solely due to the adenylylation state of the enzyme and may indicate that one of these assays is not a true reflection of the actual activities of GS *in vivo*. In particular, it is possible that the two activities may not partition

equally in cell fractions and assays of the cell-free extract only may not give a true indication of enzyme activity in the cells. Thus, the absolute GS activities presented in this chapter may not be relevant, but the relative levels between treatments and between experiments should be useful indicators of GS activity differences.

Attempts to determine the extent of adenylylation of GS in experiments 1 and 2 were inconclusive. This is probably due to sub-optimal conditions for the modified γ-GT assay used to determine adenylylation state in these experiments, rather than an indication that GS from rumen bacteria is not controlled by adenylylation. Work by Shapiro & Stadtman (1970a), Stadtman *et al.* (1970) and Bender *et al.* (1977), in which the effects of pH, divalent cations and substrates were studied in relation to GS activities, has emphasised the importance of optimal assay conditions. In the mixed rumen bacterial samples from experiments 1 and 2 the assay conditions could not be optimised for adenylylation measurements because the bacterial species resident in each rumen vary greatly, so that the properties of the resultant enzymes would also vary. It is unlikely that control mechanisms such as adenylylation can be detected under these conditions. This concept is highlighted by the diversity of enzyme properties evident within antigenically similar GS's from a number of Gram-negative bacteria (Tyler, 1978). Since a large number of bacterial species exist in the rumen, a greater diversity of enzyme properties would also be expected to occur in this environment. Such a range of enzyme properties between rumen bacteria is confirmed by pure culture studies with *Succinivibrio dextrinosolvens* and *Ruminobacter (Bacteroides) amylophilus*, which indicate that these species have GS enzymes that are subject to adenylylation control (Hespell, 1984; Patterson & Hespell, 1985). Studies with *Selenomonas ruminantium* have shown that GS is not controlled by adenylylation in this species (Smith *et al.*, 1980, 1981; Hespell, 1984).

2.4.2 VARIATION IN ENZYME ACTIVITY LEVELS FROM DAY-TO-DAY

2.4.2.1 Day-to-day Variation in Experiment 1

The aim of experiment 1 was to determine the effects (if any) of rumen ammonia and free protein levels on the activity of the ammonia-assimilating enzymes *in vivo*. GS activity data from this experiment were unreliable due to problems with boiled enzyme controls and the data are therefore not included in this thesis. The more reliable GS activity values measured in

experiment 2 (control values were very low) show a large amount of variation between days for each diet (Table 2.14) which did not correlate with the fluctuations of any of the rumen fluid parameters measured at the same time. Similarly, the NADH- and NADPH-dependent GOGAT, GDH and AlaDH activities fluctuated greatly from day-to-day in all of the diets in both experiments, as did the AsS activities measured in experiment 2 only, and the intracellular levels of glutamic acid, glutamine, aspartic acid and alanine measured in both experiments.

As mentioned previously (section 2.3.2.8), interpretation of the amino acids data is difficult, since the values were not expressed on a per mg protein basis, thus it is not possible to compare the values between diets, nor between the two experiments. Nevertheless, the fact that the same volume of rumen fluid was used to prepare each cell pellet means that conclusions can tentatively be drawn from data within any particular diet, especially for samples taken from diets D and E on the same day, since these diets showed relatively little variation in rumen fluid parameters within and between days (Table 2.13 between days and Tables 2.15 & 2.16 & Figures 2.6, 2.7 & 2.8 within days). Leedle *et al.* (1982) showed that fairly similar numbers of bacteria can be sampled throughout the day in diurnal experiments, suggesting that the number of bacteria in each sample taken on any one day in experiment 2 may be similar, since adequate precautions and consistent methods were maintained.

Work by Moir & Somers (1957), Warner (1962, 1966a,b, c) and Bryant & Robinson (1968) highlights that this assumption is not always correct, especially if changes in water and feed intake occur. Russell (1984) and Baker (1985) point out that, even if the same number of cells are present in each sample, the composition of the rumen changes with the time elapsed since feeding so that samples from the microbial population would be expected to reflect these changes (i.e. although the numbers of bacteria may be the same, the proportion of various species may change). These studies (Russell, 1984; Baker, 1985) also suggest that differences are likely to be much greater between rations, especially when there are different feed intakes and feeding frequencies. This is because there is a greater probability of sampling a different range of bacterial cells for each sample affected by the diets, and of sampling a different proportion of the microbial species from each animal. Samples taken on different days for any one animal were also assumed to contain the same number of cells, but this assumption is not as valid as for samples taken on the same day from the same animal. Nevertheless, by

making this assumption it is apparent that the levels of intracellular amino acids from day-to-day show no relationship to rumen fluid parameters.

In experiment 1 the variation in enzyme activities between days may have been influenced by the changes in fermentation rates within each diet resulting from uncontrolled animal factors, such as fluctuations in feed and water intake, and rumination by the animal. These factors obviously can affect the microorganisms directly by altering the levels of nitrogen, energy and carbon sources available to them, but they also have more subtle effects by changing the distribution of microbial species (Thorley *et al.*, 1968; Latham *et al.*, 1971), thereby also affecting enzyme activities of rumen samples. As a consequence of these effects, namely feed intake and feeding frequency, sampling from day-to-day could result in different proportions of microbial species being assayed for enzyme activity (Moir & Somers, 1957; Bryant & Robinson, 1968; Leedle *et al.*, 1982; Cecava *et al.*, 1990). Changes in the activities of enzymes which are associated with individual organisms can be used to confirm changes in the rumen bacterial population (Palmquist & Baldwin, 1966), and this also may be possible for enzymes such as are present here which are not necessarily unique to one organism. Possible changes in rumen volume may also have some effects on the species composition of the rumen microbial communities (Wohlt *et al.*, 1976), and therefore also on the enzyme activities from day-to-day. Sampling time, which also affects the species composition (Warner, 1962, 1966a, b, c; Bryant & Robinson, 1968; Wohlt *et al.*, 1976; Leedle *et al.*, 1982), was constant throughout this experiment and should not have affected the microbial flora.

2.4.2.2 Variation from Day-to-day in Experiment 2

The fermentation pattern and proportions of various bacterial species within the rumen are not usually constant throughout the day, nor from day-to-day, in animals fed once daily (Moir & Somers, 1957; Warner, 1962, 1966a, c; Bryant & Robinson, 1968; Leedle *et al.*, 1982). Since these factors may have influenced variations in enzyme activities from day-to-day in experiment 1, a more controlled ruminal fermentation, in which such variations were at least greatly reduced, was devised for experiment 2. The diurnal levels of rumen parameters determined for diets D and E (Table 2.11) show that the fermentation of feed materials throughout the day, and between days, was fairly constant for the two diets. In contrast, the

"normal" diets (fed once daily) studied by Moir & Somers (1957) [pH, ammonia and volatile fatty acids], Warner (1962), Warner (1966a, c) [concentrations of various microorganisms], Wohlt *et al.* (1976) [ammonia] and Leedle *et al.* (1982) [pH, ammonia, carbohydrates and cell numbers] show much larger variations in the parameters measured. The diurnal data (section 2.3.3.1), feed degradation rates (section 2.3.3.2) and, to a lesser extent, the culture counts (section 2.3.3.3), determined for diets D and E indicate that the feeding and infusion regimes of experiment 2 were partially successful in creating two very different rumen fermentation patterns, each with its own fairly constant rumen fluid parameters throughout and between days. These fermentation patterns appear to be more stable than those for the concentration of various microorganisms achieved with a sheep fed every three hours (Warner, 1966b).

Rumen fluid pH values in the animals fed diets D and E were within the normal range (5 - 7.5 : Czerkawski, 1986), however the ammonia levels as a result of diet D were extremely low (0.09 to 0.79 mM) due to the very poor quality basal ration offered to the animal. These levels are below the normal range of ammonia concentrations (2 to 40 mM according to Hespell (1984); 2 to 12 mM according to Czerkawski (1986)), however Schaefer *et al.* (1980) found that the predominant species of rumen bacteria are efficient scavengers of ammonia with very high affinities for ammonia (5 to 10 μM) and many can attain 95 % of their maximum growth rate in the presence of less than 1 mM ammonia. The effective growth of microorganisms at such a low level of rumen ammonia may be partly due to microenvironment effects raising the immediate ammonia levels for the microorganisms (Hobson & Wallace, 1982b; Odle & Schaefer, 1987), or due to active transport mechanisms enabling ammonia "scavenging" (Russell & Strobel, 1987), or a combination of these factors.

Although infused with one mole of NH_4Cl per day, the slightly variable diurnal ammonia level for diet E (Table 2.11) was just below the optimal range for microbial growth, which appears to be between 5 and 15 mM (Schaefer *et al.*, 1980). The actual optimal ammonia concentration is a controversial topic (Schaefer *et al.*, 1980) because it is undoubtedly dependent upon other dietary conditions (Hobson & Wallace, 1982b). The ammonia level for diet E (less than 4 mM) was higher than for diet D, but was much lower than had been expected, given that one mole of NH_4Cl, when infused daily into the rumen of a test animal, resulted in a rumen ammonia concentration between 35 and 50 mM. These data, and the variability of carbohydrates and free protein concentrations between days (Table 2.13), show that even though fairly constant

fermentation patterns were achieved with the artificial diets of experiment 2, variation in some rumen parameters still occurs. The variation was probably caused by animal factors, such as those discussed for experiment 1, technical factors, such as variation in the point of sampling within the rumen (Wohlt *et al.*, 1976), and assay inaccuracies, but this variation did not consistently influence the activity of the ammonia-assimilating enzymes.

When planning experiment 2 it was decided to artificially produce a low nitrogen and low energy diet, in addition to a high nitrogen and higher energy diet. The low nitrogen/ low energy diet was successfully created (diet D), in which the microbial community could only obtain their carbohydrate and nitrogen requirements from the very low quality chopped wheaten straw fed *ad libitum*, or from body reserves (the animal lost about 8 kg during the experiment). The diet and consequent rumen fluid parameters were significantly different for diet E compared with the low nitrogen/low energy diet (Table 2.13), and this animal gained about 4 kg during the latter stages of the experiment. Nevertheless, this was not the high nitrogen/high energy diet planned, since ammonia levels were below 4 mM, and the carbohydrate level was about 220 μg glucose (ml rumen fluid)$^{-1}$ for samples taken during the bolus experiments (but less than 100 μg ml^{-1} during diurnal sampling). The carbohydrates contents of diet D were about 115 and 100 μg ml^{-1}, respectively. The reason for the relatively low ammonia and energy conditions in diet E may have been due to the infusion of carbohydrates and NH_4Cl, resulting in a higher fermentation rate in diet E (see feed degradation data in section 2.3.3.2 and culture counts data in section 2.3.3.3). Although more nitrogen and energy were available to the microbes for growth in diet E, and a different fermentation pattern and microbial population resulted, the ammonia values were not "high" (e.g. less than 5 mM compared to the intended 20-30 mM; carbohydrate content was about 100 -200 μg glucose per ml rumen fluid). As a consequence, diet E produced a high fermentation rate, probably associated with high ruminal energy and nitrogen levels, but did not have high resident values for these parameters.

2.4.2.3 Comparisons Between Experiments 1 and 2

Although the activities of the ammonia-assimilating enzymes appear to fluctuate independently to the daily levels of rumen pH, ammonia, free protein and carbohydrates values from day-to-day in experiments 1 and 2, the average GS activities for diets D and E

(experiment 2) appeared to be influenced by rumen ammonia levels. In fact, diet D, which resulted in an extremely low rumen ammonia concentration (0.09 - 0.79 mM), had about a five-fold higher GS activity than diet E, which had a rumen ammonia level between 1.8 and 6.2 mM (Table 2.13). Similarly, there were some general similarities and differences in enzyme activities between diets A, B and C (experiment 1), such as 2.5-fold higher NADH-dependent GOGAT activities for diets A and B (ammonia levels were 26.9 and 32.6 mM, respectively) than for diet C (ammonia concentration of 1.7 mM). In addition, the NADH-dependent GDH activity was three-fold higher for diet B than diet C, but was only 1.5-fold higher than for diet A. The NADPH-dependent activities were similar between diets for GOGAT and GDH. Salem *et al.* (1973) also showed that GS, as well as NADH-dependent GDH, activities, were higher in diets containing 50 % urea-nitrogen than diets with zero or 100 % urea-nitrogen. In addition, Erfle *et al.* (1977) showed that GS activity was induced *in vitro* in mixed rumen bacterial cultures when the ammonia concentration decreased from 5 mM. They did not find a relationship between ammonia concentration and GDH activities. In contrast, Wallace (1979) found no differences in GS or NADH-dependent GDH activities for rumen bacterial samples taken from a diet in which the rumen ammonia concentration was increased from 6.1 to 13.4 mM by urea supplementation, but the NADPH-dependent GDH and NADH-dependent AlaDH activities were lower in samples from the latter rumen.

Thus, the data from experiments 1 and 2 support the view that, although the ammonia concentration in the rumen apparently has some effect on the activity of at least some of the ammonia-assimilating enzymes, this parameter cannot explain all the changes in enzyme activity. It is likely that pH, ammonia, free protein and carbohydrates levels are not the only parameters influencing the activity of the ammonia-assimilating enzymes.

2.4.3 NICOTINAMIDE ADENINE DINUCLEOTIDE SPECIFICITIES OF THE AMMONIA-ASSIMILATING ENZYMES IN THE RUMEN

2.4.3.1 Relative Proportions of NADH- and NADPH-dependent Activities

An important feature of the enzyme data presented for experiments 1 and 2 is that the NADH-dependent activities of GOGAT, GDH and AlaDH were much greater than those for NADPH-dependent activities. Chalupa *et al.* (1970), Wallace (1979) and Lenártová *et al.*

(1985) have previously shown that the NADH-dependent GDH activity is higher than that for NADPH in rumen bacteria (i.e. *in vivo* studies) and Lenártová *et al.* (1985) have shown the same trend for GOGAT activities. In contrast, the mixed culture work of Erfle *et al.* (1977) indicated that the NADPH-dependent GDH activity was higher than the corresponding NADH activity. No previous reports exist on both NADH- and NADPH-dependent AlaDH activities. Wallace (1979) detected NADH-dependent activity only.

The NADPH-dependent activities of GOGAT, GDH and AlaDH were low (compared to the NADH-dependent activities) under the widely-different rumen conditions of experiments 1 and 2. In contrast, the NADH-dependent activities of these enzymes, and therefore also the ratio of the NADH- to the NADPH-dependent activities, often fluctuated in response to undetermined factors. Palmquist & Baldwin (1966) found that the NADH to NADPH ratio for GDH changed in rumen bacterial samples with increasing ammonia concentration or, more accurately, with a change from a forage to a concentrate diet. Similarly in experiment 1, this ratio seems to increase (due to an increase in NADH-dependent activity) as the rumen ammonia concentration increases.

2.4.3.2 Possible Roles for the NADH and NADPH-dependent Activities

Brown *et al.* (1974), in a review of microbial inorganic nitrogen metabolism, indicated that microorganisms can possess GDH's with specificity for one of the nicotinamide adenine dinucleotides, or a non-specific requirement. Although bacteria (and mammalian sources) which possessed dual coenzyme specificity had not been reported at that time, *Bacteroides fragilis* has now been shown to have a GDH with dual coenzyme specificity (Yamamoto *et al.*, 1984). Similar results have been reported by Corman & Inamdar (1970), Doherty (1970), Fahien & Cohen (1970), Smith *et al.* (1975), Tyler (1978) and Kumar & Nicholas (1984). Given the plethora of bacterial species in cell-free extracts assayed in experiments 1 and 2 it is unlikely that the sole form of GDH is either two enzymes with distinct nicotinamide adenine dinucleotide specificities, or one enzyme with dual specificity. More importantly the data indicate that GOGAT and AlaDH also have NADH- and NADPH-dependent activities, raising the possibility that a similar range of specificities may occur in the rumen for these enzymes as probably occurs for GDH.

The NADPH-dependent activity of GDH is involved in the biosynthesis of glutamate (i.e. the reversible GDH reaction operates in the direction : 2-oxoglutarate + ammonia → glutamate + water), whereas the NADH-dependent activity has a catabolic function under low ammonia and/or high amino acid conditions (Brown *et al.*, 1974; Smith *et al.*, 1975; Tyler, 1978; Martinez-Bilbao *et al.*, 1988). The GDH activity data for experiments 1 and 2 indicate that in the rumen, NADH-dependent GDH activity predominates in the microbial population because of high ammonia levels and is probably involved in biosynthetic activity assimilating ammonia-nitrogen to amino-nitrogen. An alternative explanation is that amino acids present in the rumen may be inducing the high NADH-dependent activities. The levels of free amino acids in the rumen are very low (Allison, 1969, 1970; Church, 1979d; Mercer & Annison, 1976; Hobson & Wallace, 1982b), however glutamate is a major constituent of most feedstuffs (e.g. about 20 - 30 % of protein in wheat; about 10 % of protein in lucerne - Ostrowski-Meissner, 1987). Thus, the degradation of feed proteins should release glutamate into the rumen, which could be responsible for "driving" the catabolic activity of GDH (NADH-dependent activity), rather than the ammonia concentration. This has been reported for *Clostridium botulinum* 113B, in which the enzyme has apparently evolved for the release of excess ammonia and to rapidly replenish 2-oxoglutarate as a substrate in transamination reactions (Hammer & Johnson, 1988).

Caballero *et al.* (1989) found that AlaDH has both an aminating activity, to assimilate ammonia (biosynthetic), and a deaminating activity, to supply organic carbon to cells growing on L-alanine as the sole carbon and nitrogen source (catabolic) in phototrophic non-sulphur purple bacteria. Whether one or both of the nicotinamide adenine dinucleotides is concerned with each of these activities is not clear. Brown *et al.* (1974) suggested that the essential physiological function of AlaDH is the degradation of L-alanine to produce pyruvate, which can then be used as a carbon and energy source (the latter of the activities outlined by Caballero *et al.*, 1989). GOGAT "*. . . appears to catalyse a unidirectional reaction . . .*" (i.e. does not have biosynthetic and catabolic activities with either or both of NADH or NADPH), according to Brown *et al.* (1974).

2.4.3.3 The Importance of Various Ammonia-Assimilating Enzymes

Further speculation on the mechanisms and reasons for the NADH- and NADPH-dependent activities of the ammonia-assimilating enzymes is unwarranted considering the very

diverse microbial population from which the cell-free extracts were prepared. Nevertheless, it should be noted that the very low activities of AlaDH (i.e. relative to those for GDH and GOGAT : less than one-tenth) and AsS (relative to GS activity : less than one-fifth) detected in these experiments suggests that these enzymes may not play significant roles in ammonia assimilation in rumen bacteria. Although such a statement must be made cautiously without supporting enzyme kinetic data (e.g. V_{max}), the premise is supported by the high K_m-values normally associated with AlaDH (Yoshida & Freese, 1965; Brown *et al.*, 1974; Stewart, 1980; Moreno-Vivián *et al.*, 1983). As suggested by Hespell (1984), these enzymes may serve to expand the ammonia-assimilatory capabilities of the rumen flora, providing alternatives to the two apparently major ammonia-assimilatory pathways (GS/GOGAT and GDH), but data in this thesis suggest they are usually of minor importance in overall ammonia assimilation in the rumen.

The present data largely confirm the observations made from similar studies (Burchall *et al.*, 1964b; Hoshino *et al.*, 1966; Joyner & Baldwin, 1966; Palmquist & Baldwin, 1966; Chalupa *et al.*, 1970; Griffith & Carlsson, 1974; Erfle *et al.*, 1977; Bhatia *et al.*, 1978, 1980; Jenkinson *et al.*, 1979; Wallace, 1979; Smith *et al.*, 1980, 1981; Hobson & Wallace, 1982b; Lenártová *et al.*, 1985; Patterson & Hespell, 1985). A deficiency in these studies is that the activities of all of the major ammonia-assimilating enzymes in the rumen have not been measured at the same time *in vivo*, or in *in vitro* mixed rumen bacterial cultures. Previous workers also did not detect NADH-dependent AlaDH activity. Thus, the present data show that GS, AsS and NADH- and NADPH-dependent GOGAT, GDH and AlaDH activities are all present concurrently in the rumen microbial population, supporting the statement made by Hespell (1984) that the same enzymes of ammonia assimilation are found in bacteria from the rumen as in bacteria from other environments.

2.4.4 EFFECTS OF AMMONIA AND/OR GLUCOSE BOLUSES ON THE AMMONIA-ASSIMILATING ENZYMES (EXPERIMENT 2)

2.4.4.1 Summary of the Effects

As mentioned previously (section 2.4.2), the feeding and infusion regimes used in experiment 2 resulted in two diets with very different rumen fermentation patterns, each with its own fairly constant rumen parameters throughout the day and between days. Diet D resulted

in an extremely low rumen ammonia level (0.09 to 0.79 mM), whereas that produced by diet E was between 1.8 and 6.2 mM (Table 2.13). These differences are also partially reflected in GS activities, which were about five-fold higher for samples from the animal fed diet D than diet E.

Despite the lack of a correlation between day-to-day activities of GS and rumen fluid parameter (especially ammonia) concentrations in bacterial samples from these two animals, boluses of ammonia added to the rumen of D, either alone or with glucose, resulted in a dose-dependent decrease in GS activity, suggesting a rapid regulation of activity in response to ammonia (Figures 2.6 & 2.7). The low ammonia bolus (0.005 moles NH_4Cl) resulted in a transient decrease of 17 and 30 % in GS activity (γ-GT and forward assay activities, respectively). The high ammonia bolus (0.5 moles NH_4Cl), however, resulted in about a 60 % decrease in both activities four hrs after its addition (the ammonia concentration was still greater than 50 mM compared to 0.34 mM before addition). The ammonia plus glucose bolus (0.5 moles of each) also resulted in about a 60 % decrease in both activities four hrs after addition, and an 80 % decrease after eight hrs, when the ammonia concentration was still 19.7 mM (0.73 mM before addition of the bolus). These large decreases in GS activity after adding the ammonia and ammonia plus glucose boluses occurred gradually over the duration of the elevated rumen ammonia concentrations, rather than occurring abruptly. In addition, the relative proportions of intracellular glutamic acid and aspartic acid appeared to change after the ammonia and ammonia plus glucose boluses given to the animal fed diet D.

In contrast, a consistent effect on GS activity was not observed in the animal fed diet D in response to the glucose bolus, nor were there any effects of ammonia (0.5 moles only was used), glucose, or ammonia plus glucose, boluses evident for the rumen population created by diet E (Table 2.16). Similarly, AsS, GOGAT, GDH and AlaDH activities were not affected by the bolus additions in either animal.

2.4.4.2 Conclusions from Experiment 2

The conclusions from this experiment are that ruminal GS activity is sensitive to rapid increases in ammonia concentration, but only when the GS activity in the bacteria is high, usually as a consequence of very low ammonia levels. This is the first report of such regulation in rumen bacteria, although Erfle *et al.* (1977) reported from *in vitro* mixed rumen bacterial

cultures that a "... *response in specific activity of GS to changes in NH_4^+ concentration occurred rapidly* ..." This conclusion largely refers to induction of GS activity. Clearly the work reported in this thesis on the effects of increasing the rumen ammonia concentration, is a more comprehensive study, indicating that the response in specific activity of GS truly is rapid (i.e. within 30 min. of a change in ammonia level). In addition, the lack of effect of the boluses administered to the animal fed diet E suggests that GS activity in the rumen can reach a low basal level which can be increased (e.g. diet D and shown by Erfle *et al.* (1977)), but not decreased further (i.e. constitutively present ?). Under these conditions the bacteria must be able to assimilate sufficient ammonia for their needs via other assimilatory pathways. The other assimilatory enzymes studied do not appear to be sensitive to increases in ammonia and/or glucose levels in the rumen. Control mechanisms for these enzymes therefore are not as effective as for GS, or may not be related to ammonia or energy levels in the rumen.

In addition, this experiment indicates that rumen parameter values, in particular the ammonia concentration, can have direct effects upon enzyme activities of the rumen population, whereas experiment 1, as well as the day-to-day values from experiment 2, indicate that enzyme activity fluctuations from day-to-day in the rumen are independent of the levels of these rumen parameters. This may indicate that day-to-day fluctuations in rumen parameters, which were determined from averaged values from rumen samples, do not actually impact upon most of the microbial flora in the rumen because the microorganisms are protected from such relatively small changes by the microenvironment in which they exist. When the concentrations of these parameters are changed sufficiently, such as occurred with a superimposed pulse-dose (bolus) of ammonia and glucose in experiment 2, the microenvironments were then affected and GS activity responded to these sudden environmental changes. This premise is supported by Owens & Bergen (1983), who suggest that high ammonia concentrations may be needed to penetrate microenvironments. Similarly, long term minor changes in parameters, such as occur in animals fed different diets, may negate short term shielding provided by the microenvironments.

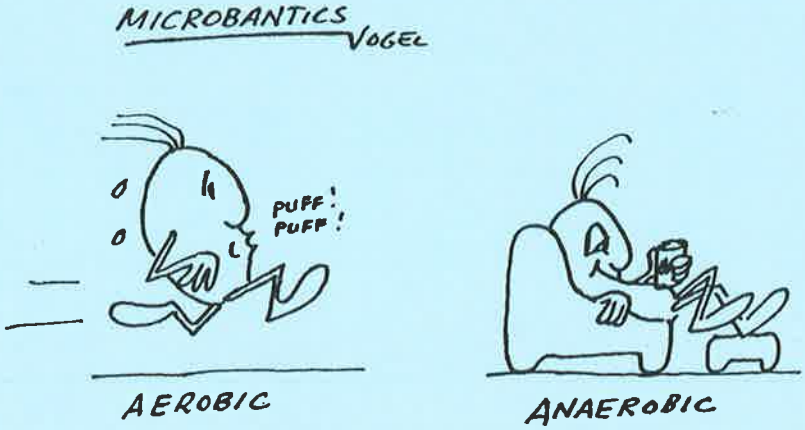
Hespell (1984) suggested that the levels of ammonia-assimilating enzymes measured in mixed rumen bacterial extracts may be due to different pathways being used simultaneously in different microorganisms. This question can only be resolved by enzyme studies with bacteria grown in pure cultures, some of which form the basis of the work presented in Chapter 3.

CHAPTER 3

Ammonia Assimilation in Selected Species of Rumen Bacteria.

"The whole rumen system <u>in</u> <u>vivo</u> is too complicated for detailed analysis . . ."

(Hobson, 1976)



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Chapter 3

Ammonia Assimilation in Selected Species of Rumen Bacteria.

3.1 INTRODUCTION

The experiments described in Chapter 2 confirmed the expectations, assumptions and observations of Chalupa (1972), Erfle *et al.* (1977), Baldwin & Denham (1979), Hobson & Wallace (1982b) and Hespell (1984) that GS, GOGAT and GDH are the major ammonia-assimilating enzymes in the rumen and that AlaDH and AsS are of minor importance, at least under the conditions studied. These data also showed some aspects of the control and biosynthetic activities of these enzymes in the rumen bacterial population and highlighted some of the difficulties of *in vivo* rumen bacterial studies. The main problems with such studies are the inconsistency of rumen fluid conditions (e.g. rumen volume; microbial population; levels of nitrogen, carbon and energy) due to uncontrollable animal factors, and the unpredictable effects of sampling such a diverse and dynamic microbial population.

It has been suggested that there is an interplay between various ammonia-assimilatory pathways in the rumen (Russell & Hespell, 1981). Similarly, Hespell (1984) suggested that the levels of ammonia-assimilating enzymes and iso-enzymes in mixed rumen bacterial extracts may be due to simultaneous utilisation of different pathways in various microorganisms. Although experiments 1 and 2 provide some data to support these hypotheses, *in vivo* studies of ammonia assimilation clearly cannot indicate the fine details of the ammonia-assimilatory pathways. Thus, to investigate these hypotheses, it is necessary to study ammonia assimilation in pure cultures of many rumen bacterial species. The accumulated data for these species studied individually, and in various mixtures, may more accurately define the mechanisms and regulatory processes of ammonia assimilation in the rumen. The data should confirm or refute the present theories on ammonia assimilation in the rumen, which are based largely on the processes that occur in bacteria from other ecosystems (Hespell, 1984). In addition, the results might suggest points at which genetic manipulation could enhance the enzyme rates, and may indicate which rumen bacterial species are most important in ammonia assimilation *in vivo*.

Pure culture studies of rumen ammonia assimilation by Burchall *et al.* (1964a) and Joyner & Baldwin (1966) showed the presence of GDH in *Bacteroides ruminicola, Butyrivibrio fibrisolvens, Fibrobacter (Bacteroides) succinogenes, Megasphaera elsdenii, Ruminobacter (Bacteroides) amylophilus, Ruminococcus albus, R. flavefaciens, Selenomonas ruminantium* and *Streptococcus bovis*, encouraging Allison (1969) and Chalupa (1972) to assign the title of the major ammonia-assimilatory mechanism to this enzyme. Similarly, work by Griffith & Carlsson (1974) supported GDH as the major ammonia-assimilatory pathway in species of *Streptococci*, including *Streptococcus bovis*, despite the fact that GS and AlaDH (but not GOGAT) activities were also detected.

A more recent and detailed study of ammonia assimilation in *Ruminobacter amylophilus* by Jenkinson *et al.* (1979) also found that GDH was a major ammonia-assimilatory mechanism. Jenkinson *et al.* (1979) also found GS and AsS activities in *Ruminobacter amylophilus*, and discovered that this species does not strictly follow the "classical" route of ammonia assimilation outlined in the Literature Review (section 1.9.2). GS activity was induced at ammonia concentrations below about 0.5 mM and inactivated at levels above about 1 mM, however GDH was constitutively present and had a relatively low K_m-value for ammonia (1 - 2 mM). In addition, GOGAT and AlaDH activities could not be detected in this bacterium, but the lack of GOGAT activity in this species may be due to an inability by workers to find an appropriate electron donor, rather than an indication of no activity *per se* (Hespell, 1984).

GOGAT activities also have not been reliably detected in *Succinivibrio dextrinosolvens*, possibly for similar reasons to those outlined for *Ruminobacter amylophilus* (Patterson & Hespell, 1985). Similarly, AlaDH activity has not been found in this species (Patterson & Hespell, 1985), and assays for AsS activity have apparently not been performed. Nevertheless, ammonia assimilation via GS and GDH still appears to follow a fairly classical pattern in which GS activity is high, and GDH activity is low, under low ammonia conditions, and the opposite pattern is observed for growth in the presence of ample ammonia (Hespell, 1984; Patterson & Hespell, 1985).

In contrast to these relatively limited studies of ammonia assimilation, the properties and regulation of the major ammonia-assimilatory enzymes in rumen bacteria have been most comprehensively studied in *S. ruminantium*. As observed for the two species already discussed, and more fully considered in the Literature Review (section 1.9.4.3), the "classical"

route of ammonia assimilation, via GS/GOGAT at ammonia concentrations below about 1 mM, and via GDH above this concentration, also appears to be responsible for ammonia assimilation in this rumen bacterium (Smith *et al.*, 1980, 1981; Hespell, 1984). These two pathways could account for all of the ammonia assimilated in *S. ruminantium*, rendering other potential pathways to very minor roles (Hespell, 1984). Activities of AlaDH, AsS, and other potential ammonia-assimilating enzymes have not been reported, but this appears to be due to a lack of study, indicating a gap in our knowledge of possible alternative ammonia-assimilating pathways in this bacterium.

Thus, the small number of rumen bacteria already studied possess basically the same type of ammonia-assimilatory enzymes as those found in bacteria from other environments, with GS/GOGAT and GDH pathways apparently the primary routes of assimilation (Hespell, 1984; see Literature Review, section 1.9.4.5). Nevertheless, other enzymes, such as AsS, AlaDH, other amino acid dehydrogenases, aspartate ammonia-lyase, carbamoyl-phosphate synthase, carbamate kinase and transaminases, may also be involved in ammonia assimilation in the rumen, especially considering the diversity of growth conditions and microbial species in the rumen (Chalupa *et al.*, 1970; Chalupa, 1972; Buttery, 1976; Hobson & Wallace, 1982b; Hespell, 1984).

The experiments outlined in this chapter were therefore designed to test the hypothesis that the major ammonia-assimilatory enzymes found in organisms from non-rumen environments, the rumen in general, and *Ruminobacter amylophilus, S. ruminantium* and *Succinivibrio dextrinosolvens* in particular, are also present, and responsible for ammonia assimilation, in a number of other important rumen bacterial species. These studies concentrated on GS, GOGAT and GDH as the major ammonia-assimilatory enzymes, as well as AlaDH, which has been reported to be responsible for ammonia assimilation in some non-rumen microorganisms when GDH is absent (Dunn & Klucas, 1973; Miflin & Lea, 1976; Herbert *et al.*, 1978; Brown, 1980; Kenealy *et al.*, 1982; Moreno-Vivián *et al.*, 1983; Bhatnagar *et al.*, 1986b; Brãna *et al.*, 1986).

Species initially chosen for investigation in pure culture were included in the study because they were representative of the important nutrient niches in the rumen, and often comprise significant proportions of the rumen microbial community (Literature Review, section 1.6.4). Most studies reported in this chapter concentrated on *B. ruminicola* subsp. *brevis* strain BA25 and *S. ruminantium* subsp. *lactilytica* strain MP72. *S. ruminantium* subsp. *ruminantium* strain ME13 and *Butyrivibrio fibrisolvens* strain 49 were also included, the latter as a

representative of an amino acid/peptide dependent bacterium, in contrast to the other species listed which were able to utilise ammonia as their sole source of nitrogen. Similarly, *B. ruminicola* subsp. *ruminicola* was studied as another example of an amino acid/peptide dependent organism even though Krieg & Holt (1984) report that this species is able to use ammonia as a nitrogen source. Variations between strains do occur.

The hypothesis stated above was initially tested by studying the effect of inhibitors of GS and GOGAT on bacterial growth at increasing concentrations of NH_4Cl, and contrasting these with the effects when amino acids and peptides were nitrogen sources. Growth inhibition by MSX or azaserine, by the mechanisms outlined in the Literature Review (section 1.10), indicates a vital role for GS or GOGAT, respectively, under the prevailing conditions, hence these experiments were aimed at determining whether GS and GOGAT were as vital for cell growth in the selected rumen bacteria as is the case in many non-rumen microorganisms (Fowden *et al.*, 1967; Norris & Lea, 1976).

The presence and activities of GS, GOGAT, GDH and AlaDH were subsequently studied in *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 grown under a similar range of NH_4Cl concentrations in the media, and results were contrasted with enzyme levels when cells were grown with amino acids and peptides as nitrogen sources. In addition, the activities of these enzymes in cells from batch cultures harvested at early log, mid log, late log and stationary phase of growth were measured, and K_m-values were determined for some of these enzymes in *B. ruminicola* BA25.

The aim of these experiments was to delineate the ammonia-assimilatory processes in *B. ruminicola* BA25, *S. ruminantium* MP72, *B. ruminicola* subsp. *ruminicola, Butyrivibrio fibrisolvens* 49 and *S. ruminantium* ME13. Nevertheless, ammonia is only one of the nitrogen sources available in the rumen and, despite the fact that most rumen bacterial species are able to grow with ammonia as the sole source of nitrogen, this may not be their preferred nitrogen source (Bryant & Robinson, 1961, 1962, 1963; Allison, 1970; Hobson & Wallace, 1982b; Russell, 1984). *B. ruminicola* subsp. *brevis* and *B. ruminicola* subsp. *ruminicola* have been shown to utilise peptide- or ammonia-nitrogen, but not significant quantities of free amino acid-nitrogen or other low molecular weight nitrogen compounds (Bryant & Robinson, 1963; Pittman & Bryant, 1964; Hobson & Wallace, 1982b). Similarly, *Butyrivibrio fibrisolvens, S. ruminantium, Streptococcus bovis* and *Succinivibrio dextrinosolvens* may require specific

amino acids for effective growth (Bryant & Robinson, 1963; Russell, 1984), however the relative preference of these microorganisms for various nitrogen sources when they are available concurrently, as occurs in the rumen, is still not clear.

Thus, the final experiments described in this chapter test the following hypothesis : "When provided with a variety of nitrogen sources (protein, peptides, amino acids and ammonia), *B. ruminicola* BA25 and *S. ruminantium* MP72 preferentially utilise peptide nitrogen, but can also grow with all other sources as the sole nitrogen source". This hypothesis, which has obvious implications in terms of the role of each species in the rumen environment, was studied by monitoring the growth of batch cultures of *B. ruminicola* BA25 and *S. ruminantium* MP72 on a minimal growth medium supplemented with 0.05 % or 0.5 % [w/v] glucose and the various nitrogen sources alone and in combinations. The two glucose levels were included in the experiment to detect the use of any of the nitrogen sources as alternative energy sources when glucose had been depleted in the media. This phenomenon is discussed in the Literature Review (section 1.7.2).

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

3.2.1.1 Organisms

Bacteroides ruminicola subsp. *brevis* strain BA25, *Selenomonas ruminantium* subsp. *lactilytica* strain MP72 and *S. ruminantium* subsp. *ruminantium* strain ME13 were isolated from rumen fluid sampled from sheep fed a lucerne diet (1 kg lucerne chaff daily).

Bacteroides ruminicola subsp. *ruminicola*, *Bacteroides ruminicola* subsp. *brevis* strains B_14 & GA33, *Butyrivibrio fibrisolvens* strain 49 and *S. ruminantium* subsp. *lactilytica* strain HD_4 were obtained from the culture collection of Dr R. B. Hespell, U.S.D.A., Peoria, Illinois, U.S.A.

Bacteroides fragilis (strain unknown) and *Escherichia coli* strain HB101 were obtained from our culture collection.

3.2.1.2 Chemicals and Biochemicals

Adenosine 5'-diphosphate (sodium salt [Na-ADP]), L-aspartic acid, azaserine, bovine albumin (Fraction V [BSA]), casein, Coomassie Brilliant Blue G250, L-cysteine hydrochloride, L-glutamic acid γ-mono-hydroxamate (γ-glutamyl hydroxamate), L-glutamine, hydroxylamine monohydrochloride, 2-mercaptoethanol, L-methionine DL-sulphoximine [MSX], ß-nicotinamide adenine dinucleotide (ß-NADH : reduced form), ß-nicotinamide adenine dinucleotide phosphate (ß-NADPH : reduced form), ninhydrin (2,2-dihydroxyl-1, 3-indanedione), 2-oxoglutarate, pyruvic acid (sodium salt), sodium arsenate, vitamins {thiamine-hydrochloride (vit. B_1), Ca-D-pantothenate, nicotinic acid, riboflavin (vit. B_2), pyridoxine-monohydrochloride (vit. B_6), *p*-aminobenzoic acid, d-biotin (vit. H), folic acid and cyanocobalamin (vit. B_{12})}, HEPES (biological buffer) and Tris(hydroxymethyl)amino-methane (Tris-HCl buffer) were obtained from Sigma Chemical Company, St. Louis, U.S.A.

Brain Heart Infusion dehydrated media, Bacto-Casamino Acids, Proteose Peptone, Trypticase, Bacto-Tryptone and Bacto-Yeast Extract were purchased from DIFCO Laboratories, Detroit, U.S.A. Resazurin, hemin-1, 4-napthoquinone (bovine hemin) and 2-methylbutyric acid were purchased from Aldrich Chemical Company, Milwaukee, U.S.A. *Iso*-butyric acid, *n*-valeric acid and *iso*-valeric acid were purchased from B.D.H. Chemicals Ltd., Poole, England. *Tri*-sodium citrate was purchased from B.D.H. Chemicals (Australia Ltd.), Boronia, Australia. *n*-butyric acid was purchased from Fluka AG Buchs SG, Switzerland. Dithiothreitol was purchased from Boehringer Mannheim Australia Pty Ltd, Sydney, Australia.

All other chemicals, the best purity available, were obtained from the following sources : Ajax Chemicals (Sydney, Australia); B.D.H. Chemicals Ltd. (Poole, England); B.D.H. Chemicals, Australia Ltd. (Boronia and Kilsyth, Australia).

3.2.1.3 Buffers

Phosphate buffer (0.1 M, pH 10) was made with 500 ml of 0.2 M Na_2HPO_4 diluted to 1 litre after pH adjustment with NaOH (Russell *et al.*, 1983; Dawson, 1986).

Citrate buffer (50 mM, pH 2.0) was made with 410 ml of 0.1 M citric acid and 90 ml of 0.1 M *tri*-sodium citrate (0.1 M citrate buffer) diluted to 1 litre with R.O. water after checking the pH (Dawson, 1986; Chen *et al.*, 1987b).

Cyanide-acetate buffer was made with 0.2 mM sodium cyanide solution in acetate buffer (acetate buffer : 2700 g $NaOAc.3H_2O$ + 2 litres R.O. water + 500 ml glacial acetic acid. Make up to 7.5 litres with R.O. water. pH should be 5.3-5.4) (Rosen, 1957).

5 mM HEPES and 1.0 M stocks of Tris(hydroxymethyl)aminomethane buffer (Tris-HCl buffer) solutions were made according to the standard methods of Dawson (1986).

Buffers, and all other solutions, were made using water purified by Reverse Osmosis (R.O. water).

3.2.2 METHODS

3.2.2.1 General Growth Conditions and Culture Media

Batch cultures of all bacterial species were initiated at regular intervals from glycerol stocks held at -80°C. Working cultures were maintained by daily transfer (1 % [w/v] inoculum) onto appropriate Nitrogen Free Mineral Salts (NFMS) media supplemented with 8 mM NH_4Cl as the nitrogen source (Table 3.2), or onto routine growth medium (RGM : Table 3.1). Experimental cultures were initiated with an inoculum equivalent to 1 % [v/v] of the final culture volume from overnight cultures grown in the appropriate NFMS medium supplemented with 8 mM NH_4Cl. Except for growth of *E. coli*, working cultures (10 ml) of experimental media for growth curve experiments were grown in "Hungate" tubes (i.e. 16 x 125 mm Pyrex® screw capped culture tubes (Corning Glass Works, Corning, U.S.A.) with butyl rubber septa inserted into the caps (Bellco Biotechnology, Vineland, U.S.A.)). Growth was monitored by the change in optical density at 600 nm directly in these tubes using the Lambda 5 UV/VIS Spectrophotometer described in Chapter 2, which was fitted with a special holding cage for these round tubes. For experiments requiring larger quantities of cells or used media, batch cultures (except of *E. coli*) were grown in 55 or 150 ml serum bottles (40 or 130 ml of media respectively) sealed with butyl rubber septa (Pierce Chemical Company, Rockford, U.S.A.). Growth in these cultures was followed either with a parallel "Hungate" tube culture (10 ml), or by withdrawing an aliquot from the bottle (1 - 1.5 ml) with a disposable plastic syringe and hypodermic needle, and transferring this sub-sample to a cuvette for measurement of the optical density at 600 nm.

Bacterial transfers from liquid to liquid were performed aseptically through the butyl rubber septa of culture bottles and "Hungate" tubes using disposable plastic syringes and hypodermic needles. Although this was not carried out in the anaerobic glove box, only small amounts of oxygen entered the tubes or bottles during these transfers (i.e. a transient colour change of the oxidation/reduction dye, resazurin, included in the media, was sometimes observed). All cultures were incubated at 39°C.

Cultures of *E. coli* were grown aerobically at 39 °C in various sized Erlenmeyer flasks, depending on the volume of cells required. Transfers were performed aseptically and aerobically. Cultures were shaken during growth.

The components and preparation method for RGM are shown in Table 3.1. This medium was used for initial growth of each species from glycerol stocks, and as a medium providing amino acids and peptides as the major nitrogen sources in some experiments. Table 3.2 shows the basic components and preparation method of NFMS media for *B. ruminicola* subsp. *brevis*, *B. ruminicola* subsp. *ruminicola*, *S. ruminantium* subsp. *lactilytica*, *S. ruminantium* subsp. *ruminantium* and *Butyrivibrio fibrisolvens*. These basic media were modified from Schaefer *et al.* (1980), and were varied for individual experiments by alteration of the source or concentration of nitrogen. In experiments 3.1, 3.2, 3.3 & 3.4, the concentration of NH_4Cl, as the sole nitrogen source in NFMS media, was varied from 0 to 128 mM. In experiment 4, NH_4Cl, Casamino Acids, Tryptone and casein were provided as nitrogen sources in the basal media, which was supplemented with either 0.05 % [w/v] or 0.5 % [w/v] glucose.

Each bacterial species was initially cultured in NFMS media with 8 mM NH_4Cl as the sole nitrogen source, however *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 grew poorly in their basal media. A series of experiments was therefore carried out to develop media in which these species would grow more effectively. R_1 salts (Table 3.1), yeast extract, Trypticase or Proteose Peptone were added to the basal NFMS media and cell growth was monitored at 600 nm.

Brain-Heart Infusion (BHI) media (Table 3.3) and L-Broth (Table 3.4) were used for growth of some bacterial species in experiment 3.2.

Table 3.1 : Components of Routine Growth Medium (RGM) [a].

COMPONENT	Final Volume 100 ml
Proteose Peptone	0.3 g
Yeast Extract	0.2 g
L-Cysteine hydrochloride	0.05 g
Glucose	0.2 g
Minerals 1 [b]	5.0 ml
Minerals 2 [b]	5.0 ml
R_1 salts solution [c]	0.1 ml
Volatile fatty acids [d]	3.0 ml
Resazurin (0.1 % solution [w/v])	0.1 ml
Hemin (0.1 % solution [w/v])	0.1 ml
R.O. Water	81.7 ml
Na_2CO_3 (8 % solution [w/v]) [e]	*5.0 ml*

[a] Media was prepared without sodium carbonate (heat labile) and the pH adjusted to 6.8 with NaOH; boiled media was placed in the anaerobic glove box (CO_2 / H_2 atmosphere) and dispensed into "Hungate" tubes or serum bottles as required; tubes or bottles were then sealed and autoclaved.

[b] As described by Bryant & Burkey (1952) {Mineral 1 : 0.3 %[w/v] K_2HPO_4 ; Minerals 2 : 0.3 %[w/v] KH_2PO_4 ; 0.6 %[w/v] $(NH_4)_2SO_4$; 0.6 %[w/v] NaCl; 0.06 %[w/v] $MgSO_4$; 0.06 %[w/v] $CaCl_2$}.

[c] As described by Hespell & Canale-Parola (1970) {25 ml modified Pfennig's trace element solution (0.5 g $AlCl_3$, 0.25 g KI, 0.25 g KBr, 0.25 g LiCl, 3.5 g $MnCl_2.4H_2O$, 5.5 g H_3BO_3 (heat to dissolve), 0.5 g $ZnCl_2$, 0.5 g $CuCl_2.2H_2O$, 0.5 g $NiCl_2.6H_2O$, 0.5 g $CoCl_2.6H_2O$, 0.15 g $SnCl_2.2H_2O$ (white precipitate forms when added), 0.15 g $BaCl_2.2H_2O$, 0.25 g $NaMoO_4.2H_2O$*, 0.05 g $NaVO_3$* (heat to dissolve). Each salt was dissolved separately in R.O. water; the pH of asterisked (*) metals was adjusted to 7.0 prior to the solutions being added in order starting with $AlCl_3$; solution was brought to final volume of 1800 ml with R.O. water and pH adjusted to 3.0 - 4.0); 1 g tetrasodium ethylenediaminetetraacetic acid (EDTA), 3.75 g $CaCl_2.2H_2O$, 12.5 g $MgCl_2.6H_2O$, 0.5 g $FeSO_4.7H_2O$; made up to 100 ml with R.O. water}.

[d] As described by Hespell *et al.* (1987) { 6.85 ml acetic acid, 3.0 ml propionic acid, 1.85 ml *n*-butyric acid, 0.55 ml 2-methylbutyric acid, 0.5 ml *iso*-butyric acid, 0.55 ml *n*-valeric acid; 700 ml 0.2 M NaOH; brought to 1000 ml with R.O. water}.

[e] Added to cooled media as sterile, CO_2 equilibrated solution.

Table 3.2 : Components of Nitrogen Free Mineral Salts (NFMS) Media for growth of *B. ruminicola* subsp. *ruminicola*, *B. ruminicola* subsp. *brevis*, *S. ruminantium* subsp. *lactilytica*, *S. ruminantium* subsp. *ruminantium* and *Butyrivibrio fibrisolvens*, respectively[a]. The source and concentration of nitrogen were manipulated within the respective media depending on requirements detailed for each experiment.

COMPONENT	*B. ruminicola brevis*	*B. ruminicola ruminicola*	*S. ruminantium ruminantium & lactilytica*	*Butyrivibrio fibrisolvens*
Glucose	0.5 g	0.5 g	0.5 g	0.5 g
L-Cysteine hydrochloride	0.05 g	0.05 g	0.05 g	0.05 g
Trypticase or yeast extract [b]	-	0.1 g	-	0.15 g
Minerals 3 [c]	5.0 ml	5.0 ml	5.0 ml	5.0 ml
Pfennigs heavy metals	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Volatile fatty acids 1 [d]	1.0 ml	1.0 ml	-	-
Volatile fatty acids 2 [e]	-	-	1.0 ml	-
Volatile fatty acids 3 [f]	-	-	-	1.0 ml
B-Vitamins (complete) [g]	1.0 ml	1.0 ml	-	1.0 ml
B-Vitamins (*S. ruminantium*) [h]	-	-	1.0 ml	-
Hemin (0.1 % solution [w/v])	0.1 ml†	0.1 ml	-	-
Resazurin (0.1 % solut[n] [w/v])	0.1 ml	0.1 ml	0.1 ml	0.1 ml
NITROGEN SOURCE [i]	**	**	**	**
Na_2CO_3 (8 % solution [w/v]) [j]	*5.0 ml*	*5.0 ml*	*5.0 ml*	*5.0 ml*

[a] All media are modified from Schaefer *et al.* (1980); quantities are per 100 ml of media; after being brought to volume with R.O. water (minus heat labile sodium carbonate solution) and pH adjusted to 6.8, the media was boiled and placed in the anaerobic glove box (CO_2 / H_2 atmosphere), dispensed into "Hungate" tubes or serum bottles as required, sealed and autoclaved.

[b] 0.1 %[w/v] yeast extract for *B. ruminicola* subsp. *ruminicola* and 0.15 %[w/v] Trypticase for *Butyrivibrio fibrisolvens*.

[c] Minerals 3 solution contained the following per litre : 18 g KH_2PO_4, 18 g NaCl, 0.4 g $CaCl_2$, 0.4 g $MgCl_2.6H_2O$.

[d] Combined as follows : 170 ml acetic acid, 10 ml *iso*-butyric acid, 10 ml *n*-valeric acid, 10 ml *iso*-valeric acid and 10 ml 2-methylbutyric acid; adjusted to pH 7.0 with NaOH and brought to 1000 ml with R.O. water.

[e] Combined as follows : 10 ml *iso*-butyric acid, 10 ml *n*-valeric acid; adjusted to pH 7.0 with NaOH and brought to 1000 ml with R.O. water.

[f] As for RGM (Table 3.1).

[g] Complete vitamins solution contained 20 mg thiamine-hydrochloride, 20 mg Ca-D-pantothenate, 20 mg nicotinic acid, 20 mg riboflavin, 20 mg pyridoxine-monohydrochloride, 1 mg *para*-aminobenzoic acid, 0.5 mg d-biotin, 0.125 mg folic acid and 0.2 mg cyanocobalamin brought to 1000 ml with 5 mM HEPES; solution was adjusted to pH 7.3 and autoclaved for 5 min.

[h] *S. ruminantium* vitamins solution contained 1 mg *para*-aminobenzoic acid, 0.5 mg d-biotin brought to 1000 ml with 5 mM HEPES; solution was adjusted to pH 7.3 and autoclaved for 5 min.

[i] Working cultures were grown with 8 mM NH_4Cl; this concentration was varied as indicated in the text; Casamino Acids, Tryptone and casein were also provided as nitrogen sources in some experiments, as indicated in the text.

[j] Added to cooled media as sterile, CO_2 equilibrated solution.

† Hemin added to *B. ruminicola* subsp. *brevis* media although not required for growth (Krieg & Holt, 1984).

Table 3.3 : Components of Brain Heart Infusion (BHI) Medium †.

COMPONENT	Final Volume 100 ml
Brain Heart Dehydrated Media	3.7 g
L-Cysteine hydrochloride	0.05 g
Hemin (0.1 % solution [w/v])	0.1 ml
Resazurin (0.1 % solution [w/v])	0.1 ml
R.O. Water	100 ml

† After being brought to volume, media was boiled, placed in the anaerobic glove box (CO_2 / H_2 atmosphere) and dispensed into "Hungate" tubes or serum bottles as required, which were sealed and autoclaved.

Table 3.4 : Components of L-Broth medium for growth of *E. coli* †.

COMPONENT	Final Volume 100 ml
Tryptone	1.0 g
Yeast Extract	0.5 g
NaCl	1.0 g
R.O. water	100 ml

† Media was adjusted to pH 7.5 with NaOH, brought to volume, covered and autoclaved.

3.2.2.2 Effects of Azaserine and L-Methionine DL-sulphoximine (MSX) on the Growth of Selected Species of Rumen Bacteria (Experiment 3.1)

The effects of azaserine and MSX on cell growth were studied in batch cultures of *B. ruminicola* BA25, *B. ruminicola* subsp. *ruminicola*, *Butyrivibrio fibrisolvens* 49, *S. ruminantium* MP72 and *S. ruminantium* ME13. Ten "Hungate" tubes, each containing 10 ml of the appropriate NFMS medium (Table 3.2) supplemented with 0, 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 or 128 mM NH_4Cl, respectively, as the sole source of nitrogen (yeast extract and Trypticase, which contain nitrogen sources, were also present as essential components of the NFMS media for *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49, respectively), and an additional 10 tubes of RGM (Table 3.1), which contained ammonia, amino acids and peptides as sources of nitrogen, were prepared for each bacterial species studied. Nine of each of these sets of tubes were inoculated from overnight cultures of the appropriate bacterial species grown in suitable NFMS medium supplemented with 8 mM NH_4Cl (1 % [v/v] inoculum), and the remaining tube of each set was retained for subsequent measurements of the actual ammonia concentration in the original media. The concentrations listed above are the intended levels, however these were rarely identical to the measured concentrations due to slight media formulation, and assay, inaccuracies.

After inoculation, growth was monitored in each tube at 30 or 60 min. intervals by optical density measurements at 600 nm (i.e. 9 tubes for each of 11 NH_4Cl levels, plus RGM, for each species). When obvious culture growth was apparent (see below), 100 µl of 0.1 M MSX was added to three of the tubes for each set of media, 100 µl of 0.01 M azaserine was added to another three tubes of each set, and the remaining three tubes were left as controls. "Obvious culture growth" for media supplemented with more than 4 mM NH_4Cl was defined as an increase above pre-growth optical density of 0.4 to 0.6 units (see Figure A2.1, Appendix 2 for examples of growth curves), however for media supplemented with 0, 0.25, 0.5, 0.75, 1.0, 2.0 or 4.0 mM NH_4Cl, respectively (very poor to poor growth), "obvious culture growth" did not occur for *B. ruminicola* BA25 and *S. ruminantium* MP72. Inhibitors were therefore added to these cultures at the same time as for the 8 mM NH_4Cl treatment of each species. Thus, for each "set" of "Hungate" tubes, 3 were controls, 3 were for azaserine addition, 3 were for MSX addition and 1 was for measurement of the ammonia concentration prior to growth. Following addition of the inhibitors, optical density measurements were

continued at 30 or 60 min. intervals until the control cultures reached stationary phase of growth, at which time media from triplicate tubes for each treatment of a "set" was combined, centrifuged (5,000 x *g* ; 10 min.; 10 °C), and the supernatant retained and stored at -20°C. The ammonia concentration in this clarified media at harvest, and in media prior to culture growth (original media), was measured by the method in section 2.2.2.6.

Only one species was studied on any day, and each species was studied on at least two separate occasions. Where inconclusive or conflicting growth data were obtained, a third study was carried out, but only for the troublesome treatment groups (the whole range of media ammonia levels was not repeated). Results are therefore the mean ± the standard error of the mean from at least two days. Data from each day are the mean of triplicate tubes, but were adjusted by subtracting the optical densities of tubes at zero time from subsequent values. This was done to eliminate optical density variations caused by media differences between days, and other sources of daily variation. The ammonia concentration in the media at harvest, and pre-growth, similarly is the mean ± the standard error of the mean from at least two data days. Duplicate ammonia measurements from each day of growth were made on stored clarified media samples.

The concentrations of azaserine and MSX used in these experiments were determined from a number of previous studies on other bacterial species (Azaserine - Kumar & Nicholas, 1982; Michalski & Nicholas, 1984 : MSX - Brenchley, 1973; Vairinhos *et al.,* 1983; Yamamoto *et al.,* 1984) and from one study on a rumen bacterial species (Smith *et al.,* 1981). *B. ruminicola* subsp. *ruminicola* was the first species studied, and the inhibitor concentrations used for this species (100 µl of 0.01 M azaserine and 100 µl 0.1 M MSX) were effective, hence no further testing of the dose was performed. Doses were not optimised for each species studied, but instead were kept at the same level as for *B. ruminicola* subsp. *ruminicola* so that direct comparisons between species could be made without the confounding influence of treating each species with different doses. Final culture concentrations of azaserine and MSX were 0.1 mM and 1 mM respectively. Stock solutions of both inhibitors were filter sterilised (0.22 µm Millipore filters; Millipore Corporation, Bedford, U.S.A.) and stored at 4°C until required. Inhibitors were aseptically added to the appropriate cultures by hypodermic needle through the butyl rubber septa, followed by gentle inversion of the tubes (i.e. mixing). A red tinge transiently appeared in some "Hungate" tube cultures, since the anaerobic glove box was

not used for these additions. The slight red colour had disappeared by the time the next optical density measurement was made.

3.2.2.3 Effects of Media Ammonia Concentration and Nitrogen Source on the Activity of Ammonia-Assimilating Enzymes in Three Rumen Bacterial Species (Experiment 3.2)

BACTERIAL GROWTH : The specific activities of GS, GOGAT, GDH and AlaDH, and the effects of the source and concentration of nitrogen on these specific activities, were studied in batch cultures of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49. These species were chosen because *B. ruminicola* BA25 and *S. ruminantium* MP72 were able to grow with ammonia as the sole source of nitrogen in experiment 3.1, whereas *Butyrivibrio fibrisolvens* 49 required amino acids and/or peptides as nitrogen sources for growth. *Butyrivibrio fibrisolvens* 49 was therefore a useful contrast to the other two species, whereas strains of *S. ruminantium* subsp. *ruminantium*, which are the same as *S. ruminantium* subsp. *lactilytica* except that the latter also ferments lactate and glycerol (Bryant, 1959; Hungate, 1966; Ogimoto & Imai, 1981; Krieg & Holt, 1984), have been studied by other workers (Smith *et al.*, 1980, 1981; Hespell, 1984), thus providing a reference for the levels of activity measured in these experiments.

Batch cultures of *B. ruminicola* BA25 were grown in NFMS media (Table 3.2) supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, respectively, as well as in RGM (Table 3.1). Batch cultures of *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 were grown in their respective NFMS media (Table 3.2) supplemented with 0.5, 8.0 and 128 mM NH_4Cl, as well as in RGM (Tables 3.1). For NFMS media supplemented with 0.5, 0.75, 1.0 and 2.0 mM NH_4Cl, two 40 ml cultures, two 120 ml cultures and two 10 ml "Hungate" tube cultures were grown simultaneously, whereas one 40 ml culture, one 120 ml culture and two 10 ml "Hungate" tube cultures were grown for media supplemented with 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, respectively, as well as for RGM cultures. The higher total culture volumes grown for media with low ammonia concentrations was necessary to ensure sufficient cells were harvested from these cultures, since they reached lower optical densities than cultures grown with more than 4 mM NH_4Cl, and cultures grown in RGM (i.e. they were ammonia-limited). It was essential to harvest a large number of cells

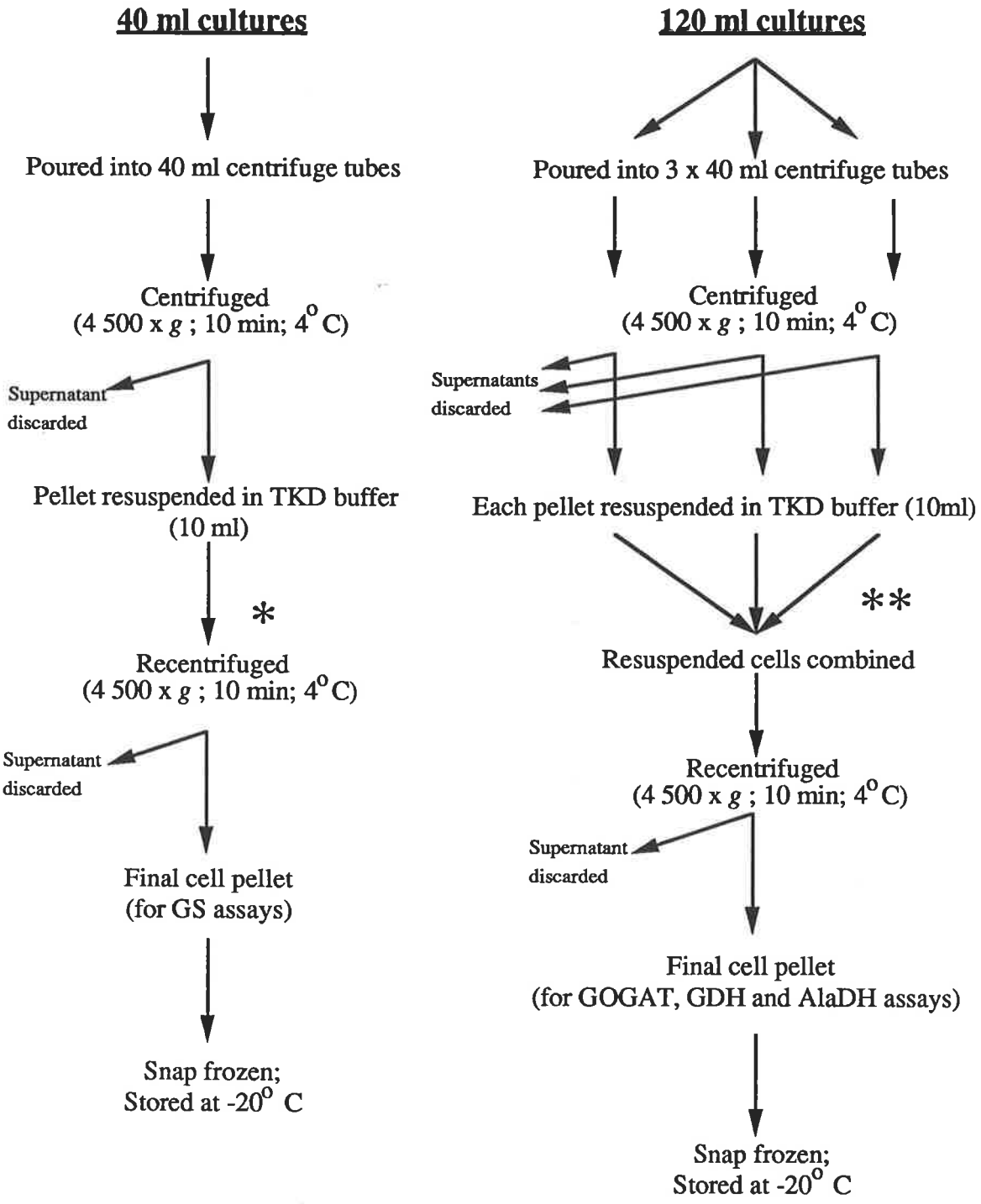
from each culture or pair of cultures so that a sufficient level of enzyme activity was present in the final cell-free extracts. The two "Hungate" tube cultures grown concurrently for each of these media were used to monitor culture growth by optical density measurements at 600 nm. All cultures were inoculated at 1 % [v/v] of the final culture volume from an overnight culture of the species in the appropriate NFMS medium supplemented with 8 mM NH_4Cl.

CELL HARVESTING : Cultures (40 & 120 ml) were harvested at mid to late log phase of growth of the "Hungate" tube cultures (see Figure A2.1, Appendix 2) by the procedure outlined in Figure 3.1. The cultures were decanted into 40 ml screw-capped polyallomer centrifuge tubes (Beckman Instruments, Inc., Palo Alto, U.S.A.) and centrifuged (4,500 x *g* ; 10 min.; 4°C) in a Sorvall® RC-5B Centrifuge (DuPont Instruments, Wilmington, U.S.A.). TKD buffer was the same as in Chapter 2 (i.e. 50 mM Tris-HCl buffer; 1 % [w/v] KCl; 1 mM dithiothreitol, pH 7.4). NFMS media for which two 40 ml cultures were grown were harvested according to the procedure outlined for one 40 ml culture (Figure 3.1), except that the two batches of resuspended cells (10 ml each) were combined before re-centrifugation (* in Figure 3.1). Similarly, media for which two 120 ml cultures were grown were harvested according to the procedure outlined for one 120 ml culture, except that the six initial cell pellets were only resuspended in a total of 40 ml of TKD buffer (i.e. 6 ml each, plus 4 ml used to rinse the tubes) prior to re-centrifugation (** in Figure 3.1). The final cell pellets were snap frozen by immersing the centrifuge tubes in liquid nitrogen for one minute, and were stored at -20°C overnight.

The optical density of "Hungate" tube cultures grown concurrently with the harvested cultures varied considerably at harvest between media with different ammonia levels. Data from experiment 3.1 was used as a guide to the growth rate for these cultures and was referred to when determining the optical density at which to harvest the cultures (i.e. to determine when cultures were at mid to late log phase of growth; see Figure A2.1, Appendix 2). A preliminary assay of GS activity in an extract of RGM grown cells showed no difference in activity levels between aerobically and anaerobically harvested cells, hence no attempt was made to keep cells anaerobic during harvesting procedures.

PREPARATION OF CELL-FREE EXTRACTS : Using the procedures outlined above, a cell pellet was produced for GS assays, as well as one for GOGAT, GDH and AlaDH assays, for each type of media. The cell pellet earmarked for GS assays from each type of

Figure 3.1 : Culture harvesting procedures for Experiment 3.2.



media was resuspended in GS "extraction" buffer (2 ml; composed of 40 mM Tris-HCl buffer, 0.3 mM $MnCl_2$ & 2 mM 2-mercaptoethanol added immediately prior to resuspension (pH 6.8)), and the pellet earmarked for GOGAT, GDH and AlaDH assays was resuspended in GOGAT/GDH/AlaDH "extraction" buffer (6 ml; composed of 50 mM Tris-HCl buffer, 0.1 mM EDTA & 2 mM 2-mercaptoethanol added immediately prior to resuspension (pH 7.0)) on the day after the cells were harvested. These cell suspensions were each passed twice through the French Pressure Cell (10,000 p.s.i.; 10°C) to disrupt the cells and the resulting suspensions were centrifuged (18,000 x *g* ; 20 min.; 4°C) to remove cell debris. Supernatants [cell-free extracts] were stored on ice if they were to be assayed on the same day, or at 4°C overnight otherwise (modified from Patterson & Hespell, 1985).

ENZYME ASSAYS : GS, GOGAT, GDH and AlaDH activities were assayed in bacterial cell-free extracts as outlined in Chapter 2 (section 2.2.2.7), except that only the γ-GT assay was used to measure GS activity. Optimal assay mixtures, determined in a series of preliminary assays with cell-free extracts of *B. ruminicola* BA25, and summarized in Tables 3.5, 3.6, 3.7 & 3.8, were modified from those used in Chapter 2. As mentioned above, different "extraction" buffers were used in the preparation of cell-free extracts for GS assays, and in the preparation of cell-free extracts for assay of GOGAT, GDH and AlaDH activities. The significance of this was discussed in Chapter 2. The amount of γ-glutamyl hydroxamate formed was determined by reference to a standard curve obtained with γ-glutamyl hydroxamate. The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977) using Coomassie Brilliant Blue G250 dye, with BSA as the standard. This measurement was used to calculate enzyme specific activity, expressed per mg of protein in the cell-free extract.

In addition to the experiments with NFMS media (above), γ-GT activity was measured in cells harvested from overnight batch cultures of *B. ruminicola* BA25, B_14 & GA33, *B. fragilis*, *B. ruminicola* subsp. *ruminicola*, and *S. ruminantium* HD_4 grown in BHI media (Table 3.3), and of *E. coli* HB101 grown in L-Broth (Table 3.4). Cell harvesting, disruption and cell-free extract preparation methods were as outlined above. GS γ-GT assays were for 30 min. at 39°C, not 20 min. as shown in Table 3.5 for the previous experiments, however all other methods were as outlined previously.

Table 3.5 : GS γ-GT assay components and optimum conditions for assays using cell-free extracts of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49, respectively (modified from Patterson & Hespell, 1985).

Volume (µl)	Stock Solution[a]	Final Concn
81.5	THM mixture [b]	20, 60 & 0.15 mM
300	0.1 M L-Glutamine, pH 7.0	30 mM
20	1.0 M Sodium arsenate, pH 7.0	20 mM
40	0.01 M Na-ADP, pH 7.0 (prepared fresh, as required)	0.4 mM
58.5	R.O. water	
500 - *x*	GS "extraction" buffer [c]	
x	Cell-free enzyme extract (prepared in GS extraction buffer); 100 - 200 µl normally used.	

Assay was for 20 min. at 39°C following a 10 min. preincubation. Stop solution added after 10 min. was the same as for Chapter 2 GS assays (section 2.2.2.7).

[a] All components were prepared as stock solutions prior to the experiment, unless stated otherwise.
[b] THM mixture composed of 20 µl 1.0 M Tris-HCl buffer; 60 µl 1.0 M Hydroxylamine solution (2.0 M hydroxylamine-monohydrochloride neutralised with 2.0 M NaOH); 1.5 µl 0.1 M $MnCl_2$, pH 6.8.
[c] Composed of 40 mM Tris-HCl buffer; 0.3 mM $MnCl_2$; 2 mM 2-mercaptoethanol, pH 6.8.

Table 3.6 : GOGAT assay components and optimum conditions for assays using cell-free extracts of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49, respectively (modified from Kumar & Nicholas, 1982).

Volume (µl)	Stock Solution[a]	Final Concn
25	0.2 M 2-Oxoglutarate, pH 7.0	5.0 mM
50	0.1 M L-Glutamine, pH 7.0	5.0 mM
5	10 mM NADPH or NADH (freshly prepared)	
920 - *x*	GOGAT/GDH/AlaDH "extraction" buffer [b]	
x	Cell-free enzyme extract (prepared in GOGAT/GDH/AlaDH "extraction" buffer); 50 and 400 µl normally used for NADPH and NADH respectively.	

[a] All components were prepared as stock solutions prior to the experiment, unless stated otherwise.
[b] Composed of 50 mM Tris-HCl buffer; 0.1 mM EDTA; 2 mM 2-mercaptoethanol; pH 7.6 for NADH-dependent assays and pH 8.8 for NADPH-dependent assays.

Table 3.7 : GDH assay components and optimum conditions for assays using cell-free extracts of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49, respectively (modified from Fahien & Cohen, 1970).

Volume (µl)	Stock Solution[a]	Final Concn
50	0.2 M 2-Oxoglutarate, pH 7.0	10 mM
100	1.0 M NH_4Cl	100 mM
5	10 mM NADH or NADPH (freshly prepared)	
845 - *x*	GOGAT/GDH/AlaDH "extraction" buffer [b]	
x	Cell-free enzyme extract (prepared in GOGAT/GDH/AlaDH "extraction" buffer); 25 and 400 µl normally used for NADPH and NADH respectively.	

[a] All components were prepared as stock solutions, unless stated otherwise.
[b] Composed of 50 mM Tris-HCl buffer; 0.1 mM EDTA; 2 mM 2-mercaptoethanol; pH 7.4 for NADH-dependent assays and pH 7.0 for NADPH-dependent assays.

Table 3.8 : AlaDH assay components and optimum conditions for assays using cell-free extracts of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49, respectively (modified from Yoshida & Freese, 1970).

Volume (µl)	Stock Solution[a]	Final Concn
20	0.1 M Pyruvic acid, pH 7.0	2.0 mM
100	1.0 M NH_4Cl	100 mM
5	10 mM NADH or NADPH (freshly prepared)	
875 - *x*	GOGAT/GDH/AlaDH "extraction" buffer [b]	
x	Cell-free enzyme extract (prepared in GOGAT/GDH/AlaDH "extraction" buffer); 200 and 400 µl normally used for NADPH and NADH respectively.	

[a] All components were prepared as stock solutions, unless stated otherwise.
[b] Composed of 50 mM Tris-HCl buffer; 0.1 mM EDTA; 2 mM 2-mercaptoethanol; pH 6.0 for NADH-dependent assays and pH 6.5 for NADPH-dependent assays.

GS specific activity is expressed as nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$. GOGAT, GDH and AlaDH specific activities are expressed as μmoles NAD(P)H oxidised $min.^{-1}$ $(mg\ protein)^{-1}$. Results are the mean ± the standard error of the mean of triplicate assays.

3.2.2.4 Effects of the Stage of Culture Growth at Harvest on GS γ-GT Activity (Experiment 3.3)

1) In experiment 3.2 the growth of "Hungate" tube cultures grown concurrently with 40 and 120 ml serum bottle cultures was determined by measuring the optical density in the "Hungate" tubes at 600 nm. These optical density measurements were assumed to correspond to the growth also occurring in the serum bottle cultures, thereby eliminating the need to take sub-samples from the larger cultures for growth measurements. Although this is a convenient and practical method of following growth in large cultures, it may not have been accurate in determining when to harvest the concurrent cultures, since some variation in the stage of growth in different bottles may have occurred. Such variation may have resulted in cells being harvested at different stages of growth between treatments (i.e. between media ammonia levels), and even within treatments, since more than one serum bottle culture was grown for some media types. This experiment was therefore carried out to determine whether the stage of growth at which cells are harvested affects the activity of the ammonia-assimilating enzymes.

Batch cultures of *B. ruminicola* BA25 were grown in "Hungate" tubes containing 12 ml of NFMS media supplemented with 0.5, 2.0, 8.0 or 128 mM NH_4Cl (Table 3.2), or 12 ml of RGM (Table 3.1). Sufficient "Hungate" tubes were inoculated (1 % [v/v] inoculum from overnight NFMS culture supplemented with 8 mM NH_4Cl) for each medium so that enough cells could subsequently be harvested at early log, mid log, late log and stationary phase of cell growth to ensure that adequate GS activity would be detected in the cell-free extracts. Table 3.9 shows that the total culture volume harvested was increased from the NFMS medium supplemented with 0.5 mM NH_4Cl through to RGM, but was decreased within each type of media from cells harvested at early log through to stationary phase of growth. This was because, in experiment 3.2, it was discovered that the activity of GS decreased as the media ammonia level increased. For this reason the concentration of enzyme prepared for GS assays (i.e. concentration of free protein in the cell-free extract) was increased for the higher media

ammonia levels, and RGM, by harvesting more cells, but ultimately preparing cell-free extracts in the same volume of "extraction" buffer. Cell density differences between batch cultures harvested at early log, mid log and late log/stationary phase were the reason for the decreasing total culture volume harvested through this progression of harvest times (Figure 3.9).

After inoculation, culture growth was monitored in each 12 ml culture by measuring the optical density at 600 nm at 30 min. intervals. The optical densities at which the designations of early log, mid log, late log, and stationary phase of growth were made, and at which cultures were harvested for this experiment, are shown in Table 3.9 (also see Figure A2.1 (a), Appendix 2). These designations were based on growth curves determined during experiment 3.1. Batch cultures were harvested at the appropriate times on the basis of their optical density (individual tubes were pooled at harvest) and processed into 2 ml of cell-free extract for each sample, as outlined in section 3.2.2.3. The clarified media from cell harvesting was retained for measurement of the ammonia concentration at harvest. Ammonia concentrations were also measured in the original media (see section 2.2.2.6 for method).

Cell-free extracts, prepared according to the above protocol, were assayed for GS activity by the γ-GT reaction, as described for experiment 3.2 (section 3.2.2.3). The amount of γ-glutamyl hydroxamate formed was determined by reference to a standard curve obtained with γ-glutamyl hydroxamate. γ-GT activity was expressed as nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$. Results are the mean ± the standard error of the mean of triplicate assays.

2) In addition to studying the effects of the stage of culture growth on GS γ-GT activity, the activity of GS in cell-free extracts prepared from batch cultures of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5, 4.0 and 8.0 mM NH_4Cl, and inoculated (1 % [v/v] inoculum) from an overnight culture of NFMS media supplemented with 4 mM NH_4Cl, rather than 8 mM NH_4Cl (the level routinely used for overnight cultures), was investigated. This was to determine whether the level of NH_4Cl in the inoculating culture affects subsequent cell growth and/or enzyme activity. Twelve ml "Hungate" tube batch cultures of *B. ruminicola* BA25 were harvested at mid to late log phase of growth, as described above, and GS γ-GT activity was measured in cell-free extracts prepared from the cell pellets, as described in section 3.2.2.3. Results, expressed as nmoles γ-glutamyl

Table 3.9 : Culture and harvest details for *B. ruminicola* BA25 in experiment 3.3 (Effects of the Stage of Culture Growth at Harvest on GS γ-GT Activity).

Growth Medium [a]	Phase of Growth (and Time) at Harvest	Optical Density of Cells at Time of Harvest	Total Culture Volume Harvested (ml)
0.5 mM NH_4Cl	Early log (3 hr)	~0.35	96
	Late log (5 hr)	~0.48	48
	Stationary (7 hr) [b]	0.48 (harvested 2 hr later)	48
2.0 mM NH_4Cl	Early log (2 hr)	~0.35	96
	Mid log (3.5 hr)	~0.95	72
	Late log (5 hr)	~1.05	48
	Stationary (7 hr)	1.05 (harvested 2 hr later)	48
8.0 mM NH_4Cl	Early log (2 hr)	~0.40	120
	Mid log (3.5 hr)	~1.15	84
	Late log (4.5 hr)	~1.90	60
	Stationary (7 hr)	1.90 (harvested 2 hr later)	60
128 mM NH_4Cl	Early log (4 hr)	~0.45	168
	Mid log (6 hr)	~1.19	132
	Late log (8 hr)	~1.90	84
	Stationary (10 hr)	1.90 (harvested 2 hr later)	84
Routine Growth Medium	Early log (6 hr)	~0.60 [c]	168
	Mid log (7 hr)	~1.45	132
	Late log (8 hr)	~1.63	84
	Stationary (10 hr)	1.63 (harvested 1 hr later)	84

[a] *B. ruminicola* subsp. *brevis* NFMS medium (Table 3.2) supplemented with the listed concentrations of NH_4Cl, or RGM (Table 3.1).

[b] Defined for this study as late log phase plus 2 hr, or 1 hr for RGM cultures.

[c] Unusually long lag phase for this study.

hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$, are the mean ± the standard error of the mean of triplicate assays.

3.2.2.5 K_m-values for Some Substrates of GS, GOGAT and GDH in *B. ruminicola* BA25 (Experiment 3.4)

The apparent K_m-values for hydroxylamine (γ-GT reaction of GS), L-glutamine (GOGAT) and NH_4Cl (GDH) were determined for *B. ruminicola* BA25 by the methods of Lineweaver & Burk (1934) as described by Michal (1983). Double reciprocal plots of reaction velocity (adjusted by subtraction of the control value determined with 0 mM substrate) versus the initial substrate concentration were prepared using the simple regression analysis (linear regression) function provided by the CricketGraph™ computer software package (version 1.3.1 : Cricket Software, Philadelphia, U.S.A.) for the Apple Macintosh.

K_m-values were determined under standard assay conditions (Tables 3.5, 3.6 & 3.7), with individual substrates varied over the following ranges : hydroxylamine, 0 to 120 mM; L-glutamine, 0 to 10 mM; NH_4Cl, 0 to 200 mM. Growth of batch cultures of *B. ruminicola* BA25 (12 ml per "Hungate" tube) in NFMS media (Table 3.2) supplemented with 0.5 or 2 mM NH_4Cl was monitored by optical density measurements at 600 nm. Cells harvested as for experiment 3.3, at mid to late log phase of growth, were processed to produce cell-free extracts for the assays.

3.2.2.6 Nitrogen Source Preferences for Growth by *B. ruminicola* BA25 and *S. ruminantium* MP72 (Experiment 4)

As outlined in the introduction to this chapter, the aim of this final experiment was to put the ammonia assimilation data of the previous experiments into context in terms of the nitrogen source conditions in the rumen. When initially developing NFMS media for growth of the bacterial species studied in this thesis it was found that *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 grew poorly when ammonia was the sole source of nitrogen. In experiment 4, the preferences (if any) of *B. ruminicola* BA25 and *S. ruminantium* MP72 for representatives of the various nitrogen sources available in the rumen were studied. In addition, a low and a high energy (glucose) supply were superimposed over the different nitrogen source treatments to determine whether these bacteria were able to utilise the nitrogen

sources as alternative energy sources when a direct energy source (glucose) was unavailable, or had been utilised by the bacteria.

B. ruminicola BA25 was grown in batch culture in a "set" of the appropriate NFMS media (Table 3.2). Each of the cultures of the "set" was supplemented with one of the nitrogen sources listed in Table 3.10. In addition, this species was grown in a second "set" of media in which 0.05 % [w/v] glucose, rather than 0.5 % [w/v] glucose shown in Table 3.2, was added. Two "sets" of media with 0.05 % glucose, and two "sets" with 0.5 % glucose, were prepared in 155 ml serum bottles (130 ml media per bottle). These were inoculated at 1 % [v/v] of the final culture volume from an overnight culture grown in NFMS media supplemented with 0.5 % glucose and 8 mM NH_4Cl, and growth was monitored at 30 or 60 min. intervals in each by optical density measurements (600 nm) of 1 ml aliquots aseptically withdrawn through the butyl rubber septa. One of the cultures containing 0.5 % [w/v] glucose from each treatment group was harvested at early log phase of growth and the other high glucose culture, as well as the cultures supplemented with 0.05 % [w/v] glucose, were harvested when the high glucose culture reached late log phase of growth (see Figure A2.1 (a), Appendix 2). The remaining low glucose culture was harvested up to 2 hr after the first culture. Forty ml of clarified media, produced by centrifugation of the cultures (4,500 x *g* ; 10 min.; 4°C), was stored at -20°C for subsequent measurement of the level of ammonia and Casamino Acids in the media (below). Extracellular peptides and protein in the media were separated in a further 5 ml sample of the clarified media by perchloric acid precipitation (5 ml of 5 % [v/v] perchloric acid added to the 5 ml of clarified media), followed by centrifugation (30,000 x *g* ; 30 min.; 0°C). The supernatant was stored at -20°C in a sealed vial for Tryptone assay (below), and the pellet containing precipitated protein was resuspended in 5 or 10 ml of 0.1 M phosphate buffer and similarly stored at -20°C in a sealed vial for casein assay (below).

In addition to this intensive study, growth data alone were collected for *B. ruminicola* BA25 on two other occasions by measuring the growth (optical density at 600 nm) of triplicate 12 ml "Hungate" tube cultures of the "set" of treatments listed in Table 3.10.

S. ruminantium MP72 similarly was studied using "sets" of NFMS media supplemented with either 0.05 % or 0.5 % [w/v] glucose, and the nitrogen sources listed in Table 3.10, in 12 ml "Hungate" tube cultures, rather than in 130 ml cultures. These 12 ml "Hungate" tube cultures, however, were only harvested at late log phase of growth (see Figure

A2.1 (b), Appendix 2), and clarified media was stored at -20°C for subsequent ammonia measurement (amino acids, peptides and protein were not measured) from one of the duplicate days of this experiment. This abridged form of the experimental protocol was used because the initial results from *B. ruminicola* BA25 only showed significant use of ammonia in the treatments, hence it was decided to concentrate on growth differences and ammonia levels. Cultures were inoculated at 1 % [v/v] of the final culture volume from overnight cultures of the *S. ruminantium* MP72 in NFMS media supplemented with 8 mM NH_4Cl, and growth was monitored at 600 nm at either 30 or 60 min. intervals.

AMMONIA ASSAY : Stored samples of clarified media were thawed and allowed to reach room temperature before the ammonia concentration was measured by the method described in section 2.2.2.6.

AMINO ACIDS ASSAY : The level of Casamino Acids in *B. ruminicola* BA25 media before bacterial growth, and in clarified media after harvest at early log and late log phase of growth was compared by assaying the level of mixed amino acids using the method of Rosen (1957). Triplicate samples of media were assayed for Casamino Acids by diluting 20 µl aliquots of thawed clarified media with 980 µl of 50 mM citrate buffer (Chen *et al.*, 1987b). Cyanide-acetate buffer (0.5 ml) was added to this 1 ml sample, followed by the addition of 3 % [w/v] ninhydrin solution in methyl cellosolve (0.5 ml : ethylene glycol monomethyl ether). This solution was heated for 15 min. in a 100°C water bath and, immediately after removing the solution from the water bath, isopropyl alcohol-water diluent (5 ml : isopropyl alcohol and water in a 1 to 1 ratio) was added and the solution shaken vigorously. After cooling to room temperature the optical density of the solution was measured at 570 nm. Samples were compared with a standard curve prepared with 0 - 0.5 mg of Casamino Acids in order to estimate the level of free amino acids in the samples. Results are the mean ± the standard error of the mean from the triplicate assays.

PEPTIDES ASSAY : The level of Tryptone in *B. ruminicola* BA25 media before bacterial growth, and in clarified media after harvest at early log and late log phase of growth, was compared by the method of Russell *et al.* (1983). Triplicate 10 µl aliquots of thawed

Table 3.10 : Nitrogen source treatments added to *B. ruminicola* BA25 and *S. ruminantium* MP72 NFMS media in conjunction with either 0.05 % [w/v] or 0.5 % [w/v] glucose in experiment 4 †.

NITROGEN SOURCE TREATMENT
1. 8 mM NH_4Cl
2. 1.5 % [w/v] Casamino Acids
3. 1.2 % [w/v] Tryptone
4. 1.0 % [w/v] casein
5. 8 mM NH_4Cl plus 1.5 % [w/v] Casamino Acids
6. 8 mM NH_4Cl plus 1.2 % [w/v] Tryptone
7. 8 mM NH_4Cl plus 1.0 % [w/v] casein
8. 1.5 % [w/v] Casamino Acids plus 1.2 % [w/v] Tryptone
9. 8 mM NH_4Cl plus 1.5 % [w/v] Casamino Acids plus 1.2 % [w/v] Tryptone
10. 8 mM NH_4Cl plus 1.5 % [w/v] Casamino Acids plus 1.2 % [w/v] Tryptone plus 1.0 % [w/v] casein

† Individual nitrogen sources were designed to provide the equivalent of approximately 1.5 g of nitrogen per litre and were based on work by Chen *et al.* (1987b). Final value for NH_4Cl treatment was about 1.1 g.

supernatant were diluted with 990 µl of 0.1 M phosphate buffer. The optical density of these 1 ml samples was measured at 215 nm in quartz cuvettes of 1 cm light path. The level of free peptides was estimated by reference to a standard curve produced with 0 - 0.1 mg Tryptone. Results are the mean ± the standard error of the mean from the triplicate assays.

PROTEIN ASSAY : The level of casein in *B. ruminicola* BA25 media before bacterial growth, and in clarified media after harvest at early log and late log phase of growth was compared by an assay of the level of protein using the method of Russell *et al.* (1983). The resuspended perchloric acid precipitate prepared for each sample was assayed in triplicate from thawed samples by diluting 10 µl aliquots with 990 µl of 0.1 M phosphate buffer. The optical density of these 1 ml samples was determined at 214 nm in quartz cuvettes of 1 cm light path and compared to a standard curve produced with 0 - 0.1 mg casein to estimate the level of protein in the media. Results are the mean ± the standard error of the mean from the triplicate assays.

3.2.2.7 Statistics

Analysis of variance statistics were performed using the CLR Analysis of Variance computer software package (version 1.1 : Clear Lake Research Inc., Houston, U.S.A.). This statistical test was used to test the significance of ammonia levels and/or nitrogen treatments on enzyme activities within experiment 3.2.

Student's T-tests were performed with the Statworks™ computer software package (version 1.2 : Cricket Software, Philadelphia, U.S.A.). This statistical test was used to compare media ammonia concentrations between inhibitor treatments in experiment 3.1, and to compare enzyme activities between media ammonia concentrations and nitrogen source treatments in experiment 3.2. The Student's T-test was also used to compare GS activites and ammonia concentrations between media types in experiment 3.3 (i.e. between NFMS media with various NH_4Cl concentrations, as well as RGM). In experiment 4 this test was used to compare ammonia, Casamino Acids, Tryptone and casein levels in media before and after cell growth for the various nitrogen source and glucose level treatments.

The mean ± the standard error of the mean were routinely calculated from triplicate assay data.

3.3 RESULTS

3.3.1 PRELIMINARY CULTURE GROWTH USING NFMS MEDIA

NFMS media (Table 3.2) were based on the defined media of Schaefer *et al.* (1980), with slight modifications made during preliminary growth experiments. Figure 3.2 (a, b & c) shows classical sigmoidal growth for *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13 in their respective NFMS media supplemented with 8 mM NH_4Cl as the sole source of nitrogen. These strains also grew very well in RGM (Table 3.1), in which amino acids and peptides are present as nitrogen sources, in addition to ammonia, although preliminary growth curves with this medium are not shown. *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 did not grow in NFMS media with NH_4Cl as the sole nitrogen source (Figure 3.2 (d & g)), however excellent growth of the former was evident when yeast extract or Proteose Peptone were added to the NFMS media (i.e. amino acid and peptide nitrogen, plus vitamins and other "growth factors" : Figure 3.2 (e)). Yeast extract (0.1 % [w/v]) was subsequently added to the NFMS medium recipe for *B. ruminicola* subsp. *ruminicola* (Table 3.2), since this amount supported adequate, but not maximal, growth (Figure 3.2 (f)). As a result of similar growth experiments with *Butyrivibrio fibrisolvens* 49 (Figure 3.2 (h)), the NFMS media recipe for this species (Table 3.2) was modified by the addition of 0.15 % [w/v] Trypticase.

Figure 3.2 also shows that growth of *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 was much less vigorous than for *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13. Less distinct differences in growth kinetics were evident between *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13. For example, *B. ruminicola* BA25 was more vigorous, and had a shorter lag time before growth, than the two subspecies of *Selenomonas*, however the growth rates and maximum optical densities for these subspecies were similar.

3.3.2 EFFECT OF A 1 % [v/v] INOCULUM ON THE AMMONIA CONCENTRATION IN MEDIA

Experiments involving the NFMS media listed in Table 3.2 required supplements of up to 128 mM NH_4Cl in the media, as well as additions of other nitrogen sources. Cultures for

Figure 3.2 : Batch culture growth of pure rumen bacterial species in NFMS media.

Triplicate "Hungate" tube cultures (10 ml) of the appropriate media were inoculated at 1 % [w/v] of final culture volume from an overnight RGM or BHI culture of the species being tested. Optical density measurements (600 nm) were made immediately following inoculation, and at various intervals afterwards, directly in the "Hungate" tubes using the spectrophotometer fitted with a special holding cage.

Results are the mean ± the standard error of the mean from the triplicate tubes.

a) Growth of *B. ruminicola* BA25 in NFMS medium supplemented with 8 mM NH_4Cl as the sole nitrogen source (◦).

b) Growth of *S. ruminantium* MP72 in NFMS medium supplemented with 8 mM NH_4Cl as the sole nitrogen source (◦).

c) Growth of *S. ruminantium* ME13 in NFMS medium supplemented with 8 mM NH_4Cl as the sole nitrogen source (◦).

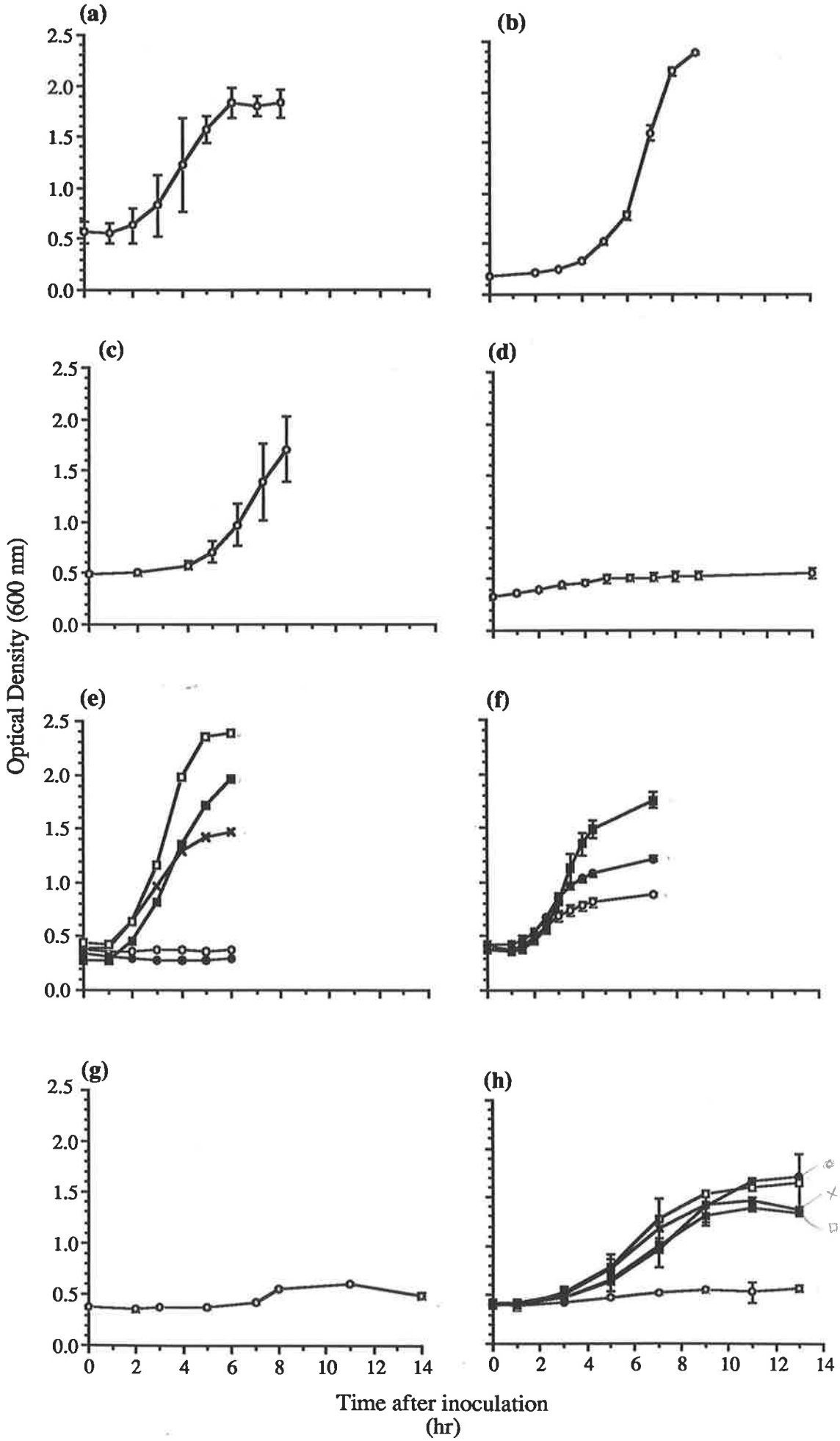
d) Growth of *B. ruminicola* subsp. *ruminicola* in NFMS medium supplemented with 8 mM NH_4Cl as the sole nitrogen source (◦).

e) Growth of *B. ruminicola* subsp. *ruminicola* in NFMS medium supplemented with 8 mM NH_4Cl (◦), 0.1 % [v/v] R_1 salts solution (•), 0.2 % [w/v] yeast extract (x), 0.3 % [w/v] Proteose Peptone (■) or 0.2 % [w/v] yeast extract plus 0.3 % [w/v] Proteose Peptone (□).

f) Growth of *B. ruminicola* subsp. *ruminicola* in NFMS medium supplemented with 0.05 % [w/v] yeast extract (◦), 0.1 % [w/v] yeast extract (•) or 0.2 % [w/v] yeast extract (■).

g) Growth of *Butyrivibrio fibrisolvens* 49 in NFMS medium supplemented with 8 mM NH_4Cl as the sole nitrogen source (◦).

h) Growth of *Butyrivibrio fibrisolvens* 49 in NFMS medium supplemented with 8 mM NH_4Cl (◦), 0.3 % [w/v] Trypticase (•), 0.15 % [w/v] Trypticase (■), 8 mM NH_4Cl plus 0.3 % [w/v] Trypticase (□) or 8 mM NH_4Cl plus 0.15 % [w/v] Trypticase (x).


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Optical Density (600 nm)
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8
10
12
14
Time after inoculation
(hr)

all experiments were usually inoculated from overnight cultures grown in NFMS media supplemented with 8 mM NH_4Cl, hence the effect of inoculation on the level of ammonia in NFMS media was measured in an aliquot of the basal media without its nitrogen source supplement. A 1 % [v/v] inoculum from RGM significantly raised the ammonia concentration in the aliquot of NFMS media from 0.203 mM (± 0.0055) to 0.275 mM (± 0.0030) ($P < 0.01$).

3.3.3 EFFECTS OF AZASERINE AND MSX ON THE GROWTH OF SELECTED SPECIES OF RUMEN BACTERIA (Experiment 3.1)

3.3.3.1 *B. ruminicola* BA25

Comparisons of the control growth (i.e. no inhibitor added) of *B. ruminicola* BA25 in NFMS media supplemented with NH_4Cl, clearly show that this species does not reach its maximum growth (i.e. maximum optical density, measured at 600 nm) until the level of this supplement was about 8 mM (Table 3.11). The level of ammonia remaining in the media at harvest was negligible for each of the NFMS media supplemented with less than 8 mM NH_4Cl, whereas the concentration of ammonia was about 2 mM for control growth in media supplemented with 8 mM NH_4Cl (Table 3.11). The level of ammonia remaining in the media at harvest increased for control cultures grown in NFMS media supplemented with 16, 32 and 128 mM NH_4Cl (Table 3.11).

The effects of MSX and azaserine as inhibitors are clearly evident in Table 3.11, which shows the optical density at harvest of *B. ruminicola* BA25 cultures (also see Figure A2.1 (a), Appendix 2). Azaserine, which inhibits GOGAT activity, appeared to inhibit even the very poor growth evident when NFMS media was supplemented with only 0.25 mM NH_4Cl. As the concentration of NH_4Cl in the media was increased, the differences in growth between azaserine inhibited cultures and control cultures become more marked, with very clear effects on growth evident when the concentration of NH_4Cl was greater than 4 mM. MSX, which is a potent inhibitor of GS, did not appear to inhibit growth of *B. ruminicola* BA25 when NFMS media was supplemented with 0, 0.25, 0.5, 0.75 or 1.0 mM NH_4Cl, however growth was clearly inhibited when the media was supplemented with more than 2 mM NH_4Cl, and both compounds were inhibitory even when the level of supplementary NH_4Cl was 128 mM.

The actual concentrations of ammonia in the NFMS media (the designations of 0.25, 0.5, 0.75, . . . are the intended ammonia levels only), as well as the level of ammonia remaining when growth was terminated in control, MSX and azaserine treated cultures, are also shown in Table 3.11. Significant differences between the pre-growth ammonia concentrations, and the concentrations when growth was terminated in control, MSX and azaserine treated cultures, were evident for cultures designated as supplemented with between 0.25 and 8 mM NH_4Cl inclusive ($P < 0.05$). Similar differences were not evident for cultures supplemented with 0 and 128 mM NH_4Cl, or for RGM cultures ($P > 0.05$), however pre-growth and control treatment levels were different for cultures supplemented with 16 and 32 mM NH_4Cl ($P < 0.05$). A significant difference was also evident in ammonia concentrations between the control, and the MSX and azaserine treatments, for cultures supplemented with 8 mM NH_4Cl ($P < 0.05$), and between the control and azaserine treatments for cultures supplemented with 16 and 32 mM NH_4Cl. For cultures supplemented with less than 8 mM NH_4Cl, similar trends were not significant ($P > 0.05$), probably due to low sample numbers ($n = 2$, except for cultures supplemented with 2, 4 or 8 mM NH_4Cl, where $n = 3$).

In contrast, batch culture growth in RGM, which has ammonia, amino acids and peptides as nitrogen sources instead of NH_4Cl, was not inhibited by either azaserine or MSX (Table 3.11), and the concentration of ammonia in this media (present in Minerals 2 and a contaminant of the Proteose Peptone and yeast extract) did not decrease as a consequence of bacterial growth in control or treatment cultures ($P > 0.05$), as occurred when ammonia was the sole nitrogen source.

3.3.3.2 *S. ruminantium* MP72 and *S. ruminantium* ME13

Tables 3.12 & 3.13 show that *S. ruminantium* MP72 and *S. ruminantium* ME13 also did not reach their growth potentials in NFMS media until the NH_4Cl supplement was about 8 mM. Growth in these species was very poor when the NH_4Cl concentration was below 1 mM, and their growth kinetics in the NFMS media were very different to those of *B. ruminicola* BA25, with the latter having a much shorter lag time than the subspecies of *Selenomonas*. The growth kinetics, and the effects of MSX and azaserine, however, were very similar for these two subspecies (see Figure A2.1 (b & c), Appendix 2), hence the following results consider *S. ruminantium* MP72 and *S. ruminantium* ME13 simultaneously.

Table 3.11 : Effects of azaserine and MSX addition on the growth of cultures of *B. ruminicola* BA25, and on ammonia concentrations in the media.

Nine "Hungate" tube cultures (10 ml each) of the appropriate media were inoculated at 1 % [v/v] of final culture volume from an overnight culture of *B. ruminicola* BA25 in NFMS media supplemented with 8 mM NH_4Cl. Optical density (600 nm) was measured immediately following inoculation, and at 30 or 60 min. intervals thereafter, directly in the "Hungate" tubes using a special holding cage fitted into the spectrophotometer. When obvious growth was evident from these optical density measurements (after 3 hr with an increase in optical density of 0.4 to 0.6 units), 100 µl of 0.1 M MSX was added to three of the tubes and 100 µl of 0.01 M azaserine was added to three other tubes. The remaining three cultures were continued as controls. Growth was continued until stationary phase in the control cultures, when the used media was harvested. Clarified media was stored for ammonia concentration measurements, as outlined in section 3.2.2.2.

Optical density values are the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated (see legend of Table). These values were calculated from the mean of triplicate tubes for the growth measurements on individual days. Optical density values were adjusted by subtraction of the pre-inoculation optical density values, hence all data is relative to an original optical density of zero.

Ammonia level in the media is expressed as the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated. Values were compared using the Student's T-test. Values with different superscipts indicate differences at the 5 % level of significance.

	MEDIA *	"0" mM NH_4Cl	"0.25" mM NH_4Cl	"0.5" mM NH_4Cl	"0.75" mM NH_4Cl	"1.0" mM NH_4Cl	"2.0" mM NH_4Cl
Optical Density at Harvest †	Control	0.13 ± 0.039	0.23 ± 0.049	0.34 ± 0.037	0.49 ± 0.007	0.55 ± 0.083	0.91 ± 0.040 £
	+ MSX	0.13 ± 0.039	0.25 ± 0.042	0.33 ± 0.009	0.45 ± 0.084	0.57 ± 0.056	0.77 ± 0.034 £
	+ Azaserine	0.10 ± 0.036	0.19 ± 0.027	0.22 ± 0.029	0.31 ± 0.094	0.36 ± 0.058	0.49 ± 0.093 £
Ammonia Concentration (mM) #	Pre-growth	0.310 ± 0.0692 a	0.434 ± 0.0008 a	0.665 ± 0.0910 a	0.831 ± 0.0132 a	1.08 § a	2.03 ± 0.231 £a
	Control	0.033 ± 0.0197 a	0.006 ± 0.0001 b	0.006 ± 0.0009 b	0.017 ± 0.0110 b	0.012 ± 0.0047 b	0.006 ± 0.0017 £b
	+ MSX	0.030 ± 0.0219 a	0.006 ± 0.0001 b	0.007 ± 0.0012 b	0.067 ± 0.0392 b	0.074 ± 0.0669 b	0.129 ± 0.1103 £b
	+ Azaserine	0.033 ± 0.0247 a	0.020 ± 0.0137 b	0.094 ± 0.0878 b	0.186 ± 0.1244 b	0.082 ± 0.0582 b	0.403 ± 0.3343 £b

	MEDIA *	"4.0" mM NH_4Cl	"8.0" mM NH_4Cl	"16" mM NH_4Cl	"32" mM NH_4Cl	"128" mM NH_4Cl	RGM
Optical Density at Harvest †	Control	1.34 ± 0.031 £	1.60 ± 0.058 £	1.59 ± 0.013	1.58 ± 0.011	1.55 ± 0.011	1.26 ± 0.039
	+ MSX	0.82 ± 0.203 £	1.08 ± 0.137 £	0.67 ± 0.272	0.90 ± 0.307	0.81 ± 0.168	1.31 ± 0.038
	+ Azaserine	0.47 ± 0.136 £	0.51 ± 0.129 £	0.45 ± 0.225	0.55 ± 0.293	0.56 ± 0.001	1.21 ± 0.013
Ammonia Concentration (mM) #	Pre-growth	3.92 ± 0.202 £ a	7.75 ± 0.467 £ a	14.8 ± 1.09 a	30.1 ± 1.66 a	122.5 ± 7.58 a	5.28 ± 0.499 ¥a
	Control	0.122 ± 0.1029 £b	2.04 ± 0.145 £ b	8.24 ± 0.008 b	21.0 ± 0.27 b	97.6 ± 3.97 a	5.64 ± 0.341 a
	+ MSX	1.36 ± 0.559 £ b	4.19 ± 0.188 £ c	11.0 ± 0.76 abc	23.0 ± 0.39 abc	93.5 ± 5.17 a	5.72 ± 0.282 a
	+ Azaserine	1.50 ± 0.812 £ b	5.05 ± 0.507 £ c	11.4 ± 0.43 ac	23.2 ± 0.16 ac	95.4 ± 3.11 a	5.51 ± 0.519 a

* NFMS medium (Table 3.2) plus specified quantity of NH_4Cl, or Routine Growth Medium (Table 3.1).

† Measured at 600 nm and adjusted by subtraction of pre-inoculation Optical Density values (mean ± the standard error of the mean).

Values expressed as mean ± the standard error of the mean.

§ n = 1; £ n = 3; ¥ n = 7.

The growth data for the two subspecies of *Selenomonas*, and the ammonia concentrations in the media when growth was terminated (Tables 3.12 & 3.13), show that azaserine and MSX both inhibited growth even when the concentration of ammonia was only about 0.2 mM. At higher concentrations of ammonia in the media, up to about 30 mM, the extent of inhibition was more pronounced, however growth of the cultures supplemented with 128 mM NH_4Cl was not inhibited by MSX, whereas azaserine still effectively inhibited growth. Some differences between the concentration of ammonia in harvested media and pre-growth levels were evident for media supplemented with less than 16 mM NH_4Cl for both strains ($P < 0.05$), however cultures supplemented with 32 and 128 mM NH_4Cl did not have different ammonia levels before and after growth ($P > 0.05$). In addition, significant differences between the level of ammonia in control cultures, and in MSX and azaserine cultures, were evident ($P < 0.05$), but not between MSX and azaserine cultures ($P > 0.05$). Growth in RGM, and the level of ammonia before and after growth, were virtually unaffected by addition of either inhibitor.

3.3.3.3 *B. ruminicola* subsp. *ruminicola*

B. ruminicola subsp. *ruminicola* did not grow with NH_4Cl as the sole source of nitrogen (Figure 3.2 (d)), hence 0.1 % [w/v] yeast extract was added to the original NFMS media (Table 3.2), in addition to the range of NH_4Cl concentrations used for the previous bacterial species in this study. The optical density values at harvest for this species (Table 3.14) show that growth was almost identical in NFMS media regardless of the level of NH_4Cl added. Despite this observation, which indicates that *B. ruminicola* subsp. *ruminicola* growth is not stimulated by the addition of NH_4Cl (e.g. compare the control optical density values at harvest in Table 3.14), growth was slightly inhibited by both MSX and azaserine in all of these batch cultures (approx. 30 - 50 %; also see Figure A2.1 (d), Appendix 2). RGM supported much greater growth than any of the NFMS media (i.e. greater final optical density), and growth appears to be inhibited to a small extent by azaserine and MSX (approx. 25 - 30 % : Table 3.14), although growth was slowed in these cultures, rather than virtually halted as when the other species were inhibited. Growth of control cultures, and MSX and azaserine treated cultures, of *B. ruminicola* subsp. *ruminicola* resulted in an increase in the concentration of

Table 3.12 : Effects of azaserine and MSX addition on the growth of cultures of *S. ruminantium* MP72, and on ammonia concentrations in the media.

Nine "Hungate" tube cultures (10 ml each) of the appropriate media were inoculated at 1 % [v/v] of final culture volume from an overnight culture of *S. ruminantium* MP72 in NFMS media supplemented with 8 mM NH_4Cl. Optical density (600 nm) was measured immediately following inoculation, and at 30 or 60 min. intervals thereafter, directly in the "Hungate" tubes using a special holding cage fitted into the spectrophotometer. When obvious growth was evident from these optical density measurements (after 4.5 hr with an increase in optical density of 0.4 to 0.6 units), 100 µl of 0.1 M MSX was added to three of the tubes and 100 µl of 0.01 M azaserine was added to three other tubes. The remaining three cultures were continued as controls. Growth was continued until stationary phase in the control cultures, when the used media was harvested. Clarified media was stored for ammonia concentration measurements, as outlined in section 3.2.2.2.

Optical density values are the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated (see legend of Table). These values were calculated from the mean of triplicate tubes for the growth measurements on individual days. Optical density values were adjusted by subtraction of the pre-inoculation optical density values, hence all data is relative to an original optical density of zero.

Ammonia level in the media is expressed as the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated. Values were compared using the Student's T-test. Values with different superscipts indicate differences at the 5 % level of significance.

	MEDIA *	"0" mM NH_4Cl	"0.25" mM NH_4Cl	"0.5" mM NH_4Cl	"0.75" mM NH_4Cl	"1.0" mM NH_4Cl	"2.0" mM NH_4Cl
Optical Density at Harvest †	Control	0.14 ± 0.049	0.28 ± 0.010	0.35 ± 0.033	0.53 ± 0.013	0.63 ± 0.028	1.04 ± 0.007
	+ MSX	0.06 ± 0.114	0.14 ± 0.093	0.13 ± 0.094	0.19 ± 0.108	0.21 ± 0.096	0.25 ± 0.186
	+ Azaserine	0.11 ± 0.065	0.15 ± 0.063	0.12 ± 0.093	0.15 ± 0.095	0.18 ± 0.098	0.18 ± 0.130
Ammonia Concentration (mM) #	Pre-growth	0.019 ± 0.0006 [a]	0.179 ± 0.0170 [a]	0.363 ± 0.0575 [a]	0.563 ± 0.0070 [a]	0.760 ± 0.0400 [a]	1.43 ± 0.145 [a]
	Control	0.011 ± 0.0055 [a]	0.020 ± 0.0088 [b]	0.005 ± 0.0003 [b]	0.005 ± 0.0012 [b]	0.012 ± 0.0050 [b]	0.108 ± 0.1022 [b]
	+ MSX	0.055 ± 0.0340 [ab]	0.139 ± 0.1260 [ab]	0.299 ± 0.2100 [ab]	0.411 ± 0.2280 [ab]	0.548 ± 0.2797 [ab]	1.23 ± 0.474 [ab]
	+ Azaserine	0.065 ± 0.0080 [b]	0.141 ± 0.1110 [ab]	0.260 ± 0.2030 [ab]	0.407 ± 0.2620 [ab]	0.515 ± 0.2850 [ab]	1.22 ± 0.355 [ab]

	MEDIA *	"4.0" mM NH_4Cl	"8.0" mM NH_4Cl	"16" mM NH_4Cl	"32" mM NH_4Cl	"128" mM NH_4Cl	RGM
Optical Density at Harvest †	Control	1.249 ± 0.0475	1.697 ± 0.0433 [£]	1.933 ± 0.2175	1.786 ± 0.0375	1.081 ± 0.2517 [£]	1.63 ± 0.053
	+ MSX	0.138 ± 0.0392	0.213 ± 0.0543 [£]	0.165 ± 0.0667	0.187 ± 0.0483	1.004 ± 0.2235 [£]	1.54 ± 0.048
	+ Azaserine	0.223 ± 0.1467	0.303 ± 0.1332 [£]	0.417 ± 0.0100	0.179 ± 0.0442	0.076 ± 0.0197 [£]	1.39 ± 0.081
Ammonia Concentration (mM) #	Pre-growth	2.92 ± 0.355 [a]	8.07 ± 0.966 [a]	16.0 ± 2.40 [a]	31.8 ± 2.13 [a]	82.9 ± 2.24 [£a]	5.28 ± 0.499 [¥a]
	Control	0.401 ± 0.3953 [b]	0.950 ± 0.1226 [£b]	7.55 ± 0.024 [ab]	23.7 ± 1.44 [a]	81.2 ± 6.82 [£a]	5.51 ± 0.483 [a]
	+ MSX	3.00 ± 0.438 [a]	6.33 ± 0.391 [£ac]	13.6 ± 0.52 [ac]	28.6 ± 1.04 [a]	80.8 ± 11.19 [£a]	5.89 ± 0.265 [a]
	+ Azaserine	2.81 ± 0.633 [ab]	5.90 ± 0.453 [£ac]	12.7 ± 0.29 [ac]	27.8 ± 1.30 [a]	68.4 ± 5.88 [£a]	5.66 ± 0.045 [a]

* NFMS medium (Table 3.2) plus specified quantity of NH_4Cl, or Routine Growth Medium (Table 3.1).

† Measured at 600 nm and adjusted by subtraction of pre-inoculation Optical Density values (mean ± the standard error of the mean).

\# Values expressed as mean ± the standard error of the mean.

£ n = 3; ¥ n = 7.

Table 3.13 : Effects of azaserine and MSX addition on the growth of cultures of *S. ruminantium* ME13, and on ammonia concentrations in the media.

Nine "Hungate" tube cultures (10 ml each) of the appropriate media were inoculated at 1 % [v/v] of final culture volume from an overnight culture of *S. ruminantium* ME13 in NFMS media supplemented with 8 mM NH_4Cl. Optical density (600 nm) was measured immediately following inoculation, and at 30 or 60 min. intervals thereafter, directly in the "Hungate" tubes using a special holding cage fitted into the spectrophotometer. When obvious growth was evident from these optical density measurements (after 6 hr with an increase in optical density of 0.4 to 0.6 units), 100 µl of 0.1 M MSX was added to three of the tubes and 100 µl of 0.01 M azaserine was added to three other tubes. The remaining three cultures were continued as controls. Growth was continued until stationary phase in the control cultures, when the used media was harvested. Clarified media was stored for ammonia concentraion measurements, as outlined in section 3.2.2.2.

Optical density values are the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated (see legend of Table). These values were calculated from the mean of triplicate tubes for the growth measurements on individual days. Optical density values were adjusted by subtraction of the pre-inoculation optical density values, hence all data is relative to an original optical density of zero.

Ammonia level in the media is expressed as the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated. Values were compared using the Student's T-test. Values with different superscipts indicate differences at the 5 % level of significance.

	MEDIA *	"0" mM NH_4Cl	"0.25" mM NH_4Cl	"0.5" mM NH_4Cl	"0.75" mM NH_4Cl	"1.0" mM NH_4Cl	"2.0" mM NH_4Cl
Optical Density at Harvest †	Control	0.33 ± 0.010	0.53 ± 0.060	0.60 ± 0.041	0.83 ± 0.116	0.930 ± 0.0333	1.278 ± 0.0242
	+ MSX	0.29 ± 0.011	0.28 ± 0.012	0.26 ± 0.033	0.29 ± 0.013	0.289 ± 0.0242	0.449 ± 0.0592
	+ Azaserine	0.30 ± 0.033	0.38 ± 0.028	0.34 ± 0.044	0.38 ± 0.024	0.432 ± 0.0150	0.377 ± 0.0133
Ammonia Concentration (mM) #	Pre-growth	0.262 ± 0.0431 [a]	0.442 ± 0.0315 [a]	0.544 ± 0.0354 [a]	0.716 ± 0.1042 [a]	1.01 ± 0.016 [a]	1.85 ± 0.145 [a]
	Control	0.010 ± 0.0018 [b]	0.017 ± 0.0054 [b]	0.012 ± 0.0021 [b]	0.028 ± 0.0149 [b]	0.027 ± 0.0128 [b]	0.203 ± 0.0670 [b]
	+ MSX	0.024 ± 0.0146 [b]	0.080 ± 0.0436 [b]	0.151 ± 0.1157 [ab]	0.601 ± 0.1191 [a]	0.754 ± 0.1020 [a]	1.31 ± 0.028 [c]
	+ Azaserine	0.043 ± 0.0330 [ab]	0.053 ± 0.0286 [b]	0.379 ± 0.1191 [ab]	0.594 ± 0.1363 [ab]	0.686 ± 0.1441 [a]	1.49 ± 0.105 [ac]

	MEDIA *	"4.0" mM NH_4Cl	"8.0" mM NH_4Cl	"16" mM NH_4Cl	"32" mM NH_4Cl	"128" mM NH_4Cl	RGM
Optical Density at Harvest †	Control	1.34 ± 0.072	1.61 ± 0.274	1.38 ± 0.088	1.54 ± 0.019	0.96 ± 0.140	1.56 ± 0.002
	+ MSX	0.40 ± 0.018	0.35 ± 0.096	0.29 ± 0.028	0.42 ± 0.097	0.70 ± 0.108	1.50 ± 0.029
	+ Azaserine	0.53 ± 0.008	0.55 ± 0.098	0.41 ± 0.096	0.42 ± 0.012	0.10 ± 0.033	1.39 ± 0.017
Ammonia Concentration (mM) #	Pre-growth	3.49 ± 0.355 [a]	7.63 ± 0.558 [a]	15.3 ± 1.33 [a]	30.5 ± 3.07 [a]	118.4 ± 11.00 [a]	5.28 ± 0.499 [¥a]
	Control	1.72 ± 0.140 [b]	4.38 ± 1.130 [a]	12.1 ± 0.30 [a]	26.0 ± 0.96 [a]	122.2 ± 4.55 [a]	6.15 ± 0.047 [a]
	+ MSX	3.17 ± 0.066 [ac]	6.88 ± 0.788 [a]	13.6 ± 0.57 [a]	27.3 ± 0.96 [a]	114.2 ± 12.53 [a]	6.23 ± 0.483 [a]
	+ Azaserine	2.95 ± 0.018 [c]	6.47 ± 0.635 [a]	13.3 ± 1.00 [a]	27.5 ± 0.57 [a]	117.9 ± 8.10 [a]	6.48 ± 0.453 [a]

* NFMS medium (Table 3.2) plus specified quantity of NH_4Cl, or Routine Growth Medium (Table 3.1).

† Measured at 600 nm and adjusted by subtraction of pre-inoculation Optical Density values (mean ± the standard error of the mean).

Values expressed as mean ± the standard error of the mean.

¥ n = 7.

ammonia in the media above pre-inoculation levels for most of the cultures supplemented with less than 1 mM NH_4Cl ($P < 0.05$). At higher media ammonia concentrations no differences were evident between the ammonia levels before growth and the residual levels at harvest ($P > 0.05$), except for the culture supplemented with 32 mM NH_4Cl, in which the level of ammonia in the control culture at harvest exceeded the level prior to growth.

3.3.3.4 *Butyrivibrio fibrisolvens* 49

Butyrivibrio fibrisolvens 49 required the addition of 0.15 % [w/v] Trypticase to the NFMS media, rather than NH_4Cl, to promote growth. Addition of only 0.05 % [w/v] Trypticase to the NFMS media did not result in maximal cell growth, as shown in Table 3.15 (0 mM/0.05 % Tryp. column), and the addition of between 0.5 and 128 mM NH_4Cl did not stimulate growth above the level achieved with 0.15 % [w/v] Trypticase only (Table 3.15). Azaserine was slightly inhibitory (approx. 20 %) to the growth of this species in modified NFMS media also supplemented with between 0.5 and 128 mM NH_4Cl, however MSX did not restrict growth (Table 3.15 and Figure A2.1 (e), Appendix 2). Growth in RGM was poor in this species and no inhibitory effects were evident (Table 3.15). Significant differences between ammonia concentrations were not evident for RGM or NFMS media supplemented with NH_4Cl ($P > 0.05$), however some small differences were evident in cultures supplemented with 0.05 % [w/v] Trypticase, 0.15 % [w/v] Trypticase or no nitrogen source (Table 3.15 : $P < 0.05$).

3.3.4 EFFECTS OF MEDIA AMMONIA CONCENTRATION AND NITROGEN SOURCE ON THE ACTIVITY OF AMMONIA-ASSIMILATING ENZYMES IN THREE RUMEN BACTERIAL SPECIES (Experiment 3.2)

3.3.4.1 Glutamine Synthetase Activity

Figure 3.3 shows that an inverse relationship exists between the specific activity of GS and the concentration of NH_4Cl in NFMS media for *B. ruminicola* BA25, although analysis of variance statistics do not show a significant effect of media ammonia concentration on GS specific activity ($P = 0.12$). Experiment 3.3, which will be discussed later, was planned as a consequence of the variation evident in the present experiment. The GS activities presented

Table 3.14 : Effects of azaserine and MSX addition on the growth of cultures of *B. ruminicola* subsp. *ruminicola*, and on ammonia concentrations in the media.

Nine "Hungate" tube cultures (10 ml each) of the appropriate media were inoculated at 1 % [v/v] of final culture volume from an overnight culture of *B. ruminicola* subsp. *ruminicola* in NFMS media supplemented with 8 mM NH_4Cl. Optical density (600 nm) was measured immediately following inoculation, and at 30 or 60 min. intervals thereafter, directly in the "Hungate" tubes using a special holding cage fitted into the spectrophotometer. When obvious growth was evident from these optical density measurements (after 3.5 hr with an increase in optical density of 0.4 to 0.6 units), 100 µl of 0.1 M MSX was added to three of the tubes and 100 µl of 0.01 M azaserine was added to three other tubes. The remaining three cultures were continued as controls. Growth was continued until stationary phase in the control cultures, when the used media was harvested. Clarified media was stored for ammonia concentration measurements, as outlined in section 3.2.2.2.

Optical density values are the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated (see legend of Table). These values were calculated from the mean of triplicate tubes for the growth measurements on individual days. Optical density values were adjusted by subtraction of the pre-inoculation optical density values, hence all data is relative to an original optical density of zero.

Ammonia level in the media is expressed as the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated. Values were compared using the Student's T-test. Values with different superscipts indicate differences at the 5 % level of significance.

	MEDIA *	"0" mM NH_4Cl	"0.25" mM NH_4Cl	"0.5" mM NH_4Cl	"0.75" mM NH_4Cl	"1.0" mM NH_4Cl	"2.0" mM NH_4Cl
Optical Density at Harvest †	Control	0.77 ± 0.075	0.70 ± 0.088	0.74 ± 0.133	0.85 ± 0.124	0.76 ± 0.056	0.74 ± 0.016
	+ MSX	0.54 ± 0.118	0.46 ± 0.138	0.32 ± 0.159	0.32 ± 0.174	0.41 ± 0.153	0.45 ± 0.198
	+ Azaserine	0.58 ± 0.025	0.42 ± 0.182	0.42 ± 0.122	0.41 ± 0.062	0.42 ± 0.093	0.41 ± 0.069
Ammonia Concentration (mM) #	Pre-growth	0.268 ± 0.0937 ^{a}	0.481 ± 0.0445 ^{a}	0.684 ± 0.0093 ^{a}	0.934 ± 0.0266 ^{a}	1.08 ± 0.016 ^{a}	1.93 ± 0.035 ^{a}
	Control	0.693 ± 0.0069 ^{b}	0.763 ± 0.0211 ^{b}	0.962 ± 0.0084 ^{b}	1.23 ± 0.068 ^{ab}	1.42 ± 0.004 ^{b}	1.86 ± 0.131 ^{a}
	+ MSX	0.748 ± 0.0221 ^{b}	0.920 ± 0.0054 ^{c}	1.11 ± 0.022 ^{c}	1.38 ± 0.044 ^{b}	1.55 ± 0.046 ^{b}	2.19 ± 0.210 ^{a}
	+ Azaserine	0.603 ± 0.0626 ^{ab}	0.774 ± 0.0714 ^{abc}	1.02 ± 0.080 ^{abc}	1.25 ± 0.123 ^{ab}	1.69 ± 0.122 ^{b}	2.23 ± 0.203 ^{a}

	MEDIA *	"4.0" mM NH_4Cl	"8.0" mM NH_4Cl	"16" mM NH_4Cl	"32" mM NH_4Cl	"128" mM NH_4Cl	RGM
Optical Density at Harvest †	Control	0.699 ± 0.0575	0.74 ± 0.038	0.773 ± 0.1042	0.815 ± 0.1070	0.95 ± 0.359	2.13 ± 0.046
	+ MSX	0.283 ± 0.0908	0.51 ± 0.092	0.595 ± 0.1482	0.539 ± 0.0685	0.83 ± 0.269	1.58 ± 0.111
	+ Azaserine	0.349 ± 0.0058	0.34 ± 0.026	0.519 ± 0.1875	0.609 ± 0.1110	0.66 ± 0.282	1.46 ± 0.037
Ammonia Concentration (mM) #	Pre-growth	3.41 ± 0.038 ^{a}	6.42 ± 0.014 ^{a}	12.9 ± 0.41 ^{a}	26.0 ± 0.04 ^{a}	60.6 ± 3.54 ^{a}	5.28 ± 0.499 $^{¥a}$
	Control	3.62 ± 0.186 ^{a}	6.70 ± 0.533 ^{a}	14.6 ± 0.55 ^{a}	27.6 ± 0.34 ^{b}	73.3 ± 5.57 ^{a}	5.24 ± 0.388 ^{a}
	+ MSX	3.69 ± 0.202 ^{a}	6.29 ± 1.032 ^{a}	14.6 ± 0.35 ^{a}	27.3 ± 1.16 ^{ab}	69.4 ± 2.85 ^{a}	6.59 ± 0.435 ^{a}
	+ Azaserine	3.67 ± 0.229 ^{a}	6.24 ± 0.935 ^{a}	14.1 ± 0.32 ^{a}	26.6 ± 1.11 ^{ab}	69.4 ± 1.10 ^{a}	6.26 ± 0.641 ^{a}

* NFMS medium with 0.1 % yeast extract (Table 3.2) plus specified quantity of NH_4Cl, or Routine Growth Medium (Table 3.1).

† Measured at 600 nm and adjusted by subtraction of pre-inoculation Optical Density values (mean ± the standard error of the mean).

\# Values expressed as mean ± the standard error of the mean.

¥ n = 7.

Table 3.15 : Effects of azaserine and MSX addition on the growth of cultures of *Butyrivibrio fibrisolvens* 49, and on ammonia concentrations in the media.

Nine "Hungate" tube cultures (10 ml each) of the appropriate media were inoculated at 1 % [v/v] of final culture volume from an overnight culture of *Butyrivibrio fibrisolvens* 49 in NFMS media supplemented with 8 mM NH_4Cl. Optical density (600 nm) was measured immediately following inoculation, and at 30 or 60 min. intervals thereafter, directly in the "Hungate" tubes using a special holding cage fitted into the spectrophotometer. When obvious growth was evident from these optical density measurements (after 5 hr with an increase in optical density of 0.4 to 0.6 units), 100 µl of 0.1 M MSX was added to three of the tubes and 100 µl of 0.01 M azaserine was added to three other tubes. The remaining three cultures were continued as controls. Growth was continued until stationary phase in the control cultures, when the used media was harvested. Clarified media was stored for ammonia concetration measurements, as outlined in section 3.2.2.2.

Optical density values are the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated (see legend of Table). These values were calculated from the mean of triplicate tubes for the growth measurements on individual days. Optical density values were adjusted by subtraction of the pre-inoculation optical density values, hence all data is relative to an original optical density of zero.

Ammonia level in the media is expressed as the mean ± the standard error of the mean from duplicate days (n = 2), unless otherwise indicated. Values were compared using the Student's T-test. Values with different superscipts indicate differences at the 5 % level of significance.

	MEDIA *	0 mM NH_4Cl/ 0 % Tryp.[1]	0 mM NH_4Cl/ 0.05 % Tryp.[2]	"0" mM NH_4Cl	"0.5" mM NH_4Cl	"1.0/0.15 %" mM NH_4Cl	"2.0/0.15 %" mM NH_4Cl
Optical Density at Harvest †	Control	-0.05 ± 0.009	0.29 ± 0.075	0.84 ± 0.090	0.81 ± 0.080	0.82 ± 0.169	0.81 ± 0.145
	+ MSX	-0.06 ± 0.018	0.29 ± 0.090	0.86 ± 0.071	0.80 ± 0.104	0.83 ± 0.121	0.80 ± 0.139
	+ Azaserine	-0.08 ± 0.038	0.21 ± 0.116	0.65 ± 0.166	0.69 ± 0.166	0.66 ± 0.164	0.64 ± 0.144
Ammonia Concentration (mM) #	Pre-growth	0.223 ± 0.0030 [a]	0.281 ± 0.0405 [a]	0.365 ± 0.0397 [a]	0.769 ± 0.0735 [a]	1.11 ± 0.134 [a]	2.09 ± 0.235 [a]
	Control	0.278 ± 0.0135 [ab]	0.120 ± 0.0080 [ab]	0.064 ± 0.0365 [b]	0.645 ± 0.1110 [a]	1.32 ± 0.433 [a]	2.72 ± 0.893 [a]
	+ MSX	0.320 ± 0.0233 [ab]	0.187 ± 0.0062 [ac]	0.098 ± 0.0690 [ab]	0.653 ± 0.1223 [a]	1.36 ± 0.318 [a]	2.59 ± 0.798 [a]
	+ Azaserine	0.315 ± 0.0120 [b]	0.242 ± 0.0402 [abc]	0.335 ± 0.0747 [ab]	0.932 ± 0.2857 [a]	1.52 ± 0.505 [a]	2.64 ± 0.720 [a]

	MEDIA *	"4.0" mM NH_4Cl	"8.0" mM NH_4Cl	"16" mM NH_4Cl	"32" mM NH_4Cl	"128" mM NH_4Cl	RGM
Optical Density at Harvest †	Control	0.87 ± 0.024	0.82 [§]	0.97 ± 0.056	0.95 ± 0.045	0.87 ± 0.020	0.66 ± 0.153
	+ MSX	0.84 ± 0.052	0.80 ± 0.058	0.90 ± 0.072	0.94 ± 0.010	0.92 ± 0.055	0.62 ± 0.247
	+ Azaserine	0.60 ± 0.095	0.46 ± 0.089	0.71 ± 0.129	0.70 ± 0.113	0.63 ± 0.168	0.75 ± 0.071
Ammonia Concentration (mM) #	Pre-growth	3.88 ± 0.458 [a]	7.13 ± 1.073 [a]	16.5 [§ a]	30.4 ± 1.03 [a]	105.3 ± 15.80 [a]	5.28 ± 0.499 [¥a]
	Control	5.09 ± 1.353 [a]	7.03 ± 0.710 [a]	13.7 ± 1.30 [a]	27.8 ± 0.63 [a]	68.0 ± 2.20 [a]	6.00 ± 0.994 [a]
	+ MSX	5.14 ± 1.395 [a]	7.18 ± 0.218 [a]	13.7 ± 1.48 [a]	28.3 ± 0.39 [a]	75.3 ± 1.32 [a]	5.94 ± 0.874 [a]
	+ Azaserine	4.90 ± 1.130 [a]	6.76 ± 0.745 [a]	13.7 ± 1.09 [a]	26.6 ± 0.87 [a]	72.9 ± 1.83 [a]	5.95 ± 1.032 [a]

* NFMS medium with 0.15 % Trypticase (Table 3.2) plus specified quantity of NH_4Cl, or Routine Growth Medium (Table 3.1). NFMS medium included 0.15 % [w/v] Trypticase, except 0/0 ([1]) which has 0 mM added NH_4Cl & 0 % [w/v] Trypticase, and 0/0.05 ([2])which has 0 mM added NH_4Cl & 0.05 % [w/v] Trypticase.

† Measured at 600 nm and adjusted by subtraction of pre-inoculation Optical Density values (mean ± the standard error of the mean).

Values expressed as mean ± the standard error of the mean.

§ n = 1; ¥ n = 7.

in Figure 3.9 (page 169) from late log harvested cells in experiment 3.3 were significantly different between media ammonia concentrations, indicating that the trend of GS activities being inversely related to the media ammonia level, which is evident for the current experiment, is a real effect. In fact, the less rigorous statistics of the Student's T-test indicate significant differences in GS specific activity in the current experiment between cells grown in NFMS media supplemented with 0.5 mM NH_4Cl and cells grown with 8, 16, 32 or 128 mM NH_4Cl (Figure 3.3).

When GS specific activity data for *B. ruminicola* BA25 grown in RGM were included in an analysis of variance, an effect of media type on GS activity was observed ($P = 0.048$). Student's T-tests reveal a significant difference in GS activity between RGM grown cells and cells grown in NFMS media overall ($P < 0.01$), however comparisons between individual NFMS media and RGM were only significant for NFMS media supplemented with 0.5 and 1.0 mM NH_4Cl ($P < 0.05$).

In contrast to these data for *B. ruminicola* BA25, GS γ-GT activity was not detected in cell-free extracts of either *S. ruminantium* MP72 or *Butyrivibrio fibrisolvens* 49 in NFMS media or RGM. Even when assayed for 40 min., rather than 20 min. as for *B. ruminicola* BA25, GS γ-GT activity was still not detected in *S. ruminantium* MP72.

γ-GT activities were measured in cell-free extracts prepared from *B. ruminicola* B_14, GA33 and BA25, *B. ruminicola* subsp. *ruminicola*, *B. fragilis* and *S. ruminantium* HD_4 grown in Brain-Heart Infusion medium (BHI : Table 3.3), and cell-free extracts prepared from *E. coli* HB101 grown in L-Broth (Table 3.4). These results (Table 3.16) show that activity levels measured in this laboratory were of a similar order of magnitude to literature values.

3.3.4.2 Glutamate Synthase Activity

***B. ruminicola* BA25 :** In contrast to the GOGAT activities measured in experiments 1 and 2 (Chapter 2), where NADH-dependent activities were higher than NADPH-dependent activities, the NADPH-dependent specific activities of GOGAT for *B. ruminicola* BA25 were at least 10 times higher than the NADH-dependent specific activities (Figure 3.4 : $P < 0.01$). The extremely low levels of NADH-dependent GOGAT activities measured in this bacterium indicate that activity with this cofactor is not quantitatively important, hence the

Figure 3.3 : Specific activity of GS in cell-free extracts of *B. ruminicola* BA25 grown in NFMS media supplemented with various concentrations of NH_4Cl, or in RGM.

Batch cultures of *B. ruminicola* BA25 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, as well as in RGM, and harvested at mid to late log phase of growth (Figure A2.1 (a), Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3. Cell pellets from these cultures were resuspended in GS "extraction" buffer (see section 3.2.2.3) and passed twice through the French Pressure Cell (10,000 p.s.i.; 10°C) to disrupt the cells. The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored on ice for use on the same day, or at 4°C overnight. Section 3.2.2.3 has the full details of the methods used.

The GS (γ-GT) assay is as described in Table 3.5.
The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977) using Coomassie G250 dye, with BSA as a standard.

Results are the mean ± the standard error of the mean where "n" is shown in brackets ( ) for each value.

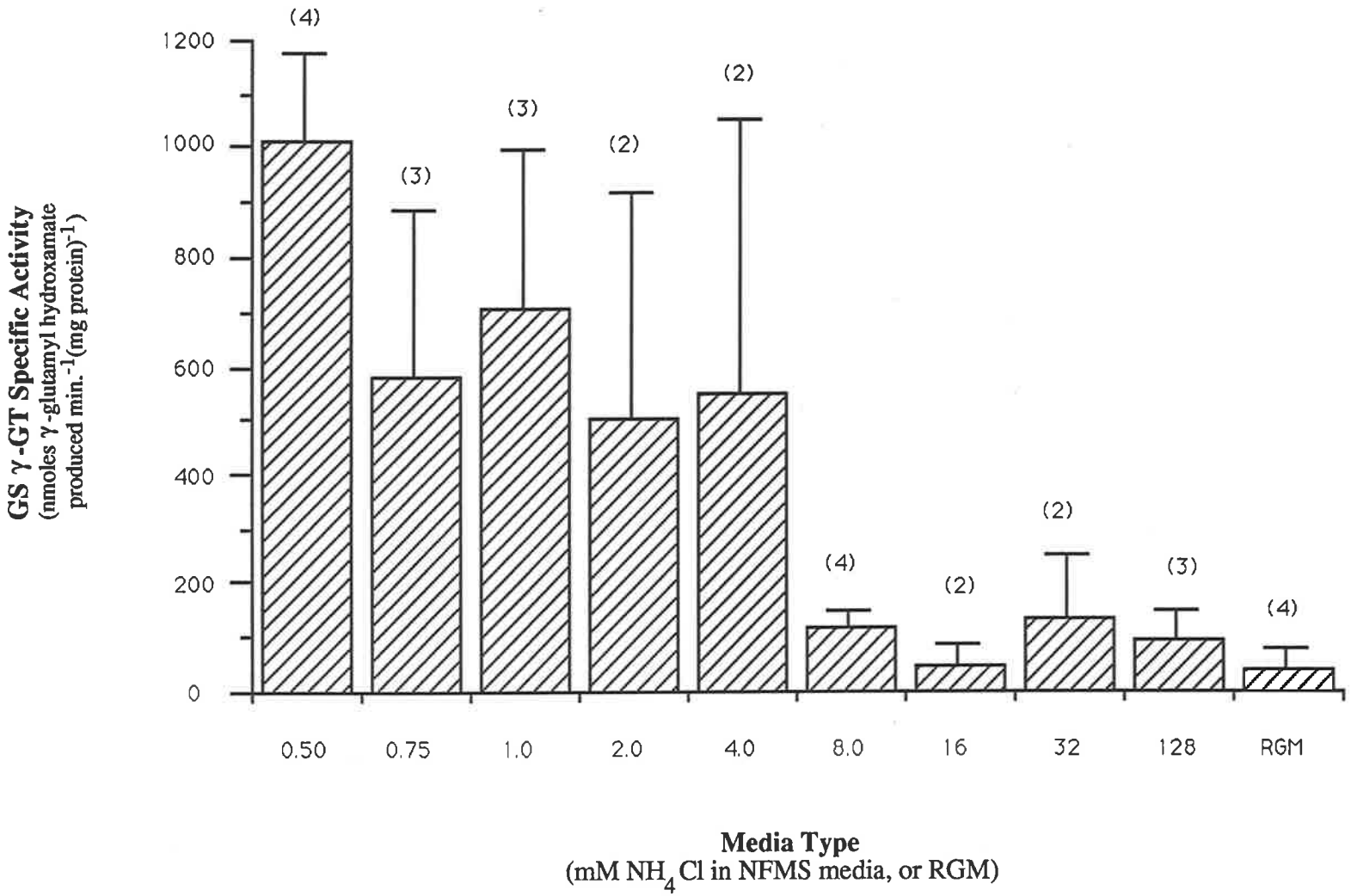

GS γ-GT Specific Activity
(nmoles γ-glutamyl hydroxamate produced min.$^{-1}$(mg protein)$^{-1}$)
1200
1000
800
600
400
200
0
(4)
(3)
(3)
(2)
(2)
(4)
(2)
(2)
(3)
(4)
0.50
0.75
1.0
2.0
4.0
8.0
16
32
128
RGM
Media Type
(mM NH_4Cl in NFMS media, or RGM)

Table 3.16 : GS activity of selected bacterial species, and some comparative literature data.

Overnight batch cultures (40 or 120 ml) of *B. ruminicola* B_14, GA33 and BA25, *B. ruminicola* subsp. *ruminicola*, *B. fragilis* and *S. ruminantium* HD_4 in BHI media, and *E. coli* HB101 in L-Broth medium, were harvested, the cells disrupted, and cell-free extracts prepared, by the methods outlined in section 3.2.2.3. GS γ-GT assays were for 30 min. at 39°C, but otherwise followed the description in Table 3.5.

Results are the mean ± the standard error of the mean where the number in brackets ( ) corresponds to "n".

Published activities are also from the γ-GT assay unless stated otherwise and are from the following literature :

[a] *B. fragilis* strain ATCC 23745 with NH_4Cl as the sole nitrogen source in defined media - Yamamoto *et al.* (1984) & Yamamoto *et al.* (1987b).

[b] *S. ruminantium* subsp. *ruminantium* strain D with the forward assay. Values of 22, 60 & 140 were obtained from cells grown in 3 mM NH_4^+, Casamino Acids & ammonia limited cultures respectively - Smith *et al.* (1980).

[c] Soy protein and urea diets respectively - Chalupa *et al.* (1970).

[d] Grass plus concentrate to barley diets - Wallace (1979).

[e] Erfle *et al.* (1977).

[f] Griffith & Carlsson (1974) - Tryptone, Yeast Extract and Casamino Acids as nitrogen sources; Glucose limited.

[g] Griffith & Carlsson (1974) - ammonia as nitrogen source; Glucose limited.

[h] Griffith & Carlsson (1974) - ammonia as nitrogen source; ammonia limited.

Bacterial Species/Strain	γ-GT Activity †	Published Activities (see footnotes for units)
B. ruminicola subsp. *brevis*		
strain B_14	4.8 ± 1.23 (5)	--
strain GA33	18.3 ± 2.18 (3)	
strain BA25	34.8 ± 8.95 (2)	
strain BA25 (RGM) §	42.4 ± 38.25 (4)	
B. ruminicola subsp. *ruminicola*	Not Detected (3)	--
B. fragilis	25.2 (1)	0.3 - 18.1 £ a
S. ruminantium subsp. *ruminantium*		17 - 311 †b
strain HD_4	30.4 ± 0.10 (2)	
S. ruminantium subsp. *lactilytica*		-
strain MP72 (RGM) §	Not Detected (2)	
Rumen Samples (from Chapter 2)		
- Sheep A	Not Detected → 8	93.7 & 121.4 †c
- Sheep B	Not Detected → 12	0 - 7.4 †d
- Sheep C	Not Detected → 14	19.4 - 350 †e
- Sheep D	337.1 ± 57.86	
- Sheep E	66.0 ± 17.35	
E. coli strain HB101	39.3 ± 4.45 (3)	
{		20 f
strain Hfr {		50 g
{		200 h

† Activity expressed as nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$.

£ Activity expressed as μmoles phosphate released $min.^{-1}$ $(mg\ protein)^{-1}$ {biosynthetic assay}.

§ Data from RGM grown cells in the first section of Experiment 3.2.

reason that a more appropriate scale was not used for NADH-dependent activities in Figure 3.4. Analysis of variance did not reveal a statistical effect of media ammonia level for either the NADH- or the NADPH-dependent GOGAT activity for this species ($P = 0.90$ & 0.69, respectively). Nevertheless, as was the case for GS activity, Student's T-tests detected differences between NADPH-dependent GOGAT activities in RGM grown cells and in cells grown in NFMS media overall (but not for NADH-dependent activities). Comparisons between NADPH-dependent GOGAT activities in RGM grown cells and cells grown in each NFMS media were only significant for media supplemented with 0.5, 2.0, 4.0, 8.0, 16 and 32 mM NH_4Cl. The data presented in Table 3.17 also suggest that a difference exists between GOGAT activities measured for cells with NH_4Cl as the sole nitrogen source (i.e. NFMS media) and from these grown with amino acids and peptides as nitrogen sources in addition to ammonia (i.e. RGM), since there is a change in the ratio of NADH-dependent activity to NADPH-dependent activity (NADH/NADPH ratio) from a mean of 0.036 for NFMS grown cells to 0.35 for RGM grown cells. The highly significant difference in ratios ($P < 0.01$) is almost entirely due to a decrease in NADPH-dependent activity in RGM grown cells relative to the levels for NFMS grown cells, rather than an increase in NADH-dependent activity. Comparisons of this ratio between individual NFMS media (i.e. different NH_4Cl levels) and RGM were not statistically significant due to the large variation in ratios for RGM batch cultures between days.

***S. ruminantium* MP72 :** Figure 3.7 (a) shows that the NADPH-dependent GOGAT activity was also significantly higher than the NADH-dependent activity in *S. ruminantium* MP72 ($P < 0.01$), even though the NADPH-dependent activity was approximately one-tenth of that measured for *B. ruminicola* BA25 ($P < 0.01$), and the NADH-dependent activity was about the same magnitude as levels observed in *B. ruminicola* BA25 ($P > 0.05$). No effects of media ammonia concentration or nitrogen source were detected for either the NADH- or the NADPH-dependent GOGAT activities ($P > 0.05$). This observation is further supported by the NADH/NADPH ratio values in Table 3.17, which were very similar for GOGAT activity in *S. ruminantium* MP72 cells grown in NFMS media supplemented with 0.5 and 8 mM NH_4Cl, and in RGM ($P > 0.05$). These similarities contrast to the differences for *B. ruminicola* BA25 grown in these types of media. The value for NFMS media supplemented with 128 mM NH_4Cl is lower than the other values, but this value is based on

one measurement, hence it is difficult to comment upon this, relative to the other values.

***Butyrivibrio fibrisolvens* 49 :** The NADPH-dependent GOGAT activities measured in cell-free extracts of *Butyrivibrio fibrisolvens* 49 (Figure 3.8 (a)) were also significantly lower than the activities in *B. ruminicola* BA25 ($P < 0.01$), and were only up to half the levels measured in *S. ruminantium* MP72 ($P < 0.05$). In contrast, NADH-dependent activities for *Butyrivibrio fibrisolvens* 49 were up to twice the value of those measured in either of the other species ($P < 0.05$) and, as was the case for *B. ruminicola* BA25 and *S. ruminantium* MP72, no effect of media type was evident ($P > 0.05$). Similarly, no effect of media type was found for the NADPH-dependent GOGAT activities. Unlike the results for *B. ruminicola* BA25 and *S. ruminantium* MP72, there also were no significant differences between the NADH- and the NADPH-dependent activities in *Butyrivibrio fibrisolvens* 49 in any media. The NADH/NADPH ratio values in Table 3.17 show that, as was the case for *S. ruminantium* MP72, the ratio for GOGAT in *Butyrivibrio fibrisolvens* 49 was not different for cells grown in NFMS media or RGM ($P > 0.05$).

NADH/NADPH Ratios : The significant differences in NADPH-dependent GOGAT specific activities between the three species studied, and the differences in NADH-dependent specific activities between *Butyrivibrio fibrisolvens* 49 and *B. ruminicola* BA25, and between *Butyrivibrio fibrisolvens* 49 and *S. ruminantium* MP72 (but not between *B. ruminicola* BA25 and *S. ruminantium* MP72), are further highlighted in Table 3.17, in which the NADH/NADPH ratios for the three species are shown. The species have significantly different NADH/NADPH ratios ($P < 0.01$), however the ratio for *B. ruminicola* BA25 is the only one apparently influenced by the nitrogen source in the media (i.e. NFMS media with NH_4Cl compared to RGM with amino acids and/or peptides present : $P < 0.01$).

3.3.4.3 Glutamate Dehydrogenase Activity

***B. ruminicola* BA25 :** As was the case for GOGAT activities measured in cell-free extracts of *B. ruminicola* BA25, the NADPH-dependent GDH activities were much higher (about 100 times) than those for NADH (Figure 3.5 : $P < 0.01$), in contrast to experiments 1 and 2 (Chapter 2), in which the NADH-dependent activity was higher than that for NADPH. Similarly, the extremely low NADH-dependent GDH activities indicated that this

Figure 3.4 : NADPH- and NADH-dependent GOGAT specific activities in cell-free extracts of *B. ruminicola* BA25 grown in NFMS media supplemented with various concentrations of NH_4Cl, or in RGM.

Batch cultures of *B. ruminicola* BA25 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, as well as in RGM, and harvested at mid to late log phase of growth (Figure A2.1 (a), Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3. Cell pellets from these cultures were resuspended in GOGAT/GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and passed twice through the French Pressure Cell (10,000 p.s.i.; 10°C) to disrupt the cells. The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored on ice for use on the same day, or at 4°C overnight. Section 3.2.2.3 has the full details of the methods used.

GOGAT assays are as described in Table 3.6.
The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

Results are the mean ± the standard error of the mean where "n" is shown in brackets ( ) for each value.

(# Note that the y-axis is split to accomodate the large standard error of the 0.75 mM value)

LEGEND :

- ■ NADPH-dependent specific activities
- □ NADH-dependent specific activities

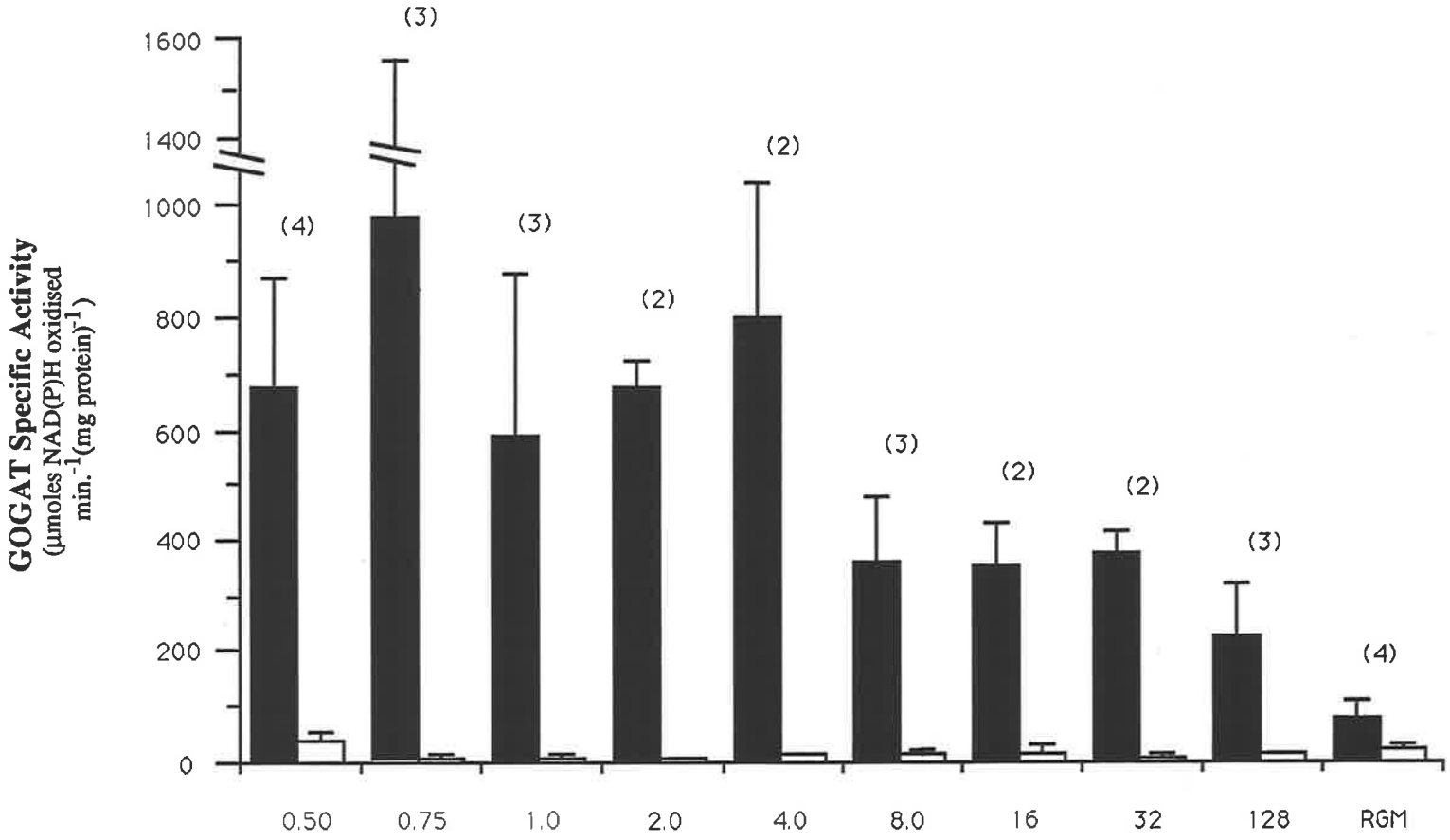

GOGAT Specific Activity
(μmoles NAD(P)H oxidised
min.$^{-1}$(mg protein)$^{-1}$)
1600
1400
1000
800
600
400
200
0
(4)
(3)
(3)
(2)
(2)
(3)
(2)
(2)
(3)
(4)
0.50
0.75
1.0
2.0
4.0
8.0
16
32
128
RGM
Media Type
(mM NH_4 Cl in NFMS media, or RGM)

Table 3.17 : Ratios of NADH-dependent activity to NADPH-dependent activity for GOGAT assayed in cell-free extracts of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 grown in NFMS media supplemented with various levels of NH_4Cl, or in RGM.

Batch cultures of *B. ruminicola* BA25 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, respectively, as well as in RGM. Batch cultures of *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 8.0 or 128 mM NH_4Cl, as well as in RGM. Cultures were harvested at mid to late log phase of growth (Figure A2.1, Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3, and cell pellets from these cultures were resuspended in GOGAT/GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and disrupted by passing the suspension twice through the French Pressure Cell (10,000 p.s.i.; 10°C). The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored overnight at 4°C. Section 3.2.2.3 has the full details of the methods used.

GOGAT assays are as described in Tables 3.6.
The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977) using Coomassie G250 dye, with BSA as a standard.

Results are the mean ± the standard error of the mean, where "n" is as shown in Figure 3.4 for *B. ruminicola* BA25, n = 3 for *S. ruminantium* MP72 except the value with the asterisk (*) which is from a single measurement, and n = 2 for *Butyrivibrio fibrisolvens* 49.

Media	NADH/NADPH Ratio		
	***B.ruminicola* BA25**	***S.ruminantium* MP72**	***Butyrivibrio fibrisolvens* 49**
0.5 mM NFMS	0.048 ± 0.0224	0.569 ± 0.0984	1.429 ± 0.2542
0.75 mM NFMS	0.013 ± 0.0026	-	-
1.0 mM NFMS	0.022 ± 0.0075	-	-
2.0 mM NFMS	0.014 ± 0.0020	-	-
4.0 mM NFMS	0.021 ± 0.0070	-	-
8.0 mM NFMS	0.060 ± 0.0158	0.561 ± 0.0938	1.865 ± 0.6924
16 mM NFMS	0.048 ± 0.0245	-	-
32 mM NFMS	0.028 ± 0.0145	-	-
128 mM NFMS	0.069 ± 0.0121	0.169 *	1.577 ± 0.2400
NFMS Mean	*0.036 ± 0.0070*	-	-
RGM	0.350 ± 0.1848	0.496 ± 0.0281	1.103 ± 0.2863

cofactor was not quantitatively important for GDH activity, hence a more appropriate scale was not used in Figure 3.5. No statistical effect of media ammonia concentration was detected by analysis of variance for either the NADH- or the NADPH-dependent GDH activities of *B. ruminicola* BA25 ($P = 0.88$ & 0.47, respectively), however significant differences between the activity for NFMS grown cells overall (i.e. NH_4Cl as the sole nitrogen source) and RGM grown cells were evident for both cofactors ($P < 0.05$). Similar differences between RGM grown cells and cells grown in each NFMS media were not evident for NADH-dependent activities, but were found for NADPH-dependent activities measured in cells harvested from batch cultures of NFMS media supplemented with 0.5, 0.75, 2.0, 4.0, 8.0, 16 and 32 mM NH_4Cl.

The NADH/NADPH ratio values in Table 3.18 also suggest that a difference exists between GDH activity measured from cells with ammonia as the nitrogen source (NFMS media supplemented with NH_4Cl) and cells grown with amino acids and/or peptides in addition to ammonia (i.e. RGM), since this ratio changed from a mean value of 0.015 for NFMS grown cells to 0.152 for RGM grown cells. As was the case for GOGAT activity, this highly significant difference ($P < 0.01$) was mainly due to a large decrease in NADPH-dependent activity, although the difference was also influenced by a small increase in NADH-dependent activity in RGM grown cells above the levels in NFMS grown cells. Individual comparisons of this ratio between each NFMS medium (i.e.supplemented with each level of NH_4Cl), and RGM were only significant between the NFMS medium supplemented with 0.5 mM NH_4Cl and RGM, due to large variations in ratios between days.

***S. ruminantium* MP72 :** The NADPH-dependent activities measured for *S. ruminantium* MP72, and presented in Figure 3.7 (b), were between one-fifth and one-tenth of the levels measured for *B. ruminicola* BA25 ($P < 0.05$), whereas the NADH-dependent activities were higher than those measured for *B. ruminicola* BA25 ($P < 0.01$). This figure also highlights a significant difference between the activities for each cofactor within *S. ruminantium* MP72. No effects of media ammonia concentration or nitrogen source were detected for either the NADPH- or the NADH-dependent activities ($P > 0.05$), and Table 3.18 shows that, unlike *B. ruminicola* BA25, the NADH/NADPH ratio for GDH activities in *S. ruminantium* MP72 did not change due to the level or source of nitrogen provided for growth of the bacteria ($P > 0.05$).

***Butyrivibrio fibrisolvens* 49 :** The NADPH- and NADH-dependent GDH activities measured in cell-free extracts of *Butyrivibrio fibrisolvens* 49 (Figure 3.8 (b)) were also significantly lower than for *B. ruminicola* BA25 ($P < 0.01$), but were not significantly different to the levels in *S. ruminantium* MP72. In *Butyrivibrio fibrisolvens* 49, the NADPH-dependent GDH activity was higher than the NADH-dependent activity, and no effects of media ammonia concentration or nitrogen source were detected ($P > 0.05$).

NADH/NADPH Ratio : As for *S. ruminantium* MP72, the NADH/NADPH ratio for GDH activity in *Butyrivibrio fibrisolvens* 49 did not change with a change in the nitrogen source provided in the media ($P > 0.05$). In fact, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 had similar ratio values, which were significantly different to the values for *B. ruminicola* BA25. The GDH activity of the latter was the only one of these activities apparently influenced by the nitrogen source in the growth medium.

3.3.4.4 Alanine Dehydrogenase Activity

***B. ruminicola* BA25 :** As was the case for GOGAT and GDH, both NADPH- and NADH-dependent activities were detected for AlaDH in cell-free extracts of *B. ruminicola* BA25 (Figure 3.6), however the magnitude of these activities was much lower than the GOGAT and GDH activities. The NADPH-dependent activities for AlaDH were up to 10 times greater than the NADH-dependent activities ($P < 0.01$), in contrast to experiments 1 and 2 (Chapter 2) in which the NADH-dependent activities of this enzyme(s) were higher in mixed rumen bacterial samples. Significant effects of media ammonia concentration and nitrogen source in the media were detected for the NADPH-dependent AlaDH activities by analysis of variance ($P = 0.001$), however Student's T-tests revealed that NADPH-dependent activities in RGM grown cells were only significantly different to activities of cells grown in NFMS media supplemented with 2 or 4 mM NH_4Cl. Student's T-test comparisons of the activities from cells grown in NFMS media supplemented with various levels of NH_4Cl showed that differences in NADPH-dependent AlaDH activity were significant between the following NFMS media : 0.5 and 2 mM, 4 & 8 mM; 1 and 4 mM; 2 and 128 mM; 4 and 8, 16, 32 & 128 mM; 8 and 16 & 128 mM; 32 and 128 mM. No effects of media ammonia concentration or nitrogen source were detected for NADH-dependent AlaDH activities ($P > 0.05$), and similarly, analysis of variance

Figure 3.5 : NADPH- and NADH-dependent GDH specific activities in cell-free extracts of *B. ruminicola* BA25 grown in NFMS media supplemented with various concentrations of NH_4Cl, or in RGM.

Batch cultures of *B. ruminicola* BA25 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, as well as in RGM, and harvested at mid to late log phase of growth (Figure A2.1 (a), Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3. Cell pellets from these cultures were resuspended in GOGAT/GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and passed twice through the French Pressure Cell (10,000 p.s.i.; 10°C) to disrupt the cells. The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored on ice for use on the same day, or at 4°C overnight. Section 3.2.2.3 has the full details of the methods used.

GDH assays are as described in Table 3.7.
The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

Results are the mean ± the standard error of the mean where "n" is shown in brackets ( ) for each value.

LEGEND :

- ■ NADPH-dependent specific activities
- □ NADH-dependent specific activities

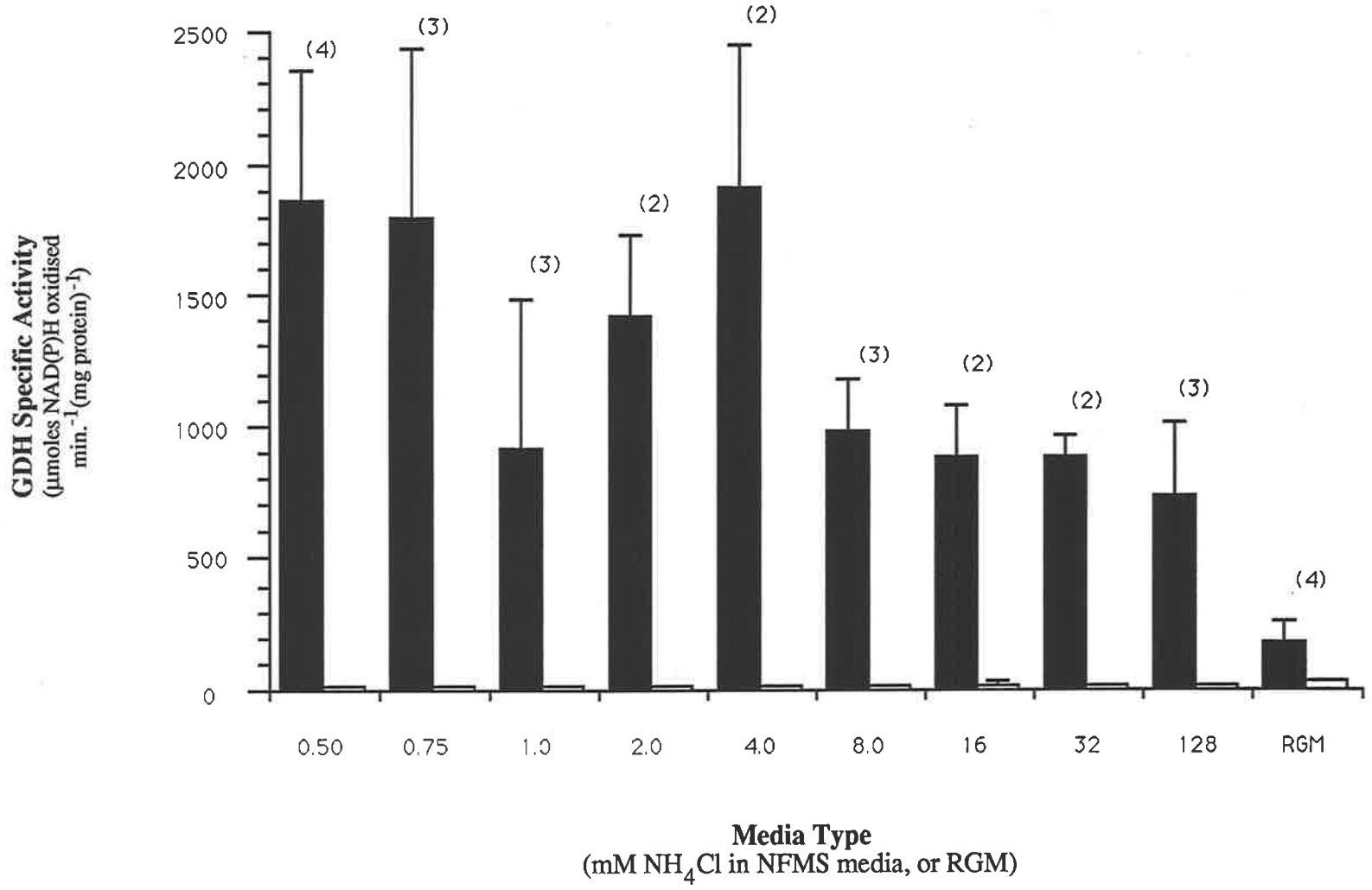

GDH Specific Activity
(μmoles NAD(P)H oxidised min.$^{-1}$(mg protein)$^{-1}$)
2500
2000
1500
1000
500
0
(4)
(3)
(3)
(2)
(2)
(3)
(2)
(2)
(3)
(4)
0.50
0.75
1.0
2.0
4.0
8.0
16
32
128
RGM
Media Type
(mM NH_4Cl in NFMS media, or RGM)

Table 3.18 : Ratios of NADH-dependent activity to NADPH-dependent activity for GDH assayed in cell-free extracts of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 grown in NFMS media supplemented with various levels of NH_4Cl, or in RGM.

Batch cultures of *B. ruminicola* BA25 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, respectively, as well as in RGM. Batch cultures of *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 8.0 or 128 mM NH_4Cl, as well as in RGM. Cultures were harvested at mid to late log phase of growth (Figure A2.1, Appendix 2) of two parallel "Hungate" tube cultures (10 ml) as outlined in section 3.2.2.3, and cell pellets from these cultures were resuspended in GOGAT/GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and disrupted by passing the suspension twice through the French Pressure Cell (10,000 p.s.i.; 10°C). The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored overnight at 4°C. Section 3.2.2.3 has the full details of the methods used.

GDH assays are as described in Tables 3.7.
The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

Results are the mean ± the standard error of the mean, where "n" is as shown in Figure 3.5 for *B. ruminicola* BA25, n = 3 for *S. ruminantium* MP72 except the value with the asterisk (*) which is from a single measurement, and n = 2 for *Butyrivibrio fibrisolvens* 49.

Media	NADH/NADPH Ratio		
	***B. ruminicola* BA25**	***S. ruminantium* MP72**	***Butyrivibrio fibrisolvens* 49**
0.5 mM NFMS	0.006 ± 0.0015	0.248 ± 0.0535	0.224 ± 0.0390
0.75 mM NFMS	0.006 ± 0.0009	-	-
1.0 mM NFMS	0.046 ± 0.0371	-	-
2.0 mM NFMS	0.011 ± 0.0026	-	-
4.0 mM NFMS	0.008 ± 0.0014	-	-
8.0 mM NFMS	0.015 ± 0.0018	0.209 ± 0.0054	0.294 ± 0.1908
16 mM NFMS	0.017 ± 0.0073	-	-
32 mM NFMS	0.010 ± 0.0012	-	-
128 mM NFMS	0.015 ± 0.0030	0.188 *	0.197 ± 0.0922
NFMS Mean	*0.015 ± 0.0041*	-	-
RGM	0.152 ± 0.0764	0.357 ± 0.1487	0.188 ± 0.0434

did not detect significant differences for the NADH/NADPH ratios of this enzyme(s) (Table 3.19). Nevertheless, Student's T-tests did show that the ratio for NFMS grown cells (i.e. combined values) was lower than the ratio for RGM grown cells ($P < 0.05$), but the ratio for individual NFMS media was not significantly lower than that for RGM grown cells, due to the large variation between measurements. An explanation for this large variation in NADH/NADPH ratios is difficult to supply, but may relate to inaccuracies in the assays at such low activity values. Some comparisons between individual NFMS media were significant, however they will not be detailed for reasons discussed later.

***S. ruminantium* MP72 :** The NADPH- and NADH-dependent activities of AlaDH for *S. ruminantium* MP72 were similar (Figure 3.7 (c) : $P > 0.01$), unlike the GOGAT and GDH activities for this species. The NADPH-dependent activity was, in fact, not significantly different to the same activity for *B. ruminicola* BA25 ($P > 0.05$), but the NADH-dependent activities were up to four times higher than for *B. ruminicola* BA25 ($P < 0.01$). No effects of media ammonia concentration or nitrogen source were detected for either the NADPH- or the NADH-dependent activities ($P > 0.05$), as found for GDH, and the NADH/ NADPH ratios in Table 3.19 were variable.

***Butyrivibrio fibrisolvens* 49 :** NADPH-dependent AlaDH activities measured in cell-free extracts of *Butyrivibrio fibrisolvens* 49 (Figure 3.8 (c)) were significantly lower than those for *S. ruminantium* MP72 ($P < 0.01$), but were not lower than those for *B. ruminicola* BA25 ($P > 0.05$), due to large variation for this species. The NADH-dependent activities for *Butyrivibrio fibrisolvens* 49, however, were higher than for *B. ruminicola* BA25 ($P < 0.01$), and were lower than for *S. ruminantium* MP72 ($P < 0.01$). Nevertheless, the NADPH- and NADH-dependent activities for *Butyrivibrio fibrisolvens* 49 were not significantly different from each other ($P > 0.05$). As was found for *S. ruminantium* MP72, no effects of media ammonia concentration or nitrogen source were found for either the NADPH- or the NADH-dependent activities ($P > 0.05$). Although the NADH/NADPH ratios for this species appeared to vary markedly between different growth conditions, no statistical differences due to ammonia concentration in the media or the nitrogen source were evident ($P > 0.05$).

NADH/NADPH Ratio : The NADH/NADPH ratio for *Butyrivibrio fibrisolvens* 49 was significantly higher than the ratio measured for *B. ruminicola* BA25, but was not different to the ratio for *S. ruminantium* MP72.

Figure 3.6 : NADPH- and NADH-dependent AlaDH specific activities in cell-free extracts of *B. ruminicola* BA25 grown in NFMS media supplemented with various concentrations of NH_4Cl, or in RGM.

Batch cultures of *B. ruminicola* BA25 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, as well as in RGM, and harvested at mid to late log phase of growth (Figure A2.1 (a), Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3. Cell pellets from these cultures were resuspended in GOGAT/GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and passed twice through the French Pressure Cell (10,000 p.s.i.; 10°C) to disrupt the cells. The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored on ice for use on the same day, or at 4°C overnight. Section 3.2.2.3 has the full details of the methods used.

AlaDH assays are as described in Table 3.8.
The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

Results are the mean ± the standard error of the mean where "n" is shown in brackets ( ) for each value.

LEGEND :

- ■ NADPH-dependent specific activities
- □ NADH-dependent specific activities

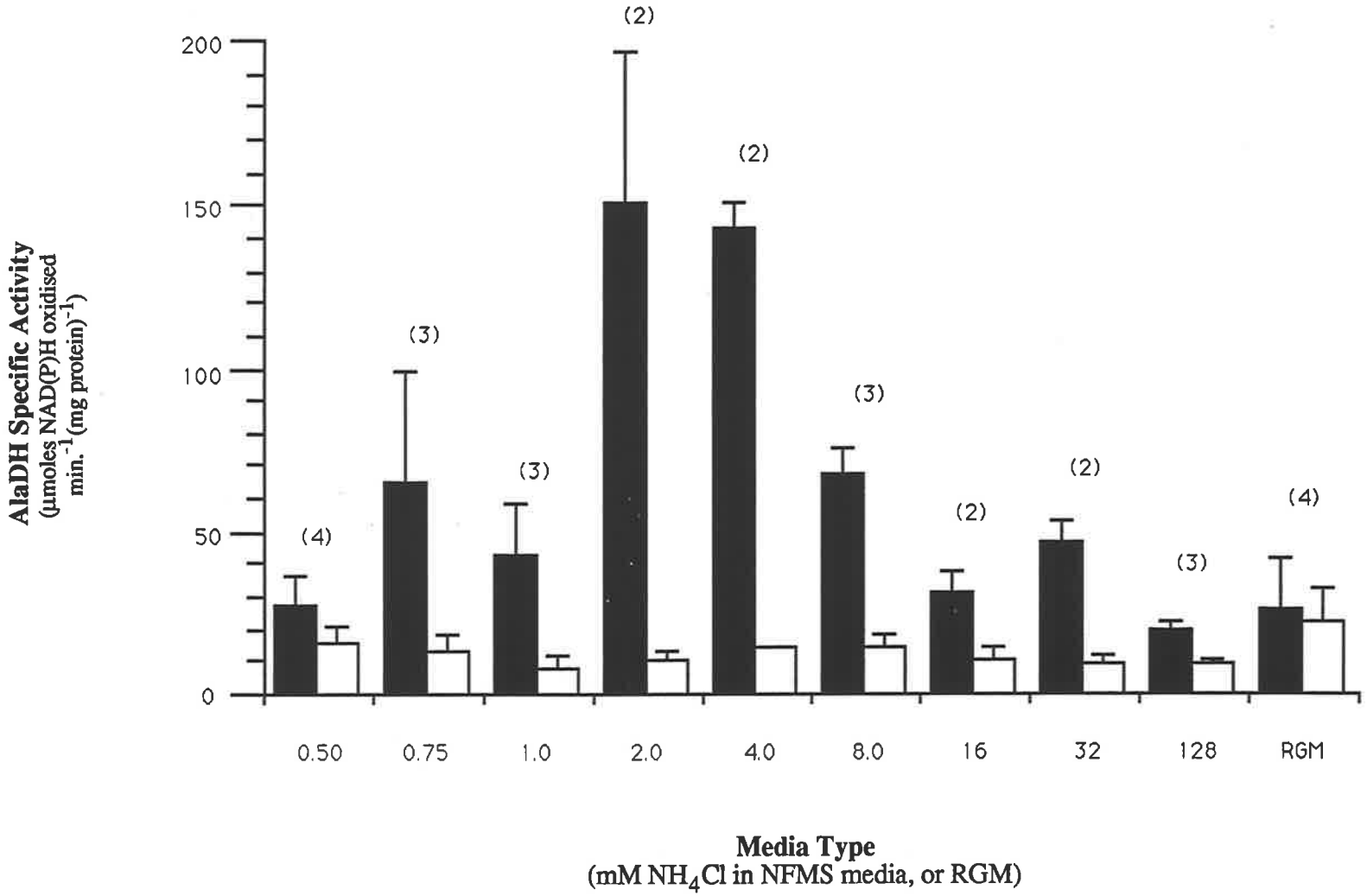

AlaDH Specific Activity
(μmoles NAD(P)H oxidised
min.$^{-1}$(mg protein)$^{-1}$)
200
150
100
50
0
(4)
(3)
(3)
(2)
(2)
(3)
(2)
(2)
(3)
(4)
0.50
0.75
1.0
2.0
4.0
8.0
16
32
128
RGM
Media Type
(mM NH_4Cl in NFMS media, or RGM)

Table 3.19 : Ratios of NADH-dependent activity to NADPH-dependent activity for AlaDH assayed in cell-free extracts of *B. ruminicola* BA25, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 grown in NFMS media supplemented with various levels of NH_4Cl, or in RGM.

Batch cultures of *B. ruminicola* BA25 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 0.75, 1.0, 2.0, 4.0, 8.0, 16, 32 and 128 mM NH_4Cl, respectively, as well as in RGM. Batch cultures of *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 8.0 or 128 mM NH_4Cl, as well as in RGM. Cultures were harvested at mid to late log phase of growth (Figure A2.1, Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3, and cell pellets from these cultures were resuspended in GOGAT/GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and disrupted by passing the suspension twice through the French Pressure Cell (10,000 p.s.i.; 10°C). The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored overnight at 4°C. Section 3.2.2.3 has the full details of the methods used.

AlaDH assays are as described in Tables 3.8.
The level of free protein in cell free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

Results are the mean ± the standard error of the mean, where "n" is as shown in Figure 3.6 for *B. ruminicola* BA25, n = 3 for *S. ruminantium* MP72 except the value with the asterisk (*) which is from a single measurement, and n = 2 for *Butyrivibrio fibrisolvens* 49.

Media	NADH/NADPH Ratio		
	***B. ruminicola* BA25**	***S. ruminantium* MP72**	***Butyrivibrio fibrisolvens* 49**
0.5 mM NFMS	0.558 ± 0.1475	1.826 ± 0.3738	1.003 ± 0.8005
0.75 mM NFMS	0.311 ± 0.1211	-	-
1.0 mM NFMS	0.265 ± 0.1253	-	-
2.0 mM NFMS	0.071 ± 0.0076	-	-
4.0 mM NFMS	0.097 ± 0.0007	-	-
8.0 mM NFMS	0.202 ± 0.0284	0.565 ± 0.0810	1.525 ± 0.1502
16 mM NFMS	0.332 ± 0.0512	-	-
32 mM NFMS	0.187 ± 0.0442	-	-
128 mM NFMS	0.519 ± 0.1402	0.856 *	1.670 ± 0.6018
NFMS Mean	*0.282 ± 0.0265*	-	-
RGM	1.038 ± 0.6049	1.298 ± 0.5231	1.488 ± 0.5281

Figure 3.7 : Specific activity of GOGAT, GDH and AlaDH in cell-free extracts of *S. ruminantium* MP72 grown in NFMS media supplemented with 0.5, 8 or 128 mM NH_4Cl, or in RGM.

Batch cultures of *S. ruminantium* MP72 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 8.0 and 128 mM NH_4Cl, respectively, as well as in RGM, and harvested at mid to late log phase of growth (Figure A2.1 (b), Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3. Cell pellets from these cultures were resuspended in either GS or GOGAT/ GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and passed twice through the French Pressure Cell (10,000 p.s.i.; 10°C) to disrupt the cells. The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored on ice for use on the same day, or at 4°C overnight. Section 3.2.2.3 has the full details of the methods used.

GS (γ-GT)†, GOGAT, GDH and AlaDH assays are as described in Tables 3.5, 3.6, 3.7 & 3.8. The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

Results are the mean ± the standard error of the mean where n = 3, except for the values with an asterisk (*), where only one sample was measured.

a) NADPH- and NADH-dependent GOGAT specific activities. The y-axis is one-tenth of the scale in Figure 3.4.

b) NADPH- and NADH-dependent GDH specific activities. The y-axis is one-tenth of the scale in Figure 3.5.

c) NADPH- and NADH-dependent AlaDH specific activities. The y-axis is the same scale as in Figure 3.6.

† GS γ-GT activity was not detected in cell-free extracts from this species.

LEGEND :

- ■ NADPH-dependent specific activities
- □ NADH-dependent specific activities

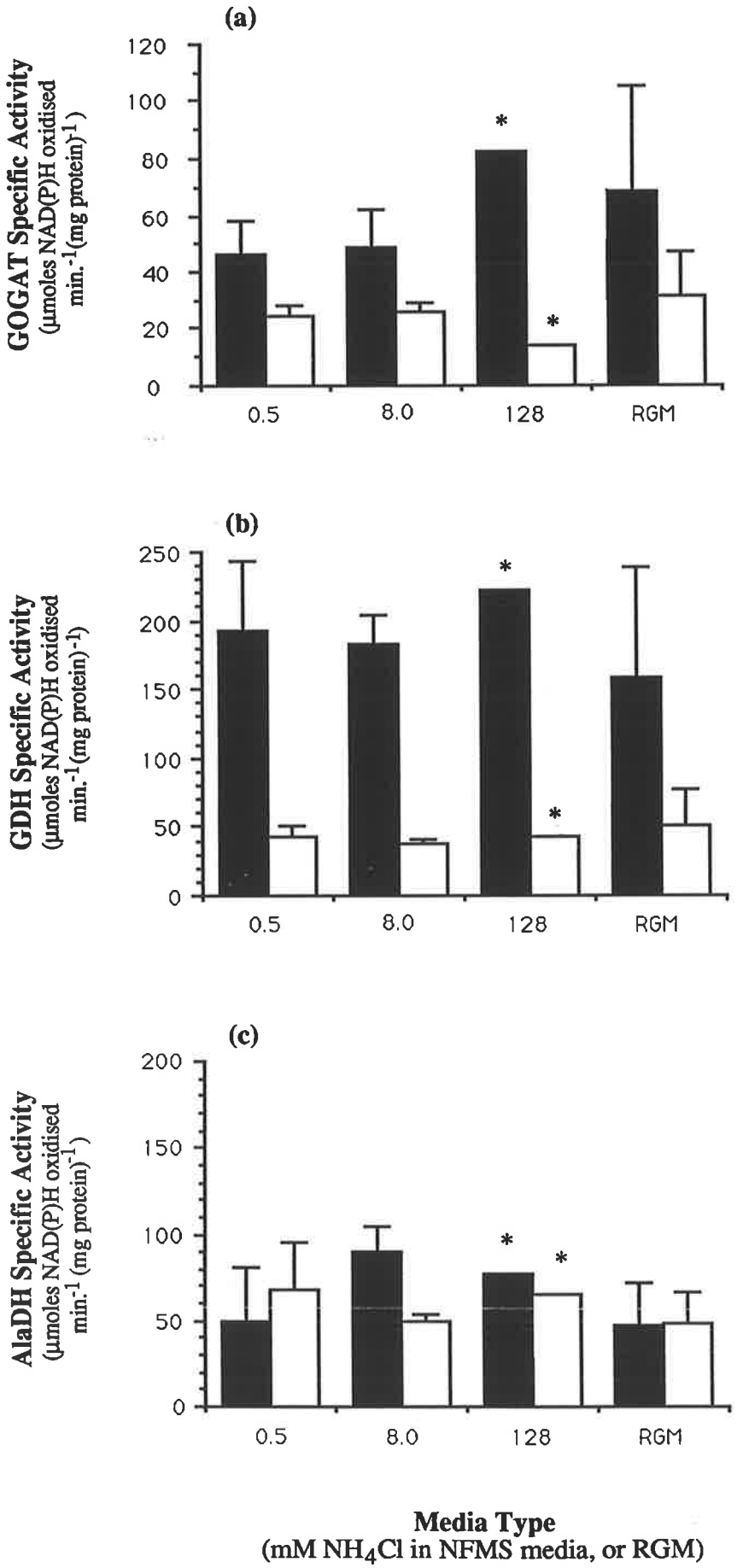

(a)
GOGAT Specific Activity
(μmoles NAD(P)H oxidised min.-1(mg protein)-1)
120
100
80
60
40
20
0
*
*
0.5
8.0
128
RGM
(b)
GDH Specific Activity
(μmoles NAD(P)H oxidised min.-1(mg protein)-1)
250
200
150
100
50
0
*
*
0.5
8.0
128
RGM
(c)
AlaDH Specific Activity
(μmoles NAD(P)H oxidised min.-1 (mg protein)-1)
200
150
100
50
0
*
*
0.5
8.0
128
RGM
Media Type
(mM NH4Cl in NFMS media, or RGM)

Figure 3.8 : Specific activity of GOGAT, GDH and AlaDH in cell free extracts of *Butyrivibrio fibrisolvens* 49 grown in NFMS media supplemented with 0.5, 8 or 128 mM NH_4Cl, or in RGM.

Batch cultures of *Butyrivibrio fibrisolvens* 49 (40 and 120 ml) were grown in NFMS media supplemented with 0.5, 8.0, and 128 mM NH_4Cl, respectively, as well as in RGM, and harvested at mid to late log phase of growth (Figure A2.1 (e), Appendix 2) of two parallel "Hungate" tube cultures (10 ml), as outlined in section 3.2.2.3. Cell pellets from these cultures were resuspended in either GS or GOGAT/GDH/AlaDH "extraction" buffer (see section 3.2.2.3) and passed twice through the French Pressure Cell (10,000 p.s.i.; 10°C) to disrupt the cells. The resulting suspensions were centrifuged to remove cell debris and the supernatants (cell-free extracts) were stored on ice for use on the same day, or at 4°C overnight. Section 3.2.2.3 has the full details of the methods used.

GS (γ-GT)†, GOGAT, GDH and AlaDH assays are as described in Tables 3.5, 3.6, 3.7 & 3.8. The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

Results are the mean ± the standard error of the mean where n = 2.

a) NADPH- and NADH-dependent GOGAT specific activities. The y-axis is the one-tenth of the scale in Figure 3.4.

b) NADPH- and NADH-dependent GDH specific activities. The y-axis is one-fifth of the scale in Figure 3.5.

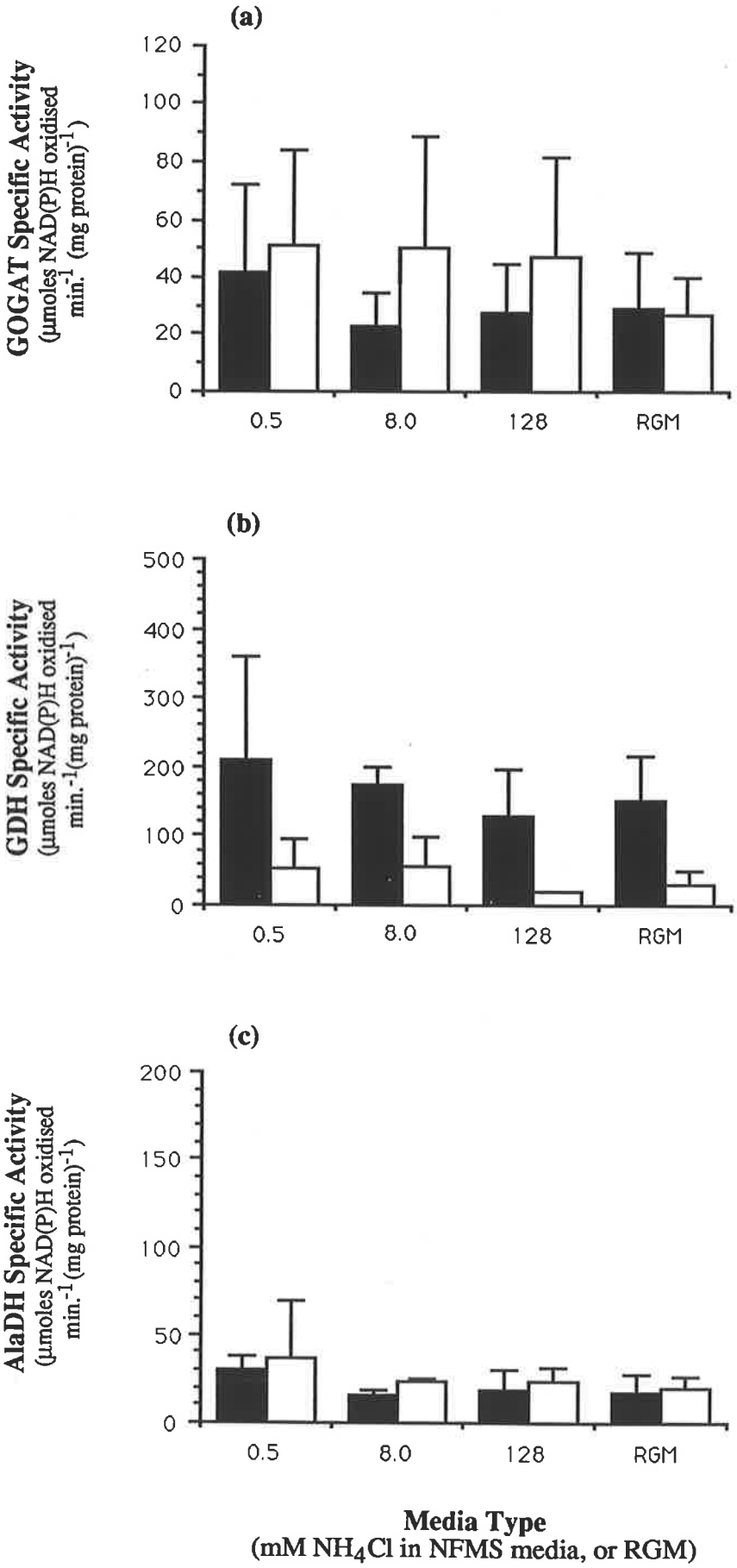
c) NADPH- and NADH-dependent AlaDH specific activities. The y-axis is the same scale as in Figure 3.6.

† GS γ-GT activity was not detected in cell-free extracts from this species.

LEGEND :

■ NADPH-dependent specific activities

□ NADH-dependent specific activities


(a)
GOGAT Specific Activity
(μmoles NAD(P)H oxidised
min.-1 (mg protein)-1)
120
100
80
60
40
20
0
0.5
8.0
128
RGM
(b)
GDH Specific Activity
(μmoles NAD(P)H oxidised
min.-1(mg protein)-1)
500
400
300
200
100
0
0.5
8.0
128
RGM
(c)
AlaDH Specific Activity
(μmoles NAD(P)H oxidised
min.-1(mg protein)-1)
200
150
100
50
0
0.5
8.0
128
RGM
Media Type
(mM NH4Cl in NFMS media, or RGM)

3.3.5 EFFECTS OF THE STAGE OF CULTURE GROWTH AT HARVEST ON GS γ-GT ACTIVITY (Experiment 3.3)

3.3.5.1 Stage of Culture Growth Experiment

In the previous experiment (3.2) the GS activities measured in cell-free extracts varied markedly from day-to-day for *B. ruminicola* BA25, making it very difficult to accurately assess the effects of the ammonia concentration in the media, or the nitrogen source available in the media, on these activities. In experiment 3.2, the optical densities of 40 and 120 ml serum bottle cultures were followed by measuring concurrent 10 ml "Hungate" tube cultures. Although these optical density measurements were assumed to correspond to the growth also occurring in the serum bottle cultures, this may not have always been the case, hence some variation in the stage of growth at which cultures were harvested may have occurred. It is likely that the stage of growth at which cells are harvested affects the enzyme activities subsequently measured in cell-free extracts, consequently this may be a source of the variation in GS activity measured in experiment 3.2. The aim of experiment 3.3, therefore, was to measure the γ-GT activity of GS in cell-free extracts prepared from *B. ruminicola* BA25 cells harvested from batch cultures at early log, mid log, late log and stationary phases of culture growth to determine whether, in fact, the stage of growth at which cells are harvested affects the activity of GS (see Figure A2.1 (a), Appendix 2 for the designations of early log, mid log, late log and stationary phase for this species).

The first feature of the data presented for experiment 3.3 in Figure 3.9 (a), and more clearly shown in Figure 3.10, is the gradual and significant decrease in GS activity for *B. ruminicola* BA25 cells harvested at late log phase of culture growth as the initial concentration of NH_4Cl in the media was increased from 0.5 to 128 mM ($P < 0.01$). As mentioned in section 3.3.4.1, these data support the trend (non-significant) observed in experiment 3.2 for GS activity being inversely related to the media ammonia concentration. Similar significant decreases in GS activity are also evident for cells harvested at early log phase of culture growth ($P < 0.01$), with the exception of the 0.5 versus 2 mM comparison ($P > 0.05$), and for cells harvested at mid log phase of culture growth ($P < 0.05$). A significant decrease in GS activity was also found for cells harvested at stationary phase of culture growth ($P < 0.01$), with the exception of the 8 versus 128 mM comparison ($P > 0.05$). For each of the four harvest times,

cells from RGM grown cultures had significantly lower GS activities than those from NFMS media supplemented with 0.5, 2 and 8 mM NH_4Cl ($P < 0.01$), with the exception of cells harvested at early log phase of culture growth from NFMS media supplemented with 8 mM NH_4Cl ($P > 0.05$). The GS activities for cells harvested at early log, mid log or late log phase of culture growth in NFMS media supplemented with 128 mM NH_4Cl were not significantly higher than those harvested at the same time from RGM grown cultures ($P > 0.05$). There was, however, a significant difference between the GS activities of cells grown in these two media when harvested at stationary phase of culture growth ($P < 0.01$).

The second feature of the data in Figure 3.9 (a) is that there were no significant differences in GS activity between cells harvested at early log and mid log phase ($P > 0.05$) for the NFMS media supplemented with 0.5, 2, 8 and 128 mM NH_4Cl. In only NFMS media supplemented with 128 mM NH_4Cl was there a significant difference in GS activity between cells harvested at late log and stationary phase of culture growth ($P < 0.05$). In contrast, the GS activities in the early log/mid log sub-group were significantly lower than in the late log/ stationary phase sub-group for cells grown in NFMS media supplemented with 0.5 and 2 mM NH_4Cl, and there were less distinct differences between the sub-groups for cells grown in NFMS media supplemented with 8 and 128 mM NH_4Cl.

The levels of ammonia present in NFMS media supplemented with 0.5, 2 and 8 mM NH_4Cl prior to inoculation were significantly higher than those remaining at each of the four stages of culture growth ($P < 0.05$: three harvest times only for 0.5 mM cultures), and significant differences were also evident between many of these stages (Figure 3.9 (b & c)). Similarly, the ammonia concentrations in the media prior to inoculation, and those at mid log, late log and stationary phase of culture growth, were significantly different for NFMS media supplemented with 128 mM NH_4Cl, however, ammonia concentrations before inoculation and at early log phase, were not significantly different. The residual ammonia in media from cultures harvested at early log, mid log and late log phase of growth were not significantly different, which is a similar trend to that for the GS activities (Figure 3.9 (a)).

The concentrations of ammonia remaining in media harvested from cultures grown in NFMS media supplemented with 128 mM NH_4Cl were normally much higher than shown for this experiment (Experiment 3.3 : Figure 3.9 (b)). In fact, the levels were always above 80 mM in experiment 3.2 (section 3.3.4.1), whereas the concentration was as low as

Figure 3.9 : Effects of the stage of culture growth at harvest on GS γ-GT activity and media ammonia concentration for *B. ruminicola* BA25.

"Hungate" tube batch cultures (12 ml) of *B. ruminicola* BA25 were grown in NFMS media supplemented with 0.5, 2.0, 8.0, and 128 mM NH_4Cl, respectively, as well as in RGM, and culture growth was monitored by optical density measurements in these tubes at 600 nm at 30 min. intervals. Cultures were harvested at early log, mid log (except for 0.5 mM NFMS cultures), late log and stationary phases of culture growth (Figure A2.1 (a), Appendix 2) and the cell pellets from each harvest time processed into 2 ml of cell-free extract as outlined in section 3.2.2.3, which were stored at 4°C overnight. The clarified media from cell harvesting was retained for measurement of the ammonia concentration at harvest and the ammonia concentrations in the original media were also measured for each media. Section 3.2.2.4 has the full details of the methods used.

GS (γ-GT) assays are as described in Table 3.5. The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

GS activity values are the mean ± the standard error of the mean where n = 4 for 8 mM cultures harvested at early and mid log of culture growth, n = 5 for 8 mM cultures harvested at late log and stationary phase of cell growth and for 128 mM and RGM cultures, and n = 6 for 0.5 and 2.0 mM cultures .
Ammonia concentrations are the mean ± the standard error of the mean from triplicate measurements of each sample.

GS activity values and ammonia concentrations were compared using the Student's T-test. Different letters above the bars within each type of media indicate differences at the 5 % level of significance (see Figure 3.10 for between media comparisons).

* Mid log samples were not harvested for NFMS media supplemented with 0.5 mM NH_4Cl because cell growth under these conditions was not sufficient to enable four distinct growth phases.

a) GS γ-GT activities in cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5, 2.0, 8.0 and 128 mM NH_4Cl, and in RGM, harvested at early log, mid log, late log and stationary phases of culture growth.

b) Concentration of ammonia in NFMS media supplemented with 0.5, 2.0, 8.0 and 128 mM NH_4Cl, and in RGM, prior to inoculation with overnight cultures of *B. ruminicola* BA25 grown in NFMS media supplemented with 8 mM NH_4Cl, and remaining in the media when cells were harvested at early log, mid log, late log and stationary phases of culture growth.

c) Expanded view of ammonia concentrations in NFMS media supplemented with 0.5, 2.0 and 8.0 mM NH_4Cl prior to inoculation, and remaining in the media when cultures were harvested at early log, mid log, late log and stationary phases of culture growth.

LEGEND :

- Pre-inoculation
- Early log harvested cultures
- Mid log harvested cultures
- Late log harvested cultures
- Stationary Phase harvested cultures

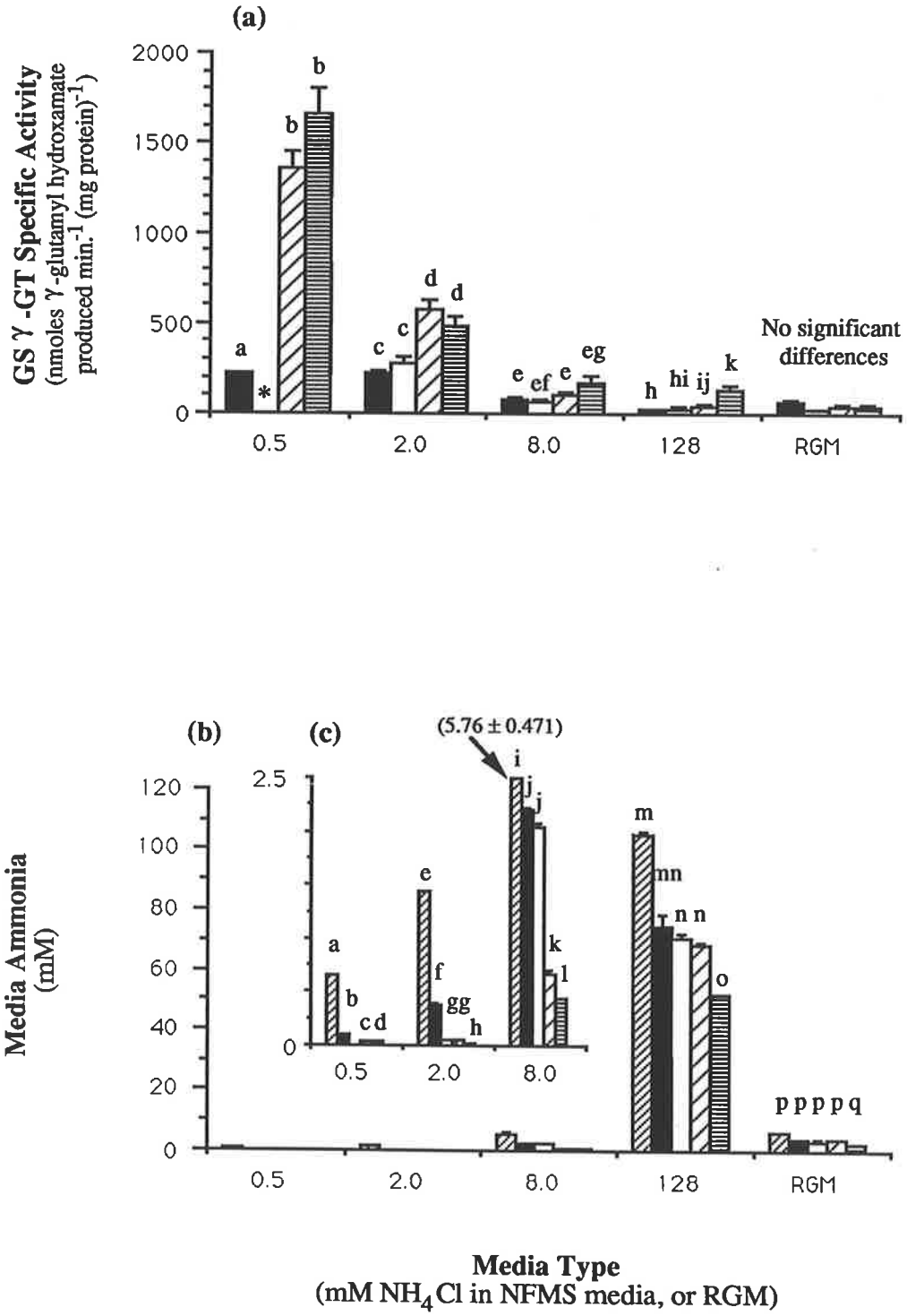

(a)
GS γ-GT Specific Activity
(nmoles γ-glutamyl hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$)
2000
1500
1000
500
0
No significant differences
0.5
2.0
8.0
128
RGM
(b)
(c)
(5.76 ± 0.471)
Media Ammonia
(mM)
120
100
80
60
40
20
0
2.5
0
0.5
2.0
8.0
0.5
2.0
8.0
128
RGM
Media Type
(mM NH_4Cl in NFMS media, or RGM)

Figure 3.10 : GS γ-GT activities in cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5, 2.0, 8.0 and 128 mM NH_4Cl, and in RGM, harvested at early log, mid log, late log and stationary phases of culture growth.

"Hungate" tube batch cultures (12 ml) of *B. ruminicola* BA25 were grown in NFMS media supplemented with 0.5, 2.0, 8.0, and 128 mM NH_4Cl, respectively, as well as in RGM, and culture growth was monitored by optical density measurements in these tubes at 600 nm at 30 min. intervals. Cultures were harvested at early log, mid log (except for 0.5 mM NFMS cultures), late log and stationary phases of culture growth (Figure A2.1 (a), Appendix 2) and the cell pellets from each harvest time processed into 2 ml of cell-free extract as outlined in section 3.2.2.3, which were stored at 4°C overnight. The clarified media from cell harvesting was retained for measurement of the ammonia concentration at harvest and the ammonia concentrations in the original media were also measured for each media. Section 3.2.2.4 has the full details of the methods used.

GS (γ-GT) assays were as described in Tables 3.5. The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

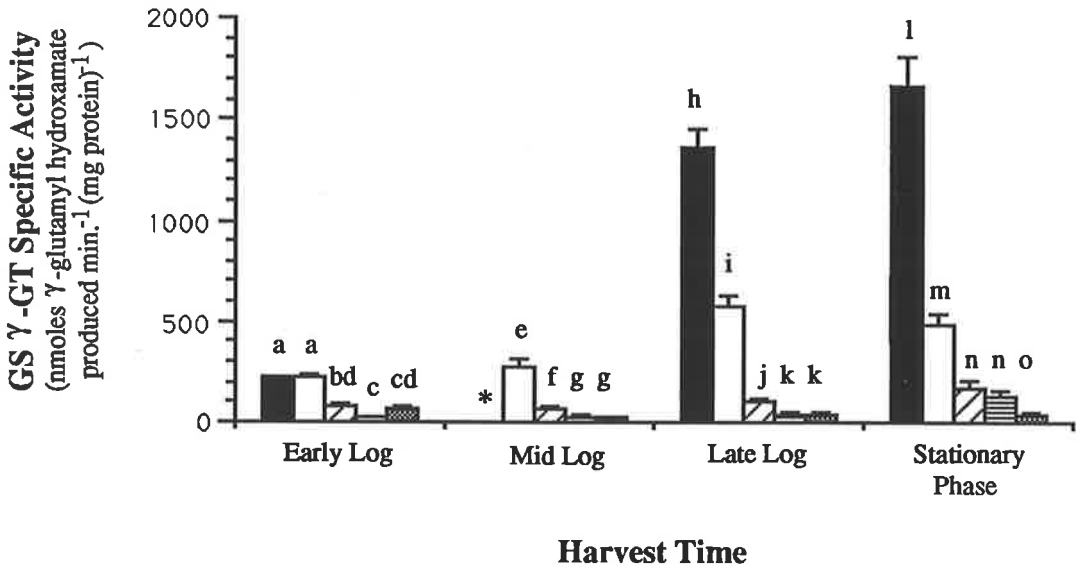
GS activity values are the mean ± the standard error of the mean where n = 4 for 8 mM cultures harvested at early and mid log of culture growth, n = 5 for 8 mM cultures harvested at late log and stationary phase of cell growth and for 128 mM and RGM cultures, and n = 6 for 0.5 and 2.0 mM cultures .

GS activity values between the media at each harvest time were compared using the Student's T-test. Different letters above the bars within each harvest time indicate differences at the 5 % level of significance (see Figure 3.9(a) for between harvest time comparisons).

* Mid log samples were not harvested for NFMS media supplemented with 0.5 mM NH_4Cl because cell growth under these conditions was not sufficient to enable four distinct growth phases.

LEGEND :

- ■ NFMS media supplemented with 0.5 mM NH_4Cl
- □ NFMS media supplemented with 2.0 mM NH_4Cl
- ◪ NFMS media supplemented with 8.0 mM NH_4Cl
- ▤ NFMS media supplemented with 128 mM NH_4Cl
- ▩ RGM


GS γ-GT Specific Activity
(nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
2000
1500
1000
500
0
a a
bd
c
cd
*
e
f g g
h
i
j k k
l
m
n n o
Early Log
Mid Log
Late Log
Stationary Phase
Harvest Time

51.5 mM in experiment 3.3. Significant differences in ammonia concentration for cultures grown in RGM were found between those harvested at stationary phase of growth and all other harvest times (including pre-inoculation levels) ($P < 0.05$).

3.3.5.2 Media Ammonia Concentration After Inoculation

An experiment was carried out to determine whether the level of ammonia in cultures used for inoculation affects subsequent GS activity in *B. ruminicola* BA25 cells harvested from experimental cultures (i.e. NFMS cultures). The data presented in Table 3.20 show that GS activities of cell-free extracts of this species grown in NFMS media supplemented with 0.5 and 8.0 mM NH_4Cl, respectively, and inoculated from an overnight culture of NFMS media supplemented with 4 mM NH_4Cl, were significantly lower than from those grown in the same media inoculated from an overnight culture of NFMS media supplemented with 8 mM NH_4Cl (61 & 40 %, respectively : $P < 0.01$). Similar differences between the 4 mM NH_4Cl inoculum data and that from experiment 3.2 also obtained from cultures inoculated from overnight cultures supplemented with 8 mM NH_4Cl were only evident for "8 mM NFMS" media due to the large standard error of the data from experiment 3.2. Thus, inoculation of NFMS media from an overnight culture in NFMS media supplemented with 4 mM, rather than 8 mM, NH_4Cl resulted in a significant decrease in GS activity in cells subsequently grown from this inoculum. The decrease appears to be of similar proportion for all media.

3.3.6 K_m-VALUES FOR SOME SUBSTRATES OF GS, GOGAT AND GDH IN *B. ruminicola* BA25 (Experiment 3.4)

The effects of various concentrations of substrates for the Mn^{++} dependent γ-GT activity of GS, the NADPH-dependent activity of GOGAT, and the NADPH- and NADH-dependent activities of GDH, were studied. The effects of hydroxylamine (NH_2OH) concentrations over a range of 0 to 120 mM on the γ-GT activity of GS are shown in Figure 3.11 (a & c) for *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 and 2.0 mM NH_4Cl, respectively. The enzyme activities increased with hydroxylamine concentrations up to about 60 mM for samples from each of the media ammonia concentrations.

Table 3.20 : GS γ-GT activities for *B. ruminicola* BA25 grown in media inoculated from overnight cultures grown in NFMS media supplemented with 4 or 8 mM NH_4Cl.

"Hungate" tube batch cultures (12 ml) of *B. ruminicola* BA25 were grown in NFMS media supplemented with 0.5, 4.0 and 8.0 mM NH_4Cl, respectively, and growth was monitored by optical density measurements in these tubes at 600 nm at 30 min. intervals. Cultures were harvested at late log phase of culture growth (Figure A2.1 (a), Appendix 2) and the cell pellets were processed into 2 ml of cell-free extract as outlined in section 3.2.2.3, which were stored at 4°C overnight. Section 3.2.2.4 (2) has the full details of the methods used.

GS (γ-GT) assays are as described in Tables 3.5. The level of free protein in cell-free extracts was determined by the method of Sedmak & Grossberg (1977), with BSA as a standard.

GS activity values are the mean ± the standard error of the mean where n = 6 for cultures inoculated from the overnight culture supplemented with 4 mM NH_4Cl and for the 0.5 mM NFMS culture inoculated from the overnight culture supplemented with 8 mM NH_4Cl. n = 5 for the 8 mM NFMS culture inoculated from the overnight culture supplemented with 8 mM NH_4Cl. n for experiment 3.2 data is as indicated in Figure 3.3.

GS activity values between each type of inoculum were compared using the Student's T-test. Different superscripts between each type of inoculum within media types indicate differences at the 5 % level of significance.

Media	GS Specific Activity†		
	Inoculated from a 4 mM overnight culture	Inoculated from an 8 mM overnight culture	
		Assayed concurrently with 4 mM inoculum samples	Data from Experiment 3.2
0.5 mM NFMS	831.4 ± 59.43 [a]	1364.2 ± 87.05 [b]	1008.0 ± 167.0 [ab]
4.0 mM NFMS	99.0 ± 16.07 [c]	-	546.4 ± 505.0 [c]
8.0 mM NFMS	41.1 ± 6.69 [d]	101.6 ± 15.90 [e]	118.8 ± 29.51 [e]

† Expressed as : nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$

The K_m-value for hydroxylamine from each culture was 4.5 mM, calculated from the double reciprocal plots in Figure 3.11 (b & d), as described in section 3.2.2.5.

The effects of various L-glutamine concentrations between 0 and 10 mM on the NADPH-dependent GOGAT activity of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl are illustrated in Figure 3.12 (a). The K_m-value for L-glutamine was 5.2 mM, calculated from the double reciprocal plot in Figure 3.12 (b), as described in section 3.2.2.5.

The effects of various concentrations of NH_4Cl between 0 and 200 mM on the NADPH- and the NADH-dependent GDH activities of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 and 2 mM NH_4Cl are shown in Figure 3.13 (a & c), respectively. The double reciprocal plots (Figures 3.13 (b & d)) of NH_4Cl concentration against oxidation of NAD(P)H gave K_m-values of 8.1 and 24.3 for the NADPH- and the NADH-dependent activities, respectively, although the NADPH-dependent activities may not follow Michaelis-Menten type kinetics.

3.3.7 NITROGEN SOURCE PREFERENCES FOR GROWTH BY *B. ruminicola* BA25 AND *S. ruminantium* MP72 (Experiment 4)

The preferred nitrogen sources for growth by *B. ruminicola* BA25 and *S. ruminantium* MP72 were studied by inoculating appropriate NFMS media supplemented with 0.05 % [w/v] or 0.5 % [w/v] glucose and 8 mM NH_4Cl, 1.5 % [w/v] Casamino Acids, 1.2 % [w/v] Tryptone, 1.0 % [w/v] casein, or combinations of these nitrogen sources (see Table 3.10). Overnight cultures grown in appropriate NFMS media supplemented with 0.5 % [w/v] glucose and 8 mM NH_4Cl were used as the inoculum.

3.3.7.1 *B. ruminicola* BA25

The data presented in Table 3.21, which highlight the growth characteristics of *B. ruminicola* BA25 at two glucose levels and with various nitrogen sources, are from only one of the three days on which growth measurements were made. Data are not presented for the other two days, since the trends are essentially the same as those in Table 3.21, although the absolute values of growth rate, change in optical density, lag time and time to stationary phase

Figure 3.11 : Effect of various concentrations of hydroxylamine on γ-GT activity of GS, and double reciprocal plots from these data for K_m determination, in *B. ruminicola* BA25 cells.

Batch cultures of *B. ruminicola* BA25 were harvested from 12 ml "Hungate" tube cultures of NFMS media supplemented with 0.5 or 2.0 mM NH_4Cl at mid to late log phase of culture growth, as determined by optical density measurements at 600 nm. Harvested cells were processed to produce cell-free extracts which were assayed under the standard conditions detailed in Table 3.5, except that the hydroxylamine concentration was varied over the range of 0 to 120 mM. Activity values were standardised by subtraction of the activity measured when 0 mM hydroxylamine was included in the assay mixture.

a) GS γ-GT activities at various concentrations of hydroxylamine for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. GS activity is expressed as the reaction velocity (V) = μmoles γ-glutamyl hydroxamate formed $min.^{-1}$.

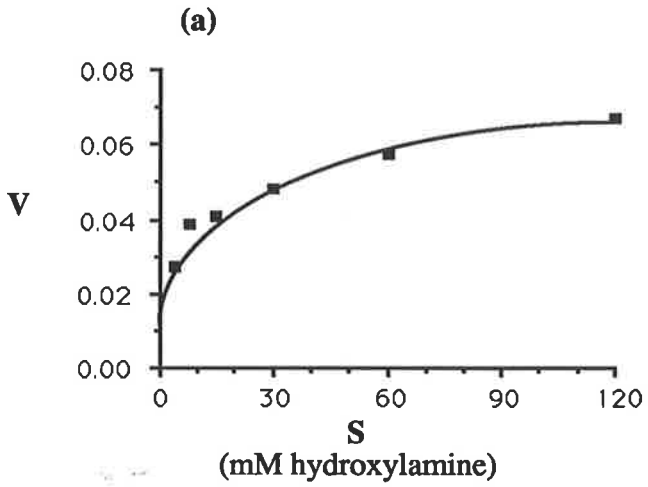
b) Lineweaver & Burk double reciprocal plot of GS reaction velocity ($\frac{1}{V}$) versus initial hydroxylamine concentration ($\frac{1}{S}$) for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. The K_m-value was determined from the regression equation :

$$\frac{1}{V} = 16.750 + 74.949 \left(\frac{1}{S}\right)$$

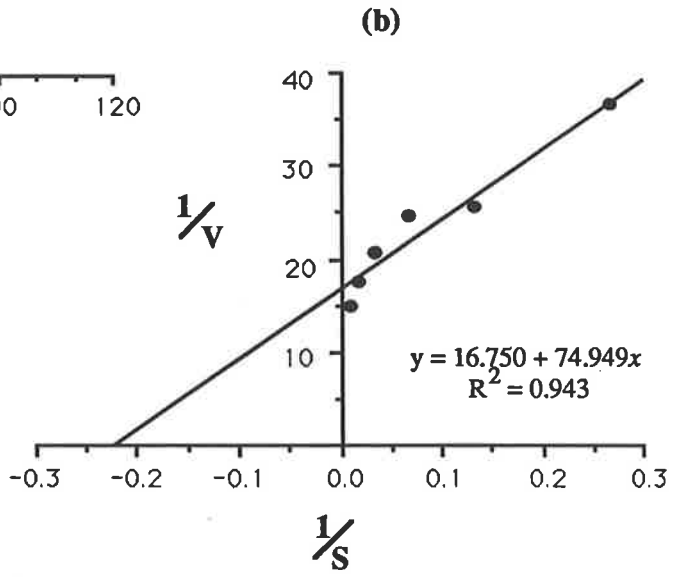
c) GS γ-GT activities at various concentrations of hydroxylamine for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 2.0 mM NH_4Cl. GS activity is expressed as the reaction velocity (V) = μmoles γ-glutamyl hydroxamate formed $min.^{-1}$.

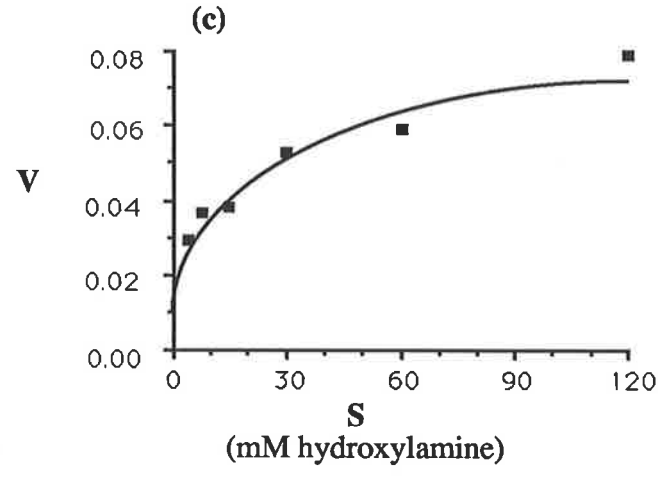
d) Lineweaver & Burk double reciprocal plot of GS reaction velocity ($\frac{1}{V}$) versus initial hydroxylamine concentration ($\frac{1}{S}$) for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 2.0 mM NH_4Cl. The K_m-value was determined from the regression equation :

$$\frac{1}{V} = 16.301 + 73.149 \left(\frac{1}{S}\right)$$



K_m = 4.5 mM
V_{max} = 0.06





K_m = 4.5 mM
V_{max} = 0.06

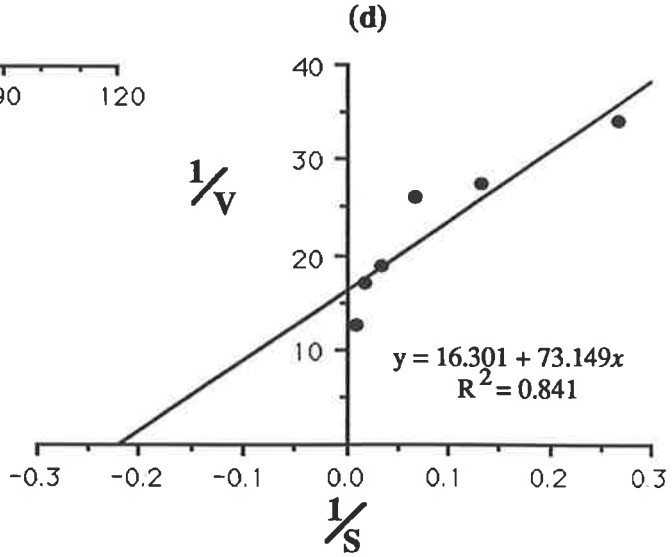


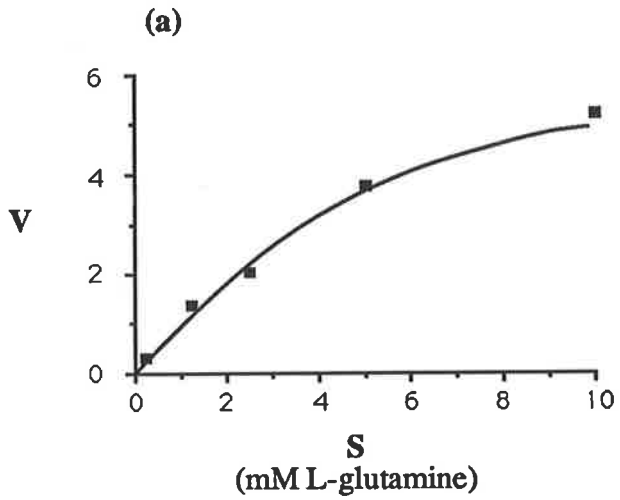
Figure 3.12 : Effect of various concentrations of L-glutamine on the NADPH-dependent activity of GOGAT, and a double reciprocal plot from these data for K_m determination, in *B. ruminicola* BA25 cells.

Batch cultures of *B. ruminicola* BA25 were harvested from 12 ml "Hungate" tube cultures of NFMS media supplemented with 0.5 mM NH_4Cl at mid to late log phase of culture growth, as determined by optical density measurements at 600 nm. Harvested cells were processed to produce cell-free extracts which were assayed under the standard conditions detailed in Table 3.6, except that the L-glutamine concentration was varied over the range of 0 to 10 mM. Activity values were standardised by subtraction of the activity measured when 0 mM L-glutamine was included in the assay mixture.

a) GOGAT NADPH-dependent activities at various concentrations of L-glutamine for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. GOGAT activity is expressed as the reaction velocity (V) = nmoles NADPH oxidised $min.^{-1}$.

b) Lineweaver & Burk double reciprocal plot of GOGAT reaction velocity ($\frac{1}{V}$) versus initial L-glutamine concentration ($\frac{1}{S}$) for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. The K_m-value was determined from the regression equation :

$$\frac{1}{V} = 0.14179 + 0.73928 \left(\frac{1}{S}\right)$$


(a)
V
6
4
2
0
0
2
4
6
8
10
S
(mM L-glutamine)

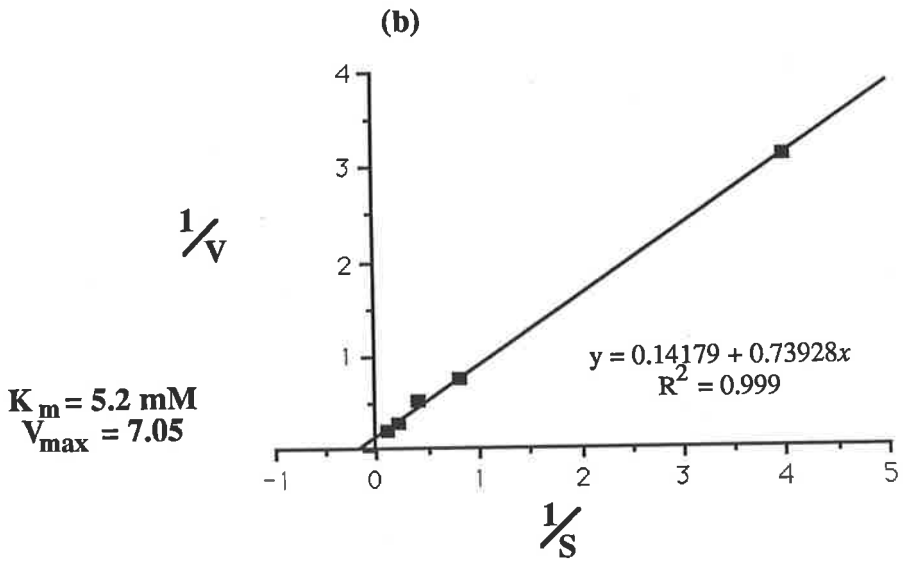

(b)
1/V
4
3
2
1
-1
0
1
2
3
4
5
1/S
y = 0.14179 + 0.73928x
R2 = 0.999
Km = 5.2 mM
Vmax = 7.05

Figure 3.13 : Effect of various concentrations of NH_4Cl on the NADPH- and the NADH-dependent activity of GDH, and double reciprocal plots from these data for K_m determination, in *B. ruminicola* BA25 cells.

Batch cultures of *B. ruminicola* BA25 were harvested from 12 ml "Hungate" tube cultures of NFMS media supplemented with 0.5 mM NH_4Cl at mid to late log phase of culture growth, as determined by optical density measurements at 600 nm. Harvested cells were processed to produce cell-free extracts which were assayed under the standard conditions detailed in Table 3.7, except that the NH_4Cl concentration was varied over the range of 0 to 200 mM. Activity values were standardised by subtraction of the activity measured when 0 mM NH_4Cl was included in the assay mixture.

a) NADPH-dependent GDH activities at various concentrations of NH_4Cl for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. GDH activity is expressed as the reaction velocity (V) = nmoles NADPH oxidised $min.^{-1}$.

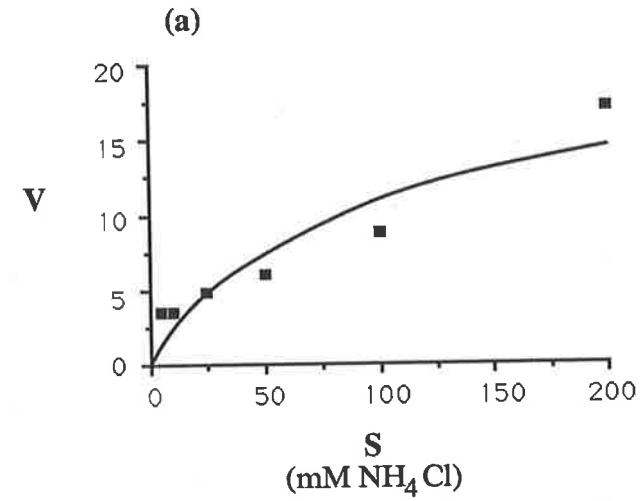
b) Lineweaver & Burk double reciprocal plot of NADPH-dependent GDH reaction velocity ($\frac{1}{V}$) versus initial NH_4Cl concentration ($\frac{1}{S}$) for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. The K_m-value was determined from the regression equation :

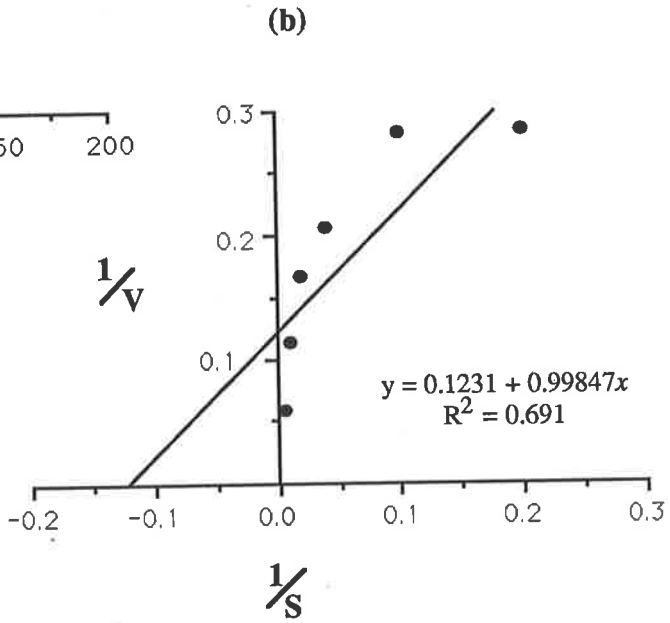
$$\frac{1}{V} = 0.1231 + 0.99847 \left(\frac{1}{S}\right)$$

c) NADH-dependent GDH activities at various concentrations of NH_4Cl for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. GDH activity is expressed as the reaction velocity (V) = nmoles NADH oxidised $min.^{-1}$.

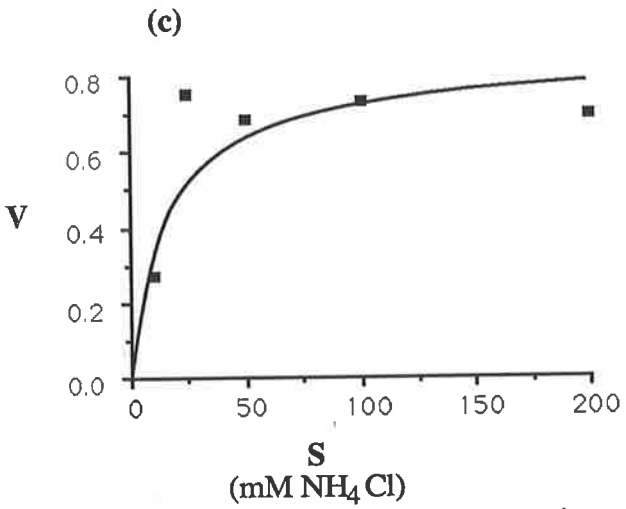
d) Lineweaver & Burk double reciprocal plot of NADH-dependent GDH reaction velocity ($\frac{1}{V}$) versus initial NH_4Cl concentration ($\frac{1}{S}$) for cell-free extracts of *B. ruminicola* BA25 cells grown in NFMS media supplemented with 0.5 mM NH_4Cl. The K_m-value was determined from the regression equation :

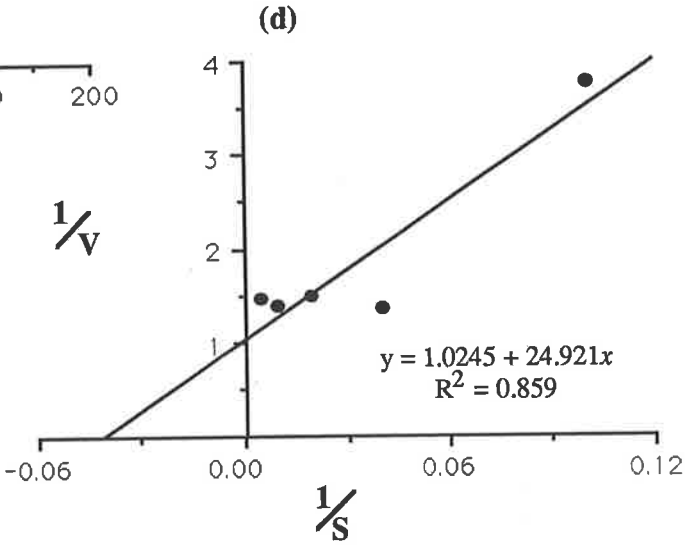
$$\frac{1}{V} = 1.0245 + 24.921 \left(\frac{1}{S}\right)$$





K_m = 8.1 mM
V_{max} = 8.12





K_m = 24.3 mM
V_{max} = 0.98

of growth were slightly different. In the case of one of these extra data "sets" the different absolute values were the result of measuring the optical densities of cultures using sub-samples of media from 130 ml serum bottle cultures, rather than using "Hungate" tube cultures whose optical densities could be measured directly in the tubes. Although the sub-sampling technique did not alter the growth of the cultures *per se*, it did result in inconsistent optical density values.

Low values were recorded for the change in optical density from inoculation to stationary phase of growth for *B. ruminicola* BA25 cultures supplemented with 0.05 % [w/v] glucose (Table 3.21). Similarly, only small (but often significant at the 5 % levels) amounts of ammonia were utilised or produced by these cultures, relative to the amounts for cultures supplemented with 0.5 % [w/v] glucose (Table 3.21), and Casamino Acids, Tryptone and casein levels (data presented in Appendix 2) were significantly different from pre-growth levels for only a few treatments (5 % level of significance). Two exceptions were cultures supplemented with NH_4Cl plus Casamino Acids and with NH_4Cl plus Casamino Acids plus Tryptone plus casein, for which the ammonia level of both more than doubled, possibly due to lysis of cells. Nevertheless, these data indicate that very poor culture growth resulted when only 0.05 % [w/v] glucose was provided in the media, hence it is apparent that *B. ruminicola* BA25 was not able to utilise any of these nitrogen sources as energy sources when the initial glucose supply in the media was exhausted.

In contrast, the large changes in optical density (> 1.5 units at 600 nm) measured for cultures supplemented with 0.5 % [w/v] glucose and one of the nitrogen sources (Table 3.21) indicates that this rumen bacterial species is able to effectively utilise each of the four nitrogen sources when adequate glucose is available. These data indicate that *B. ruminicola* BA25 achieves its maximum cell density (determined by the optical density at 600 nm) with Tryptone as the nitrogen source, followed in descending order of cell density by NH_4Cl, casein and Casamino Acids as the nitrogen source. The superior growth of *B. ruminicola* BA25 with Tryptone is further highlighted by these cultures having the highest maximum growth rates (Table 3.21), again followed in descending order of growth rate by NH_4Cl, casein and Casamino Acids. Similarly, the lag times and times from inoculation to stationary phase of culture growth (which is a function of both the lag time and the growth rate) show growth with Tryptone to have the shortest times for both parameters, followed by casein, NH_4Cl and Casamino Acids.

The values of these growth parameters for cultures in which combinations of nitrogen sources were available were intermediate between the values for each nitrogen source individually. For example, the change in optical density and maximum growth rate for cultures supplemented with NH_4Cl plus Casamino Acids, or NH_4Cl plus casein, were not as high as for NH_4Cl alone, but were higher than for Casamino Acids or casein alone. The exception to this phenomenon was for growth with NH_4Cl plus Tryptone, which achieved a higher cell density and higher maximum growth rate than when either nitrogen source was available individually.

Although maximum growth in NFMS media was therefore evident when Tryptone was at least one of the nitrogen sources, a shorter lag time and time from inoculation to stationary phase of culture growth was evident for RGM cultures (Table 3.21), however the maximum optical density of these cultures only reached about 1.59 units, probably due to a lack of glucose in this medium (only 0.2 % [w/v] glucose is initially present in RGM).

Traces of ammonia (added as NH_4Cl in some treatments), amino acids (as Casamino Acids), peptides (as Tryptone) and protein (as casein) were detected in media from all treatments due to impure stock products (e.g. Casamino Acids mainly contains free amino acids, but traces of peptides are also present), imprecise assays (especially the amino acids, peptides and protein assays), or a combination of both factors (Table 3.21 & Table A2.1, Appendix 2). When NH_4Cl, Casamino Acids, casein, or NH_4Cl plus casein, were the nitrogen sources in NFMS media supplemented with 0.5 % [w/v] glucose, a large and significant proportion (> 50 %) of the ammonia present (specifically added, or present as a contaminant) in the media was utilised by the bacteria ($P < 0.05$). When Tryptone, or Casamino Acids plus Tryptone, were the nitrogen sources, significant changes in the ammonia concentration between pre-inoculation and late log harvested samples were not evident ($P > 0.05$), however, for other combinations of nitrogen sources (i.e. NH_4Cl plus Casamino Acids, NH_4Cl plus Tryptone, NH_4Cl plus Casamino Acids plus Tryptone and NH_4Cl plus Casamino Acids plus Tryptone plus casein), large increases in the ammonia concentration were measured in the post-harvest media ($P < 0.05$).

Casamino Acids and casein levels were unaffected by growth of *B. ruminicola* BA25 in most treatments supplemented with 0.5 % [w/v] glucose ($P > 0.05$). Tryptone, in contrast, was the only nitrogen source which consistently and significantly decreased as a result

of growth for most treatments ($P < 0.05$), although, curiously, growth in media supplemented with Tryptone, and with casein, did not result in significant decreases in the level of Tryptone. Nevertheless, these data are only presented in Table A2.1 (Appendix 2), since only small proportions of the available amino acids (added as Casamino Acids), peptides (added as Tryptone) and protein (added as casein) were utilised for growth of *B. ruminicola* BA25, even when each was present as the predominant nitrogen source (i.e. when added singly). In contrast, more than 50 % of the available ammonia was utilised in some cultures, and significant quantities of ammonia were produced by others.

Growth of *B. ruminicola* BA25 in RGM resulted in a small, but significant, decrease in media ammonia concentration, no change in the level of Casamino Acids, a significant decrease in the level of Tryptone, and a decrease in the level of casein, which was initially very low (Table 3.21 and Table A2.1, Appendix 2 : 5 % level of significance). These cultures were second only in growth rate to those with Tryptone as the nitrogen source.

Since the small changes in the levels of Casamino Acids, Tryptone and casein measured for the first set of *B. ruminicola* BA25 cultures did not provide much information about the use of nitrogen sources by this species, these nitrogen sources were not measured for the other "sets" of *B. ruminicola* BA25 cultures, or for similar cultures of *S. ruminantium* MP72.

3.3.7.2 *S. ruminantium* MP72

Growth characteristics of *S. ruminantium* MP72 cultures with the glucose level and nitrogen source treatments listed in Table 3.10, as well as the ammonia concentrations in the media before growth and when cultures were harvested at late log, are shown in Table 3.22. This experiment was repeated once, however the duplicate data are not presented, since the growth characteristics were similar to those in Table 3.22, and the trends and differences between treatments were essentially the same for both sets of data.

As was the case for *B. ruminicola* BA25 cultures grown with 0.05 % [w/v] glucose, growth of *S. ruminantium* MP72 stopped early in mid log phase of culture growth for all of the nitrogen source treatments. This is shown in Table 3.22 by the relatively low values for the change in optical density from inoculation to stationary phase of culture growth. Similarly, smaller amounts of ammonia were generally utilised or produced by these cultures,

Table 3.21 : Growth parameters and ammonia levels for *B. ruminicola* BA25 cultures in NFMS media supplemented with 0.05% [w/v] or 0.5% [w/v] glucose and various nitrogen sources, individually and in combinations.

Duplicate "Hungate" tube cultures (12 ml) of *B. ruminicola* BA25, inoculated from an overnight culture in NFMS media supplemented with 8mM NH_4Cl, were grown in the appropriate NFMS medium (see Table 3.2) supplemented with one of the nitrogen source treatments listed in Table 3.10, and either 0.05 % [w/v] or 0.5 % [w/v] glucose. Growth was monitored at 30 or 60 min. intervals by optical density measurements directly in these tubes. Media ammonia concentrations were measured in samples which were not inoculated, as well as in sub-samples of media harvested at late log of culture growth in serum bottle cultures. See section 3.2.2.6 for full details.

Ammonia concentrations are the mean ± the standard error of the mean from triplicate measurements. Different superscripts within each glucose level indicate significant differences at the 5% level of significance (Student's T-tests were not performed between glucose levels or treatments).

Casamino Acids, Tryptone and casein levels measured in these samples are presented in Table A2.1 (Appendix 2).

† The NFMS medium (Table 3.2) was modified with the nitrogen source & glucose level listed.

£ Change in $O.D._{600\ nm}$ was calculated by subtracting the initial optical density (600 nm) of cultures from the optical density at stationary phase of culture growth.

§ Growth rate is expressed as the maximum rate of change of optical density of the batch cultures supplemented with 0.5 % glucose and was calculated from the sigmoidal growth plots.

¥ Lag time is the approximate time from inoculation of the batch culture to early log phase of culture growth. The value in brackets ( ) represents the approximate time from inoculation of the batch culture to stationary phase of culture growth.

TREATMENT †		Change in O.D.$_{600\ nm}$ £ (units)	Growth Rate § (units. hr $^{-1}$)	Lag Time ¥ (hr)	Ammonia Concentration	
Nitrogen Source	Glucose Conc^{n}				Sample	(mM)
NH_4Cl	0.05 %	0.19	-	-	pre late log	5.33 ± 0.027^{a} 3.95 ± 0.520^{b}
	0.5 %	1.94	0.70	5 (8)	pre late log	5.50 ± 0.188^{a} 0.60 ± 0.029^{b}
Casamino Acids	0.05 %	0.20	-	-	pre late log	4.10 ± 0.017^{a} 3.09 ± 0.018^{b}
	0.5 %	1.51	0.39	7 (12)	pre late log	4.21 ± 0.019^{a} 2.24 ± 0.061^{b}
Tryptone	0.05 %	0.38	-	-	pre late log	2.02 ± 0.019^{a} 2.70 ± 0.047^{b}
	0.5 %	2.13	1.25	4 (6)	pre late log	1.97 ± 0.007^{a} 1.90 ± 0.065^{a}
Casein	0.05 %	0.20	-	-	pre late log	0.90 ± 0.035^{a} 0.90 ± 0.016^{a}
	0.5 %	1.79	0.61	4.5 (8)	pre late log	0.71 ± 0.035^{a} 0.03 ± 0.001^{b}
NH_4Cl +	0.05 %	0.19	-	-	pre late log	4.80 ± 0.013^{a} 14.0 ± 0.15^{b}
Casamino Acids	0.5 %	1.58	0.45	4.5 (8)	pre late log	7.92 ± 0.112^{a} 11.3 ± 0.25^{b}
NH_4Cl +	0.05 %	0.47	-	-	pre late log	6.66 ± 0.278^{a} 8.75 ± 0.096^{b}
Tryptone	0.5 %	2.26	1.35	4 (6)	pre late log	5.85 ± 0.055^{a} 7.95 ± 0.309^{b}
NH_4Cl +	0.05 %	0.22	-	-	pre late log	5.21 ± 0.095^{a} 6.65 ± 0.031^{b}
Casein	0.5 %	1.85	0.95	4.5 (6)	pre late log	5.59 ± 0.139^{a} 0.01 ± 0.001^{b}
Casamino Acids +	0.05 %	0.40	-	-	pre late log	5.31 ± 0.019^{a} 6.05 ± 0.060^{b}
Tryptone	0.5 %	2.04	1.10	4 (6)	pre late log	4.96 ± 0.052^{a} 5.12 ± 0.232^{a}
NH_4Cl + Casamino	0.05 %	0.39	-	-	pre late log	8.68 ± 0.187^{a} 9.36 ± 0.102^{a}
Acids+ Tryptone	0.5 %	2.15	1.10	3.5 (5)	pre late log	8.51 ± 0.257^{a} 13.9 ± 0.03^{b}
NH_4Cl + CasAcids	0.05 %	0.22	-	-	pre late log	8.43 ± 0.209^{a} 18.3 ± 0.02^{b}
+ Tryptone + Casein	0.5 %	1.60	0.95	4.5 (6)	pre late log	9.50 ± 0.305^{a} 11.6 ± 0.06^{b}
RGM	0.2 %	1.59	1.01	1.5 (4)	pre late log	5.28 ± 0.499^{a} 4.78 ± 0.028^{a}

relative to the amounts for cultures supplemented with 0.5 % [w/v] glucose. Thus, these data indicate that *S. ruminantium* MP72, like *B. ruminicola* BA25, is also apparently unable to utilise any of the nitrogen sources as energy sources when the initial glucose supply in the media was exhausted.

Much larger changes in optical density (> 1.1 units at 600 nm) were measured for *S. ruminantium* MP72 cultures supplemented with 0.5 % [w/v] glucose and NH_4Cl, Tryptone or casein, indicating that this species is able to effectively utilise these nitrogen sources for growth when adequate energy was available. Casamino Acids, in contrast, did not induce effective growth of *S. ruminantium* MP72 when they were available as the sole nitrogen source, since the change in optical density for these cultures was only 0.40 units (cf. 0.37 units when 0.05 % [w/v] glucose was present) and their growth rate was only 0.03 units. hr^{-1} (cf. 0.29 with NH_4Cl and 0.60 with Tryptone). As occurred for *B. ruminicola* BA25 cultures, *S. ruminantium* MP72 cultures reached their highest cell density with Tryptone as nitrogen source, followed by NH_4Cl and casein. The superior growth of *S. ruminantium* MP72 with Tryptone is further highlighted by these cultures recording more than double the growth rate of NH_4Cl and casein supplemented cultures, as well as slightly shorter lag time and time from inoculation to stationary phase of culture growth (Table 3.22).

For cultures with combinations of the nitrogen sources the values of these growth parameters were generally intermediate between the values for each nitrogen source individually, as was the case for cultures of *B. ruminicola* BA25. Two exceptions to this trend were NH_4Cl plus Tryptone and NH_4Cl plus casein, when cultures reached higher cell densities and usually greater growth rates than when either nitrogen source was available individually. Of particular interest is that growth was supported when Casamino Acids was one of the nitrogen sources for growth, indicating that the components of this compound were not inhibiting cell growth.

A significant quantity of ammonia was apparently produced by all cultures of *S. ruminantium* MP72 in the presence of 0.5 % [w/v] glucose ($P < 0.05$), except for those cultures supplemented with NH_4Cl, NH_4Cl plus Casamino Acids and NH_4Cl plus casein, respectively (Table 3.22). The NH_4Cl and NH_4Cl plus casein cultures utilised 48 and 45 % of the ammonia supplied, respectively.

Growth rates of RGM cultures of *S. ruminantium* MP72 were greater than those for Tryptone only cultures (0.73 compared to 0.60 units. hr^{-1}). The RGM cultures did not reach the same cell density as the Tryptone only cultures, since they may have become glucose-limited. An interesting feature of the lag time data for *S. ruminantium* MP72 (Table 3.22) is that there is little difference in the length of the lag phases between the NFMS treatments (with the obvious exception of Casamino Acid cultures) and between the NFMS treatments and RGM for *S. ruminantium* MP72 growth. In contrast , cultures of *B. ruminicola* BA25 had shorter lag times with Tryptone as a nitrogen source than for other cultures.

3.3.7.3 Comparisons of Growth Between the Two Species

Appraisal of the growth characteristics for *B. ruminicola* BA25 (Table 3.21) and *S. ruminantium* MP72 (Table 3.22) reveals that cultures of the former generally had higher growth rates, larger changes in optical density, and shorter lag times than for *S. ruminantium* MP72 cultures. This was also observed in the preliminary growth data presented in Figure 3.2.

3.4 DISCUSSION

A summary table of the major findings for each rumen bacterial species studied in this chapter has been included in the General Discussion (Chapter 4).

3.4.1 GROWTH CHARACTERISTICS OF THE RUMEN BACTERIAL SPECIES STUDIED

Preliminary experiments to develop defined media for bacterial growth under controlled nitrogen conditions indicated that *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13 were able to grow with NH_4Cl as the sole source of nitrogen, whereas *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 did not grow unless other nitrogen sources were provided (Figure 3.2). These findings confirm the variability in nitrogen source requirements between and within species recorded by Bryant & Robinson (1961, 1962, 1963), Hungate (1966), Bryant (1973), Ogimoto & Imai (1981), Russell & Hespell (1981) and Hobson & Wallace (1982b). Nevertheless, these data appear to refute reports by Cotta &

Table 3.22 : Growth parameters and ammonia levels for *S. ruminantium* MP72 cultures in NFMS media supplemented with 0.05% [w/v] or 0.5% [w/v] glucose and various nitrogen sources, individually and in combinations.

Duplicate "Hungate" tube cultures (12 ml) of *S. ruminantium* MP72, inoculated from an overnight culture in NFMS media supplemented with 8mM NH_4Cl, were grown in the appropriate NFMS media (see Table 3.2) supplemented with one of the nitrogen source treatments listed in Table 3.10, and either 0.05 % [w/v] or 0.5 % [w/v] glucose. Growth was monitored at 60 min. intervals by optical density measurements directly in these tubes.As well as measuring the ammonia concentrations in media which had not been inoculated, cultures were harvested at late log phase of growth and samples of clarified media retained for measurement of ammonia levels. See section 3.2.2.6 for full details.

Ammonia concentrations are the mean ± the standard error of the mean from triplicate measurements. Different superscipts within each glucose level indicate significant differences at the 5% level of significance (Student's T-tests were not performed between glucose levels or treatments).

† The NFMS medium (Table 3.2) was modified with the nitrogen source & glucose level listed.

£ Change in $O.D._{600\ nm}$ was calculated by subtracting the initial optical density (600 nm) of cultures from the optical density at stationary phase of culture growth.

§ Growth rate is expressed as the maximum rate of change of optical density of the batch cultures supplemented with 0.5 % glucose and was calculated from the sigmoidal growth plots.

¥ Lag time is the approximate time from inoculation of the batch culture to early log phase of culture growth. The value in brackets ( ) represents the approximate time from inoculation of the batch culture to stationary phase of culture growth.

** Culture grew very poorly and therefore did not reach log phase of growth.

TREATMENT †		Change in O.D.$_{600\ nm}$ £	Growth Rate §	Lag Time ¥	Ammonia Concentration	
Nitrogen Source	Glucose Conc^{n}	(units)	(units. hr $^{-1}$)	(hr)	Sample	(mM)
NH_4Cl	0.05 %	0.62	-	-	pre	7.17 ± 0.038^{a}
					late log	0.01 ± 0.001^{b}
	0.5 %	1.80	0.29	4 (>10)	pre	5.02 ± 0.038^{a}
					late log	2.81 ± 0.063^{b}
Casamino Acids	0.05 %	0.37	-	-	pre	4.60 ± 0.069^{a}
					late log	5.61 ± 0.060^{b}
	0.5 %	0.40	0.03	**	pre	4.57 ± 0.033^{a}
					late log	6.59 ± 0.015^{b}
Tryptone	0.05 %	0.82	-	-	pre	3.33 ± 0.003^{a}
					late log	4.96 ± 0.030^{b}
	0.5 %	2.03	0.60	3 (9)	pre	3.11 ± 0.047^{a}
					late log	5.43 ± 0.021^{b}
Casein	0.05 %	0.54	-	-	pre	0.67 ± 0.010^{a}
					late log	0.77 ± 0.006^{b}
	0.5 %	1.13	0.26	4 (9)	pre	0.67 ± 0.013^{a}
					late log	0.81 ± 0.010^{b}
NH_4Cl +	0.05 %	0.42	-	-	pre	8.82 ± 0.252^{a}
					late log	13.6 ± 0.14 ^{b}
Casamino Acids	0.5 %	1.11	0.21	4 (10)	pre	9.68 ± 0.066^{a}
					late log	10.9 ± 0.56 ^{a}
NH_4Cl +	0.05 %	0.86	-	-	pre	8.20 ± 0.000^{a}
					late log	13.8 ± 0.18 ^{b}
Tryptone	0.5 %	2.24	0.70	3 (7)	pre	6.57 ± 0.030^{a}
					late log	14.5 ± 0.02 ^{b}
NH_4Cl +	0.05 %	0.53	-	-	pre	4.31 ± 0.038^{a}
					late log	6.91 ± 0.359^{b}
Casein	0.5 %	2.15	0.29	4 (10)	pre	5.04 ± 0.024^{a}
					late log	2.66 ± 0.046^{b}
Casamino Acids +	0.05 %	0.82	-	-	pre	8.72 ± 0.029^{a}
					late log	12.0 ± 0.25 ^{b}
Tryptone	0.5 %	1.99	0.60	3.5 (9)	pre	6.23 ± 0.044^{a}
					late log	24.0 ± 0.21 ^{b}
NH_4Cl + Casamino	0.05 %	0.88	-	-	pre	14.2 ± 0.12 ^{a}
					late log	18.4 ± 0.22 ^{b}
Acids + Tryptone	0.5 %	1.94	0.65	3.5 (9)	pre	11.0 ± 0.21 ^{a}
					late log	15.4 ± 0.47 ^{b}
NH_4Cl + Cas Acids	0.05 %	0.67	-	-	pre	11.3 ± 0.05 ^{a}
					late log	23.9 ± 0.30 ^{b}
+ Tryptone + Casein	0.5 %	1.80	0.50	3.5 (8)	pre	11.2 ± 0.12 ^{a}
					late log	22.1 ± 0.23 ^{b}
RGM	0.2 %	1.63	0.73	3 (6)	pre	5.47 ± 0.499^{a}
					late log	13.5 ± 0.03 ^{b}

Hespell (1986b) that *Butyrivibrio fibrisolvens* 49 is able to grow with ammonia as its only nitrogen source. Krieg & Holt (1984) also suggest that *Butyrivibrio fibrisolvens* (and *B. ruminicola* subsp. *ruminicola*) is able to grow with ammonia as the sole nitrogen source, however they indicate that there is some variability between strains. Mannarelli (1988) supports this premise and states "*. . . that strains presently assigned to the species Butyrivibrio fibrisolvens actually comprise a genetically heterogeneous group of bacteria.*" Thus, the strain designated here as *Butyrivibrio fibrisolvens* 49 may not be true to name, perhaps due to mutation during continual sub-culture in pure culture.

As a consequence of the lack of growth of *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 with NH_4Cl as a sole nitrogen source, yeast extract and Trypticase, respectively, were added to the otherwise "nitrogen-free" mineral salts (NFMS) media to supply at least part of the nitrogen requirement for their growth. The data in Figure 3.2 demonstrate that growth in supplemented NFMS media approached that achieved for *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13 with NH_4Cl as the sole nitrogen source. Growth was, however, restricted in the experiments outlined in this chapter by supplying sub-optimum levels of yeast extract or Trypticase, so that maximum growth was not achieved. This was done deliberately so that the effects of growth stimulation by additions of NH_4Cl, if they occurred, could be observed and measured in these bacteria, as they were for the species which grew with NH_4Cl as the sole source of nitrogen.

A 1 % [v/v] inoculum supported rapid growth in most of the media used for the studies outlined in this chapter. A preliminary experiment showed that the concentration of ammonia in media would be significantly raised by inoculation from an overnight RGM culture (from 0.203 to 0.275 mM ammonia), however the increase was not large enough to cause an overlap of the NH_4Cl concentration between treatments. For example, the culture supplemented with 0.25 mM NH_4Cl would not actually have an ammonia concentration of about 0.5 mM following inoculation. This is especially true when one considers that inoculations to start culture growth were made from overnight cultures of NFMS media initially supplemented with 8 mM NH_4Cl. Unlike RGM cultures, in which the ammonia level often increased slightly due to bacterial growth, cultures grown in NFMS media initially supplemented with 8 mM NH_4Cl had a much lower ammonia concentration after overnight growth (usually 2 to 4 mM), hence a smaller rise in ammonia level would be expected as a result of inoculation from these cultures.

Thus, although inoculation from overnight cultures increases the ammonia concentration of the media, the amount is unlikely to affect the nitrogen status of the media sufficiently to confound any experimental effects.

3.4.2 EFFECTS OF AZASERINE AND MSX ON THE GROWTH OF SELECTED SPECIES OF RUMEN BACTERIA (Experiment 3.1)

3.4.2.1 General Comments

As discussed in the Literature Review (section 1.10), MSX and azaserine are competitive inhibitors of GS and GOGAT, respectively. They have been used in many systems to study ammonia assimilation (e.g. Ronzio *et al.*, 1969 [sheep brain]; Brenchley, 1973 [*Klebsiella aerogenes*]; Miflin & Lea, 1976, Lea & Norris, 1976 & Fentem *et al.*, 1983 [plants & plant roots]; Stewart, 1980 [algae]; Kumar & Nicholas, 1982 [*Nitrobacter agilis*]; Vairinhos *et al.*, 1983 [*Rhizobium japonicum*]; Yamamoto *et al.*, 1984 [*Bacteroides fragilis*]; Rachim & Nicholas, 1985 [*Sclerotinia sclerotiorum* - fungus]; Bhatnagar *et al.*, 1986b [*Methanobacterium ivanovi*]; Westby *et al.*, 1987 [*Azospirillum brasilense*]; Kusnan *et al.*, 1987, 1989 [*Aspergillus nidulans* - fungus]), however only Smith *et al.* (1981) have used MSX to study ammonia assimilation in rumen bacteria, and there are no reports of the use of azaserine in other studies of rumen bacteria.

Miflin & Lea (1976) and Fentem *et al.* (1983) state that MSX is a potent inhibitor of GS which has no effect on GDH activity, but some small effect on GOGAT activity, hence inhibition of bacterial growth by this analogue of glutamate indicates that GS occupies a major growth pathway (Brenchley, 1973; Westby *et al.*, 1987). Similarly, inhibition of bacterial growth by azaserine, an analogue of glutamine which also does not significantly inhibit GDH activity, probably indicates a major role for GOGAT in the growth pathway of the organism, however the inhibitory effects of this analogue are not as specific as those of MSX, since azaserine also inhibits all glutamine amide transfer reactions, including GOGAT (Miflin & Lea, 1976; Fentem *et al.*, 1983). The specificity problems of inhibitors have been dealt with in Chapter 1, and are further highlighted by the more recent criticisms of inhibitor studies by Lea *et al.* (1989) and Rhodes *et al.* (1989). These workers point out that the use of inhibitors can be criticised on grounds that no inhibitor is entirely specific and may have secondary, hitherto

unknown, effects on metabolism. Not withstanding such criticism, the usually accepted roles of MSX and azaserine as inhibitors of GS and GOGAT, respectively, will be used to explain the results of experiment 3.1.

In the experiments discussed below, MSX and azaserine were added to cultures of *B. ruminicola* BA25, *S. ruminantium* MP72, *S. ruminantium* ME13, *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 at early log phase of culture growth in media with NH_4Cl only (NFMS media : 0 - 128 mM), or ammonia, amino acids and peptides (RGM), as nitrogen source(s). The aim of this preliminary work was to study the roles of GS and GOGAT as ammonia-assimilating enzymes in cells grown with various nitrogen sources.

3.4.2.2 *B. ruminicola* BA25

Growth of control (i.e. no inhibitor) cultures of *B. ruminicola* BA25 indicated that much of the ammonia present in NFMS media supplemented with more than 8 mM NH_4Cl is surplus to the growth requirements of this species when only 0.5 % glucose is present. This is clear from the results, since the optical density at harvest was maximal in cultures when the level of supplementary NH_4Cl was about 8 mM, but with higher levels of NH_4Cl these values were similar (Table 3.11). In addition, a large amount of ammonia remained in the media for each of the cultures harvested at late log phase of growth in NFMS media supplemented with at least 8 mM NH_4Cl (i.e. 8, 16, 32 & 128 mM cultures), whereas the ammonia supply was essentially depleted by the time cultures supplemented with less than 8 mM NH_4Cl were harvested. Thus, when grown in media supplemented with less than 8 mM NH_4Cl, the cells appeared to use most of the available ammonia until growth was limited by the very low ammonia concentrations, whereas at higher levels of NH_4Cl growth was limited by the level of carbon and energy (i.e. glucose) in the medium.

The growth of these control cultures of *B. ruminicola* BA25 indicates similarities to the *in vitro* findings of Satter & Slyter (1974) and Russell & Strobel (1987), where growth of mixed rumen bacterial cultures decreased significantly when the ammonia concentration of the environment was lower than about 3 to 4 mM. In contrast, Schaefer *et al.* (1980) found that *B. ruminicola* B_14 had an ammonia saturation constant (K_s) of 30 μM in batch culture, indicating that it can theoretically achieve better than 95 % of maximal growth rate in the presence of less than 1 mM ammonia (a K_s-value of 50 μM is required for 95 % of maximal growth rate at 1 mM

ammonia). Growth rate data from the current experiments is not complete, since control cultures supplemented with less than 8 mM NH_4Cl did not reach mid log phase of growth to enable accurate determinations to be made. Nevertheless, the fact that *B. ruminicola* BA25 was able to grow in cultures supplemented with very low NH_4Cl concentrations (less than 0.5 mM), prior to the depletion of its ammonia supply, indicates that active transport of ammonia is probably a feature of this bacterium.

As outlined in the Literature Review (section 1.8), definitive studies to determine the presence or otherwise of ammonia transport systems in rumen bacterial species had not been published prior to the commencement of work reported in this thesis. Russell & Strobel (1987), however, have shown that rumen bacteria establish a concentration gradient of ammonia across their cell membranes and that active ammonia transport occurs in rumen bacteria. Nevertheless, this transport mechanism may not be readily or maximally induced, causing Russell & Strobel (1987) and Russell *et al.* (1990) to suggest that active ammonia transport does not occur *in vivo*. In contrast to the work by Russell & Strobel (1987), less than 0.7 mM ammonia was left in many cultures in the present work. An active transport system therefore may be a feature of the growth of *B. ruminicola* BA25 *in vitro*, and may explain why rumen bacteria are able to scavenge ammonia (Schaefer *et al.*, 1980; Russell & Strobel, 1987). Passive diffusion of ammonia may, however, occur at high concentrations (Russell & Strobel, 1987; Mackie & White, 1990; Russell *et al.*, 1990).

When amino acids and peptides were available as nitrogen sources in addition to ammonia (i.e. in RGM cultures), growth of *B. ruminicola* BA25 began after a shorter lag time until early log phase of culture growth than was evident for NFMS cultures supplemented with only NH_4Cl. This sometimes indicates energy supply/availability differences between the cultures, but also may indicate hydrolysis and transport/uptake differences for the different nitrogen sources of NFMS media versus RGM. The ammonia levels in RGM media harvested at late log phase of growth did not differ from pre-growth levels, indicating that amino acids and/or peptides may be used as nitrogen sources for growth under these conditions, in preference to ammonia. Bryant & Robinson (1961, 1962, 1963), Pittman & Bryant (1964), Bryant (1973), Russell & Hespell (1981), Cotta & Russell (1982), Russell (1983) and Mackie & Bryant (1990) have reported the preferential use of either peptides or ammonia as nitrogen sources, rather than amino acids, for a number of rumen bacterial species, hence these data

indicate that peptides may be preferred to ammonia as a nitrogen source by *B. ruminicola* BA25. This will be discussed more fully later. RGM cultures, however, did not attain the same maximum optical density values as NFMS cultures supplemented with more than 8 mM NH_4Cl. This is probably a result of the RGM cultures becoming glucose-limited, since only 0.2 % [w/v] glucose was present in RGM, compared with 0.5 % [w/v] in NFMS cultures.

Growth of *B. ruminicola* BA25 cultures in NFMS media supplemented with 2 to 128 mM NH_4Cl was inhibited after the addition of MSX or azaserine at early log phase of growth, however growth was not inhibited in RGM cultures. Azaserine also inhibited growth in cultures supplemented with only 0.25, 0.5, 0.75 and 1.0 mM NH_4Cl and, as the level of supplementary NH_4Cl was increased from 0.25 mM, the differences in growth between control and azaserine treated cultures increased. These increases further support the view that growth was limited in cultures supplemented with low levels of NH_4Cl, since the increasing differences in growth between control and azaserine-treated cultures suggest that fewer restrictions were being placed on growth of control cultures by the addition of more NH_4Cl, whilst azaserine addition resulted in very similar optical density values in cultures regardless of the media ammonia level. In contrast, MSX addition did not result in inhibition of *B. ruminicola* BA25 growth until media ammonia levels were about 1 mM.

Measurements of ammonia concentration in the media of cultures grown in NFMS media supplemented with 8 mM NH_4Cl indicate that subtle differences may exist between control, azaserine and MSX treated cultures at some levels of supplementary NH_4Cl, since the amounts of residual ammonia present at harvest in these control cultures were significantly lower than those in the cultures treated with MSX or azaserine. Identical, but non-significant, trends were also evident for cultures supplemented with between 0.5 and 4 mM NH_4Cl, and trends (non-significant) were evident in the ammonia level between MSX and azaserine treatments for some of these cultures. Differences in ammonia concentrations between control and treated cultures further highlight the inhibitory effects of azaserine in all NFMS cultures supplemented with NH_4Cl, but also suggest that MSX addition may have resulted in some growth inhibition in cultures supplemented with 0.75 and 1.0 mM NH_4Cl, despite the growth data not reflecting this effect.

Thus, based solely on inhibitor studies, it appears that ammonia assimilation in *B. ruminicola* BA25 may not fit the ideal model suggested by Hespell (1984). Firstly, GS

apparently is not the principal assimilatory enzyme of ammonia at concentrations below about 1.0 mM, but it plays a vital role in growth at higher levels (up to at least 128 mM) when NH_4Cl is the sole nitrogen source. This conclusion can be drawn from the results, since MSX is a potent and very specific inhibitor of GS, with only minor effects on GOGAT, but not inhibiting the activity of GDH, which is the major alternative ammonia-assimilatory enzyme (Miflin & Lea, 1976; Fentem *et al.*, 1983). Thus, *B. ruminicola* BA25 may rely on other enzyme(s), possibly in addition to a GS with a lower than expected affinity for ammonia, to assimilate ammonia (probably into glutamine for use by GOGAT to ultimately produce glutamate) when the ammonia concentrations in the media are very low. In fact, Moreno-Vivián *et al.* (1983) provide an example of such an alternative pathway in *Rhodobacter (Rhodopseudomonas) capsulata* strain E1F1, where ammonia assimilation in MSX-treated cells occurred via AlaDH aminating activity coupled with alanine : 2-oxoglutarate aminotransferase activity, so that glutamate is formed as the end product of a sequence of three transamination reactions (i.e. alanine → glycine → aspartate → glutamate). This species has no aminating GDH activity.

Although this is a feasible explanation of these data, it is also possible that the apparent non-reliance of *B. ruminicola* BA25 on GS at very low media ammonia concentrations may be an artifact caused by transport problems associated with MSX. Fentem *et al.* (1983) showed that transport effects associated with MSX do exist sometimes in *Hordeum vulgare*, since MSX is able to penetrate root tissues, but was not rapidly transported into the shoot. Similarly, Kusnan *et al.* (1987) suggest a transport effect of MSX *in vivo* for *Aspergillus nidulans*. MSX does not appear to inhibit what may be an active ammonia transport system in *B. ruminicola* BA25 cells, as has been reported for several bacterial species (Kleiner, 1985). Nevertheless, such a transport mechanism, if it exists, may be necessary for growth of this species at very low ammonia concentrations and somehow interferes with MSX uptake, or facilitates faster uptake of ammonia than of MSX so that the inhibitor is ineffective in restricting GS. Alternatively, it may be that growth in MSX-treated cultures supplemented with very low concentrations of NH_4Cl may occur because amino acids and/or peptides carried over in the inoculum can be utilised by transamination and catabolism of amino acids. Further work is clearly necessary to resolve this problem.

The role of GOGAT in *B. ruminicola* BA25 also requires further clarification, since azaserine, a glutamine analogue, inhibits growth of this bacterium in NFMS cultures

supplemented with only 0.25 mM NH_4Cl. Clearly the enzymes inhibited by this analogue are vital for growth, however azaserine inhibits GOGAT activity, as well as all glutamine amide transfer reactions, but, like MSX, does not significantly inhibit GDH activity (Dunn & Klucas, 1973; Miflin & Lea, 1976). The metabolism of glutamine initially produced by GS or alternative pathways therefore may be catalysed by GOGAT, but the relatively non-specific nature of azaserine inhibition means that catalysis by a variety of glutamine amide transfer reactions cannot be discounted.

The inhibition of growth by MSX and azaserine at high media ammonia levels (up to 128 mM NH_4Cl) indicates that the GS/GOGAT pathway is the principal one involved in ammonia assimilation in *B. ruminicola* BA25 at all ammonia concentrations above about 1 mM. Although these results do not dismiss the notion that GDH is an important ammonia-assimilatory enzyme in *B. ruminicola* BA25 at high ammonia levels, the ideal model of ammonia assimilation presented by Hobson & Wallace (1982b) and Hespell (1984) is clearly not followed by this species because GS/GOGAT is a vital ammonia-assimilatory pathway even at high concentrations of ammonia in the environment. The concept that GDH is not important in ammonia assimilation has been introduced by Deuel & Prusiner (1974) [*Bacillus subtilis*], Herbert *et al.* (1978) [*Rhodopseudomonas acidophila*], Kenealy *et al.* (1982) [*Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum*], Rachim & Nicholas (1985) [*Sclerotinia sclerotiorum*], Bhatnagar *et al.* (1986a) [*Methanobacterium ivanovii*] and Braña *et al.* (1986) [*Streptomyces clavuligerus*], who all provide examples of ammonia assimilation in the absence of GDH activity.

Lomnitz *et al.* (1987) suggested that the main role of GOGAT in *Neurospora crassa* is recycling glutamine to glutamate for use by GS. Inhibition of GS and GOGAT activities therefore indicates that the products of the GS/GOGAT pathway (i.e. glutamine, and ultimately glutamate) are vital for biosynthesis by the cells, and that this is at least one of the principal ammonia-assimilatory pathways in *B. ruminicola* BA25, as is the case in soybean nodules and most plants (Pate, 1989), and in *Rhodobacter (Rhodopseudomonas) capsulata* E1F1 (Moreno-Vivián *et al.*, 1983), *Sclerotinia sclerotiorum* (Rachim & Nicholas, 1985), *Beggiatoa albus*, *Xanthobacter* H4-14, *Anabaena* sp., *Rhodopseudomonas acidiphila* (Vargas & Strohl, 1985), and *Azospirillum brasilense* (Westby *et al.*, 1987), to name only a few organisms.

The fact that neither MSX nor azaserine inhibited growth of *B. ruminicola* BA25 when added to RGM cultures at early log phase of growth indicates that GS and GOGAT are not vital for growth, and ammonia is probably not assimilated, when amino acids and peptides are available as nitrogen sources in addition to ammonia. As mentioned earlier, this premise is supported by the similar levels of ammonia in RGM prior to growth, and when the control and treated cultures were harvested at late log phase of growth. These results are supported by the work of Smith *et al.* (1981), who stated that assimilatory enzymes (GDH in particular) may not be important in growth conditions such as these, since sufficient glutamate could theoretically be produced via transamination or catabolism of the free or peptide bound amino acids.

3.4.2.3 *S. ruminantium* MP72 and *S. ruminantium* ME13

The effects of MSX and azaserine on growth of *S. ruminantium* MP72 and *S. ruminantium* ME13 appear quite similar between these sub-species, however there were differences. These were probably due to the large variation in the data, especially between days for *S. ruminantium* MP72. In addition, these effects were very similar to those observed for *B. ruminicola* BA25, except that both MSX and azaserine inhibited growth of the *S. ruminantium* sub-species in NFMS media supplemented with very low concentrations of NH_4Cl (down to 0.25 mM), but MSX did not inhibit growth of these NFMS cultures supplemented with 128 mM NH_4Cl (azaserine was still inhibitory at this concentration). Thus, although the actual growth kinetics and maximum optical densities of control cultures of the sub-species of *Selenomonas* differed from those of *B. ruminicola* BA25, growth of the sub-species, and utilisation of ammonia by them, was also maximised when about 8 mM NH_4Cl was present in the media. Smith *et al.* (1981) have previously reported growth inhibition of cultures of *S. ruminantium* with ammonia as the nitrogen source to be dependent on the dose of MSX .

Growth inhibition of *S. ruminantium* MP72 and *S. ruminantium* ME13 by MSX in cultures supplemented with very low concentrations of NH_4Cl, but not in those supplemented with 128 mM NH_4Cl, may indicate that GS is the principal ammonia-assimilating enzyme in these sub-species when very little ammonia is available for growth, but not at very high ammonia concentrations. Thus, these sub-species of *S. ruminantium* appear to assimilate ammonia by the same mechanism determined for *S. ruminantium* subsp. *ruminantium* strain D

by Smith *et al.* (1981). This contrasts to the data for *B. ruminicola* BA25 (above), indicating that the GS enzyme may be quite different between these species, possibly with a higher affinity for ammonia in the sub-species of *S. ruminantium* than in *B. ruminicola* BA25.

The data for *S. ruminantium* MP72 and *S. ruminantium* ME13 indicate that there may be differences in ammonia (and MSX ?) transport mechanisms between these sub-species and *B. ruminicola* BA25. These results do not indicate that ammonia transport (if it exists) is more effective in *S. ruminantium* MP72 and *S. ruminantium* ME13 than in *B. ruminicola* BA25, since both species grew and utilised ammonia even at very low concentrations of supplementary NH_4Cl. The results may, however, indicate that MSX entry occurs in *S. ruminantium* cells even at very low media ammonia concentrations, whereas MSX entry is somehow inhibited or interfered with in *B. ruminicola* BA25. Regardless of which explanation is correct, GS activity differs in importance in the species at very high concentrations of ammonia in the media.

The growth data for *S. ruminantium* MP72 and *S. ruminantium* ME13 also show that GOGAT is a key enzyme for growth in the two sub-species of *S. ruminantium,* even at very high ammonia concentrations. This is surprising given that GS is not essential for growth at these ammonia levels. Nevertheless, this may indicate that the enzyme(s) responsible for ammonia assimilation at very high ammonia concentrations (i.e. instead of GS, for example GDH) is not sufficiently active in this species to produce enough glutamate, and therefore requires GOGAT activity. Alternatively, these results suggest that azaserine inhibits enzymes other than GOGAT, thereby inhibiting cell growth. A curious feature of these data for cell growth in *S. ruminantium* MP72 and *S. ruminantium* ME13 is that GS appears to be vital for cell growth at ammonia levels up to 32 mM (and up to 128 mM in *B. ruminicola* BA25) despite the usually accepted role of the GS/GOGAT pathway operating when the ammonia concentration in the environment is low, and the GDH pathway when the level is high (Smith *et al.,* 1980, 1981; Hespell, 1984). The possibility that the effects of MSX are actually due to inhibition of other essential metabolic reactions can probably be dismissed for *S. ruminantium*, since Smith *et al.* (1981) showed that MSX inhibits GS activity in, as well as growth of, *S. ruminantium* D, *in vitro*. They did not, however, study the effects of azaserine on GS or GDH activity.

As was the case for *B. ruminicola* BA25, cultures of *S. ruminantium* MP72 and *S. ruminantium* ME13 grown in RGM were not inhibited by addition of MSX or azaserine at

early log phase of growth. This implies that when amino acids and peptides were available as nitrogen sources in addition to ammonia, nitrogen is assimilated by transamination or catabolism of the amino acids present in peptides, rather than by assimilation of ammonia via GS/GOGAT or other pathways. Smith *et al.* (1981) speculated that this may occur on the basis of very low GDH activity levels in *S. ruminantium* D under such conditions.

3.4.2.4 *B. ruminicola* subsp. *ruminicola*

In contrast to the previous species, which grew well with NH_4Cl as the sole nitrogen source, but also with amino acids and peptides as nitrogen sources, *B. ruminicola* subsp. *ruminicola* required amino acids and/or peptides as nitrogen sources for growth, which was not stimulated by the addition of NH_4Cl. Nevertheless, MSX and azaserine caused up to 50 % inhibition of growth in this species, indicating that, although ammonia could not be used as the sole nitrogen source, GS and GOGAT are important enzymes in nitrogen assimilation in this species. These results may, however, indicate that glutamate and glutamine were the most important amino acids used by transamination and catabolism reactions in this species, since MSX and azaserine are analogues of these compounds, respectively, and, because of a lack of specificity of azaserine for GOGAT in particular, these reactions (or other metabolically important ones) may have been inhibited. It is also feasible that MSX and azaserine could have inhibited transport of the amino acids and peptides present in the modified NFMS media used for growth of this species, thereby causing the 30 - 50 % inhibition of growth. Alternatively, the concentration of the inhibitors may not have been sufficient to cause the dramatic effects evident for the previous species. The latter explanation is suggested because the same concentrations of MSX and azaserine were used for all of the species studied, and therefore may not have been ideal for each bacterium. Further study is required to clarify these points.

The ammonia concentration in *B. ruminicola* subsp. *ruminicola* cultures increased as a consequence of growth when a low level of NH_4Cl was added to NFMS media, however this may have resulted from the release of residual ammonia from cells left in growth tubes at stationary phase of growth for many hours (i.e. bacterial autolysis). Alternatively, this rise in media ammonia concentration may have been due to bacterial activity in metabolising the amino acids and peptides present in the modified NFMS media. Russell *et al.* (1988a)

described a number of rumen bacterial species able to produce ammonia when grown in semi-continuous cultures with Trypticase (amino acids and peptides) as the "sole" nitrogen source. Argyle & Baldwin (1989) also showed evidence for the production of ammonia as a consequence of ammonia utilisation, supporting this possibility.

3.4.2.5 *Butyrivibrio fibrisolvens* 49

Butyrivibrio fibrisolvens 49 also did not grow with NH_4Cl as the sole nitrogen source, and the addition of NH_4Cl to modified NFMS media containing a low concentration of Trypticase (0.15 % [w/v]) did not stimulate growth (i.e. growth rate or maximum optical density) in this species. Azaserine caused about 20 % growth inhibition in these cultures, however, since this analogue is not completely specific for GOGAT, these results may not indicate a reliance on GOGAT activity, especially since MSX did not inhibit growth in any of the cultures. GS and GOGAT therefore do not appear to be important for growth of this species, which may rely on the transamination and catabolism reactions discussed earlier, rather than ammonia assimilation, to fulfil its nitrogen requirements.

3.4.2.6 Important Considerations from Experiment 3.1

The inhibitor experiments discussed above indicate that, of the species studied, the GS/GOGAT pathway of ammonia assimilation is important only in those which can grow with NH_4Cl as the sole nitrogen source, and in these species only when NH_4Cl is the sole nitrogen source. In the two species in which NH_4Cl was not able to provide the sole source of nitrogen for growth, and when amino acids and peptides were available as nitrogen sources, in addition to ammonia, for growth of *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13, transamination and catabolism of amino acids (free and/or present in peptides) appear to be the mechanisms whereby nitrogen is obtained for growth. For the species utilising the GS/GOGAT pathway, alternative pathways of ammonia assimilation may operate under some circumstances, however they do not appear to operate exactly as previous work might indicate (Hespell, 1984). This may reflect the deficiencies inherent in inhibitor studies (Miflin & Lea, 1976; Lea *et al.*, 1989; Rhodes *et al.*, 1989), but also indicates that

rumen bacteria do not entirely conform to the usual ammonia-assimilating pathways recorded in other microorganisms.

The results of inhibitor studies cannot be unequivocal because the inhibitors may not be specific for the enzymes in rumen bacteria and there may be hitherto unknown effects on other metabolic systems. These points are highlighted by the inhibitor results for *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49, which show that MSX and azaserine may affect enzymes other than the well known "target" enzymes. The experiments discussed below, in which the relative activities of the assimilating enzymes were determined under various levels and sources of nitrogen, should shed further light on the operation of the ammonia-assimilating pathways.

3.4.3 ACTIVITY OF AMMONIA-ASSIMILATING ENZYMES (Experiments 3.2, 3.3 & 3.4)

3.4.3.1 Introductory Remarks

In experiments 3.2, 3.3 and 3.4 GS activity was measured using the transferase reaction. Control assays incorporating boiled enzyme were included for each sample, however the activity in these samples was calculated assuming that the control assays had zero activity, even though this was not always the case. Actual control values were very variable, probably due to non-enzymic production of γ-glutamyl hydroxamate, however subtraction of these values from the "active" assays would not affect the final trends discussed below, even though the magnitude of the specific activity values would be altered. Comparison of the absolute activity levels between experiments, and with those of other workers, is therefore dubious, however, the trends should be comparable.

Despite this cautionary note on the GS activity values, it is interesting to note that those from the *in vivo* experiments for Chapter 2 (experiments 1 & 2) ranged from 0 to 550 nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$ when ammonia concentrations ranged between 0.1 and 60 mM in the rumen fluid samples. Similar GS activity values from the current *in vitro* experiments were obtained for media ammonia concentrations above about 1 mM, but were much higher for ammonia concentrations below 1 mM. In the Discussion of Chapter 2 it was pointed out that the ammonia concentration measured in rumen fluid, and that

encountered by bacteria in the rumen, could differ because of the existence of microenvironments within the rumen. It is the ammonia concentrations in these microenvironments (e.g. in particles of partly degraded food, free in the liquid, in the floating mat of rumen contents, etc.) that are more important than the rumen fluid ammonia concentration *per se* (Hobson & Wallace, 1982b; Odle & Schaefer, 1987; Owens & Zinn, 1988), however, even these ammonia concentrations, and those in culture media, may differ from those within the cells.

Active transport mechanisms specific for ammonia have been found in a number of bacterial systems (e.g. *E. coli* (Stevenson & Silver, 1977); numerous species (Kleiner, 1985); *Rhodobacter* (*Rhodopseudomonas*) *sphaeroides* (Cordts & Gibson, 1987); *E. coli* & *Klebsiella pneumoniae* (Merrick, 1988)) and in the yeast *Saccharomyces cerevisiae* (Egbosimba & Slaughter, 1987). Russell & Strobel (1987) showed that, although active ammonia transport occurs in mixed rumen bacteria, it is not readily or maximally induced. Thus, the ammonia concentration in media (and in the rumen) may not be a true reflection of the level within the cells, especially at very low media ammonia concentrations. The ammonia level within cells is, however, the most important factor in terms of enzyme activity and regulation and, in this thesis, this concentration was considered to be proportional to the media ammonia concentration. Merrick (1988) stated that specific ammonia transport systems exist in bacteria when they encounter low environmental ammonia concentrations, but at high external levels, diffusion of ammonia through the membrane is fast enough to support the nitrogen demand of the cells. This implies that a difference exists between the internal and external ammonia concentrations at low environmental ammonia concentrations, but that the internal and external concentrations are equal when high levels are present in the environment. Differences between the data presented in this thesis and those discussed by Mackie & White (1990) and Russell *et al.* (1990) suggest that this differential in uptake systems may occur in some rumen bacteria.

3.4.3.2 GS Activity and Regulation in *B. ruminicola* BA25 when NH_4Cl is the Sole Nitrogen Source for Growth

The specific activity of GS for *B. ruminicola* BA25 in experiment 3.2 (section 3.3.4) appeared to be inversely related to the concentration of NH_4Cl present in NFMS media, although this trend was not statistically significant. In experiment 3.3 (section 3.3.5) this relationship was significant in cells harvested at most stages of growth, and especially in those

harvested at mid log and at late log phase. The reason for the difference between these sets of data is probably that the GS activities for experiment 3.3 were measured in cell-free extracts of cells harvested at very definite stages of growth in NFMS media. In contrast, the GS activities for experiment 3.2 were measured in cell-free extracts prepared from cells whose growth was also closely monitored, but the stage of growth at which they were harvested may have varied from day-to-day. Student's T-test comparisons of data for experiment 3.2 showed significant differences between cells grown in NFMS media supplemented with 0.5 mM and with 8, 16, 32 and 128 mM NH_4Cl. The large standard error for these data is probably the reason an analysis of variance was not statistically significant. In contrast, all of the comparisons between NFMS media for experiment 3.3 were significant.

Smith *et al.* (1981) found that the level of GS (and urease) decreased in *S. ruminantium* D when NH_4Cl was added to nitrogen-limited cultures, indicating that the enzyme responded to the concentration of ammonia in the environment in a similar way to that shown from the data in this thesis. Patterson & Hespell (1985) showed a similar effect for *Succinivibrio dextrinosolvens* and Cacciari *et al.* (1986) found that GS activity was inversely correlated with the initial NH_4^+ concentration in the culture medium of the nitrogen-fixing bacterium, *Arthrobacter sp. "fluorescens"*. In *Rhizobium phaseoli* two forms of GS are present. These are regulated in a reciprocal manner with the GS II form repressed when ammonium is present (Bravo & Mora, 1988). Thus, GS activity in *B. ruminicola* BA25 appears to be increased by low ammonia concentrations in the media, or decreased by high ammonia concentrations, even though the effects of media ammonia concentration may have been masked by large standard errors caused by day-to-day variation in experiment 3.2. In addition, GS is probably an important route of ammonia assimilation in this species, as it is in *S. ruminantium* D and *Succinivibrio dextrinosolvens*.

In reviewing ammonia assimilation in rumen bacteria, Hespell (1984) suggested that a low level of GS activity is present in some species when the concentration of ammonia in the surrounding environment is high. This was the case *in vivo* during experiment 2 (Chapter 2), and is clearly the case for *B. ruminicola* BA25 *in vitro*. Nevertheless, growth of this species was inhibited by MSX at high concentrations of supplementary NH_4Cl, but not at very low concentrations of NH_4Cl. The lack of growth inhibition at very low ammonia concentrations is difficult to explain in light of the very high GS activity values detected under

these conditions in experiment 3.2, but it may be associated with ammonia and MSX transport into cells already discussed (section 3.4.2.1). In contrast, the apparent requirement of these cells for GS activity even at very high environmental ammonia concentrations (experiment 3.1), when GS activity is very low (experiment 3.2), may indicate that these cells have a requirement for at least a minimum level of glutamine from GS activity at all times when NH_4Cl is the sole source of nitrogen. This is not surprising considering the central role of glutamine, and glutamate, the product via GOGAT activity, in nitrogen metabolism of most bacteria (see Figure 1.3 in the Literature Review).

The GS activities in *B. ruminicola* BA25 measured at various media ammonia concentrations for experiment 3.2 suggest that changes in GS activity may occur suddenly at a threshold media ammonia level (Figure 3.3), however data from the more closely controlled experiment 3.3 indicate that this relationship may be exponential (Figure 3.9). This is a more plausible possibility, since it means that the ammonia concentration of the media, through its (largely unknown) effects on the ammonia concentration within the cell (Russell & Strobel, 1987), may act directly as the main inhibitor of GS activity, or the end products of glutamine metabolism, such as tryptophan, histidine, 2-oxoglutarate, asparagine, amino acids, AMP, glucosamine-6-phosphate and carbamoyl phosphate, may act as cumulative feedback inhibitors on the activity, as occurs in *E. coli* and a number of other bacterial species (Hubbard & Stadtman, 1967; Shapiro & Stadtman, 1970b; Stadtman *et al.*, 1970; Prusiner & Stadtman, 1973; Deuel & Prusiner, 1974; Senior, 1975; Bender *et al.*, 1977; Brown, 1980). Other forms of regulation which were not studied in these experiments, such as induction/repression of enzyme synthesis, adenylylation/deadenylylation or phosphorymation of the enzyme, and availability of appropriate divalent cations, may also affect GS in *B. ruminicola* BA25, as is the case in many other bacteria (Hubbard & Stadtman, 1967; Shapiro & Stadtman, 1970b; Stadtman *et al.*, 1970; Deuel & Prusiner, 1974; Senior, 1975; Bender *et al.*, 1977; Brown, 1980).

Smith *et al.* (1980, 1981) suggested that regulation of GS in *S. ruminantium* D, which does not have adenylylation control, may not be directly due to the ammonia level in the environment, but due to glutamine and 2-oxoglutarate levels, as occurs in a number of species of *Bacillus* (Deuel & Prusiner, 1974). This may be the major control in *S. ruminantium* D, but further work is necessary to determine details of similar fine controls for *B. ruminicola* BA25.

The inverse relationship between media ammonia concentration and GS activity in *B. ruminicola* BA25 is also evident if one compares the GS activity and ammonia concentration at each stage of growth of this bacterium for a particular medium. This is possible for experiment 3.3, in which decreasing ammonia concentrations at successive stages of cell growth in NFMS cultures were to some extent mirrored by GS activity increases. Thus, as the enzymes utilise ammonia, the concentration which is available for further assimilation decreases, and GS activity increases. Alternatively, this change in GS activity may reflect growth of the culture, such that the products of glutamine metabolism are used and therefore do not inhibit the activity of the enzyme. Mink *et al.* (1982) showed that GS activity in *S. ruminantium* D increased slightly during cell starvation, which may be a similar phenomenon to that occurring here with cells harvested at progressively later stages of culture growth.

The apparent K_m-value for hydroxylamine in GS was 4.5 mM when NH_4Cl was the sole nitrogen source for growth of *B. ruminicola* BA25. This value equates to the K_m-value for ammonia of this enzyme, due to the direct substitution of hydroxylamine for ammonia in the γ-GT assay. The K_m-value is much higher than the 0.5 mM or less suggested by Hespell (1984) for GS in rumen bacteria, indicating that the enzyme may not be the nitrogen scavenger many expect, at least in this species. This K_m-value is, however, similar to that found in *Nitrobacter agilis* (10 mM : Kumar & Nicholas, 1982), *Methanobacterium ivanovi* (6.8 mM : Bhatnagar *et al.*, 1986b), and *Bacillus polymyxa* (3.5 mM : Ojha & Kantengwa, 1989), and indicates that GS has a relatively low affinity for ammonia in *B. ruminicola* BA25. This observation supports similar circumstantial evidence from the effects of MSX on growth in experiment 3.1, which suggests that GS is not important for cell growth at very low media ammonia concentrations, but is important at all levels above about 1 mM, and even up to at least 128 mM, NH_4Cl in the NFMS media. Apart from K_m-values for ammonia of 0.56 to 0.69 mM in *Bifidobacteria* (Hatanaka *et al.*, 1987a, b), examples of very low K_m-values for GS are relatively scarce in the literature for any bacteria, despite the assumption that GS generally has a very low K_m (high affinity) (Brown *et al.*, 1974). Wallace & Cotta (1988) also suggest that GS generally has a low K_m-value for ammonia (1.8 mM), but also point out that rumen ammonia concentrations *in vivo* would rarely be so low as to require such a high affinity for ammonia. Although this assertion ignores the possibility of very low ammonia

concentrations occurring within microenvironments in the rumen, it may help explain the relatively high K_m-value for ammonia from the current work.

3.4.3.3 GS Activity in *B. ruminicola* BA25 Grown with Ammonia, Amino Acids and Peptides as Nitrogen Sources

As well as studying GS activity in *B. ruminicola* BA25 cells grown with NH_4Cl as the sole nitrogen source, the activities of this and other ammonia-assimilating enzymes were measured in cells grown in RGM, which contains ammonia, amino acids and peptides as nitrogen sources. In general, GS activity was significantly lower in cell-free extracts of cells grown in RGM than in NFMS media supplemented with NH_4Cl (experiments 3.2 and 3.3), however Student's T-test comparisons were only significant for the activities of cells grown in RGM and in NFMS media supplemented with 0.5 and 1.0 mM NH_4Cl. Ammonia is present as a nitrogen source for bacterial growth at a concentration of about 5 mM in RGM, in addition to amino acids and peptides, hence it is to be expected that the GS activity (and the activities of other ammonia-assimilating enzymes) in cells harvested from these cultures would be similar to those measured in cells harvested from NFMS cultures supplemented with between 4 and 8 mM NH_4Cl. Student's T-tests of the data from experiment 3.2 did not show significant differences between GS activities from these media, however, in experiment 3.3, cells grown in NFMS media supplemented with 8 mM NH_4Cl have a significantly higher GS activity than those grown in RGM.

This difference in GS activity between RGM grown cells and those grown in NFMS media with a similar level of ammonia is clearly due to the nitrogen source rather than the concentration of ammonia in the media, since RGM has a lower ammonia concentration than is present in NFMS media supplemented with 8 mM NH_4Cl, but the cells also have a lower GS activity. This is contrary to the inverse relationship between media ammonia concentration and GS activity discussed above, but supports the findings of Smith *et al.* (1981) who found that GS was repressed in cultures of *S. ruminantium* D with Casamino Acids as nitrogen source, due to the abundance of various amino acids and potential glutamate sources which could have a cumulative repressing effect on the enzyme. Thus, GS activity in *B. ruminicola* BA25 is probably repressed by the more complex nitrogen sources present in RGM, but is also lower simply because the other nitrogen sources may be used preferentially

by the cells. This was also suggested by Smith *et al.* (1981), since they found that sufficient glutamate could theoretically be produced by transamination (e.g. aspartate) or catabolism of amino acids (e.g. glutamine), instead of by GDH.

B. ruminicola B_14, GA33 & BA25, *B. ruminicola* subsp. *ruminicola, B. fragilis* and *S. ruminantium* HD_4 were grown in Brain-Heart Infusion (BHI) Media, which contains amino acids and peptides as nitrogen sources. Similarly, *E. coli* HB101 was grown in L-Broth medium, which contains mainly peptides as nitrogen sources. Both types of media also have a moderate concentration of ammonia. The similarities of these "enriched" media to RGM suggest that cells grown in them would have similar enzyme activities to those of RGM grown cells. This was the case for *B. ruminicola* BA25 (Table 3.16), however similar data were not obtained for the other species and strains, since none of them was grown in both RGM and BHI media. The GS activities for *E. coli* , which are similar to those reported by Griffith & Carlsson (1974), indicate that assay conditions and techniques employed in this thesis were comparable to those used by other workers. This statement takes into account the reservations expressed in section 3.4.3.1 regarding comparisons between laboratories and between workers, however the activity values measured in this thesis are of the same order of magnitude as those of other workers.

3.4.3.4 The Effect of Stage of Culture Growth at Harvest on GS Activity

The results discussed in section 3.4.3.2 for experiment 3.3 were not the primary ones envisaged for this experiment, which was aimed at determining the effect of the stage of growth on GS γ-GT activity for *B. ruminicola* BA25. This experiment showed that there is no difference in GS activity between cells harvested at early log and at mid log phase of culture growth, nor between cells harvested at late log and at stationary phase, but cells harvested at early log or mid log phase generally had a lower GS activity than those harvested at late log or stationary phase. The only comparable data in the literature are reported by Mink *et al.* (1982), where GS activity increased with cell starvation, which may occur during stationary phase of cell growth.

The differences between the early log/mid log and late log/stationary phase activities values was especially evident for cells grown in NFMS media supplemented with 0.5

or 2 mM NH_4Cl, but cells grown in RGM had similar GS activities irrespective of the stage of cell growth. These, plus other data (experiment 3.2 & later), suggest that ammonia was not assimilated by cells in this media, indicating that GS is likely to be constitutively present. Chalupa (1975) stated that proteases are constitutive in the rumen, since this enzymatic machinery is necessary for degradation which occurs under most rumen conditions. This may also be the case for GS in *B. ruminicola* BA25, as it apparently is for *Bacillus subtilis* (Ojha & Kantengwa, 1989). The bacterium therefore always has a low GS activity so that it can assimilate ammonia more readily via this major pathway when ammonia becomes the major nitrogen source available for growth in its resident rumen microenvironment.

The results discussed above for differences in enzyme activities with harvest time may explain at least some of the day-to-day variation in GS activity in cells measured in experiment 3.2, since cells were harvested between mid log and stationary phase of culture growth on a particular day in this experiment. Significant changes in GS activities from day-to-day were recorded for cells harvested from cultures grown in NFMS media supplemented with 0.5 or 2 mM NH_4Cl. Thus, the large standard errors found for experiment 3.2 were probably the result of cells not being harvested at the same stage of growth each day, and therefore GS activity varied from day-to-day, masking significant trends for GS activity (as well as other enzyme activities - see later).

3.4.3.5 The Effect of the Initial Ammonia Concentration of the Inoculating Culture on GS Activity

Experiment 3.3 also involved a minor experiment which showed that the ammonia concentration in which overnight cultures of *B. ruminicola* BA25 were grown affects the GS activity of cells grown in cultures inoculated from them. In fact, GS activities in NFMS cultures supplemented with 0.5 or 8 mM NH_4Cl were significantly lower when the inoculating culture (i.e. overnight culture) was supplemented with 4 mM NH_4Cl than with 8 mM NH_4Cl. Since the decrease in GS activity values was by a similar proportion for all media tested it is unlikely that conclusions for experiment 3.2, which were based on GS activities from cultures inoculated from 8 mM overnight cultures, would be altered, although the magnitude of the activities may be affected. This is another important factor to consider when comparing enzyme

activities, since it seems that only absolute values measured in the same laboratory can truly be compared, however trends reported from various laboratories should be comparable.

3.4.3.6 GOGAT, GDH & AlaDH Activities in *B. ruminicola* BA25 Grown with NH_4Cl as the Sole Nitrogen Source

In addition to GS activity, NADH- and NADPH-dependent GOGAT, GDH and AlaDH activities were also detected in cell-free extracts of *B. ruminicola* BA25 cells grown with various concentrations of NH_4Cl as the sole nitrogen source. In contrast to the activities of these enzymes reported in Chapter 2, the NADPH-dependent activities of GOGAT, GDH and AlaDH were all much greater than their respective NADH-dependent activities (experiment 3.2). In fact, the NADH-dependent activities were so low that they are unlikely to be quantitatively important in this species.

In preliminary experiments, fine tuning of the assays was not carried out if reasonable activities were detected. NADH and NADPH were therefore successfully used as electron donors for the assays of GOGAT, GDH and AlaDH, despite reports by Miflin & Lea (1976) and Smith *et al.* (1981) that other electron donors, such as methyl viologen/dithionite, and reduced ferredoxin, are more effective for GOGAT assays in some organisms, including plants. Patterson & Hespell (1985) similarly had difficulty in finding an appropriate electron donor for GOGAT in *Succinivibrio dextrinosolvens*, since strictly anaerobic bacteria apparently use electron carriers with lower potentials than do *E. coli* and other aerobic organisms. Kenealy *et al.* (1982) were not able to detect significant GOGAT activity in cell-free extracts of *Methanosarcina barkeri* with either NADPH, NADH or ferredoxin as electron donors, but could using hydrogen-reduced deazaflavin-factor 420 or flavin mononucleotide.

The NADPH-dependent GOGAT and GDH activities for *B. ruminicola* BA25 were about ten times greater than the AlaDH activities at all media ammonia concentrations, indicating that AlaDH may not be a quantitatively important enzyme for ammonia assimilation in *B. ruminicola* BA25 when NH_4Cl is the sole nitrogen source. This premise is supported by the very high K_m-values for ammonia generally observed for this enzyme (38 mM from *Bacillus subtilis* : Bergmeyer *et al.*, 1988; 38 - 360 mM from *Bacillus* species : Yoshida & Freese, 1965; Brown *et al.*, 1974; Stewart, 1980; Moreno-Vivián *et al.*, 1983), although some examples of lower K_m-values exist (6.7 mM in *Streptomyces aureofaciens* : Vancurová *et al.*, 1988).

Miflin & Lea (1976) similarly could find no evidence for a role for AlaDH in primary ammonia assimilation in plants, and Brown *et al.* (1974) suggested that the function of AlaDH may be in converting alanine to pyruvate, which can be readily used as a carbon and energy source.

Since AlaDH is unlikely to be an important ammonia-assimilating enzyme in *B. ruminicola* BA25, the significant effects of media ammonia concentrations on the NADPH-dependent activities (section 3.3.4.4) will not be discussed. The effects do not appear to be as simple as those for GS, and may be artifacts of the very low activities recorded. In contrast, the NADPH-dependent activities of GOGAT and GDH were very high (0.2 to 2 nmoles NADPH oxidised $min.^{-1}$ $(mg\ protein)^{-1}$), indicating that these enzymes are probably important for ammonia assimilation in this species. Neither of these activities were significantly influenced by the concentration of ammonia in the media, however trends are apparent in Figures 3.4 & 3.5 (see section 3.4.3.8). Thus, it is apparent that AlaDH is not important in assimilating ammonia in this species, however the NADPH-dependent activities of GOGAT and GDH are relevant.

Vairinhos *et al.* (1983) and Mackie & White (1990) pointed out that the presence of an enzyme does not necessarily imply that a particular pathway operates biosynthetically or is important to the organism concerned. The results from experiment 3.1 indicate that GOGAT apparently is a vital enzyme for cell growth in *B. ruminicola* BA25 when NH_4Cl is the sole source of nitrogen. This observation is reinforced by the results of experiment 3.2, where fairly high GOGAT activities were recorded at all media ammonia concentrations. In contrast, the inhibitor results from experiment 3.1 do not support the commonly held belief that rumen bacteria rely on GDH activity to assimilate ammonia when high concentrations of ammonia are available in the media, since GS and GOGAT were vital for cell growth at media ammonia levels up to 128 mM. Thus, despite the high NADPH-dependent GDH activities recorded in this experiment, it appears that this enzyme alone is not able to provide sufficient glutamate for cell growth at high media ammonia concentrations, since GS and GOGAT were still required for growth (experiment 3.1). It is possible that the enzyme is not a significant assimilator of ammonia in *B. ruminicola* BA25 despite the widely held view that GDH predominates under production conditions (Mackie & White, 1990).

Miflin & Lea (1976), Blevins (1989), Lea *et al.* (1989), Pate (1989) and Rhodes *et al.* (1989) state that, even though GDH is present in plants, it may have a negligible role in ammonia assimilation relative to the flux via the GS/GOGAT pathway, due largely to its high

K_m for ammonia. In contrast, a number of organisms do not possess GDH activity, but instead appear to rely on AlaDH in its place, or GS/GOGAT entirely (Herbert *et al.*, 1978 [*Rhodopseudomonas acidophila*]; Bhatnagar *et al.*, 1986a [*Methanobacterium ivanovii*]; Braña *et al.*, 1986 [*Streptomyces clavuligerus*]; Cacciari *et al.*, 1986 [*Arthrobacter sp. "fluorescens"*]; Caballero *et al.*, 1989 [*Rhodobacter (Rhodopseudomonas) capsulatus*]). Miflin & Lea (1976) suggested that, due to the discovery of GOGAT and its role with GS in the GS/GOGAT pathway in many organisms, it seems unlikely that GDH plays a major role in nitrogen assimilation in bacteria or higher plants, except in the relatively unusual circumstances of a high external ammonia concentration, and there is even doubt about its role under these circumstances in *B. ruminicola* BA25 (experiments 3.1 & 3.2).

The apparent K_m-value for NH_4Cl in NADPH-dependent GDH when *B. ruminicola* BA25 cells were grown with NH_4Cl as the sole source of nitrogen was 8.1 mM, compared to a value of 24.3 mM for the NADH-dependent GDH. These high K_m-values (low affinities) support the theory for a negligible ammonia assimilatory role for this enzyme when environmental ammonia concentrations are low, but also suggest that the enzyme could be important under high ammonia conditions. This K_m-value is slightly higher than the 2 to 6 mM range suggested by Hespell (1984) for rumen bacteria, and 4 mM for bacteria in general by Miflin & Lea (1976). A wide range of activities have been determined from an equally wide range of organisms (73 mM for soybean root nodule bacteroids (Dunn & Klucas, 1973); 5 to 6 mM for plants and 0.3 to 0.5 mM for green algae (Miflin & Lea, 1976); 1 to 2.1 mM for *Ruminobacter (Bacteroides) amylophilus* (Jenkinson *et al.*, 1979); 6.7 and 23 mM for *S. ruminantium* (Smith *et al.*, 1980); 6.3 to 33 mM for *Nitrobacter agilis* (Kumar & Nicholas, 1984); 5.5 mM for *Bacillus licheniformis* (Bernlohr *et al.*, 1984); 2.2 mM for *Bacillus macerans* (Kanamori *et al.*, 1987); 1 to 5 mM for *Bacteroides fragilis, B. ovatus, B. distasonis* and *B. vulgatus* (Yamamoto *et al.*, 1987b); 5.0 mM for *Candida albicans* (Holmes *et al.*, 1989)).

The apparent K_m-value for L-glutamine in NADPH-dependent GOGAT in cell-free extracts of cells of *B. ruminicola* BA25 grown with NH_4Cl as the sole nitrogen source was 2.5 mM. Some comparative values range from 0.2 to 1.8 mM for *Aerobacter aerogenes, Erwinia carotovora, Pseudomonas fluorescens, Bacillus subtilis* and *Bacillus megaterum* (Meers *et al.*, 1970), 0.25 mM for *E. coli* and 1.8 mM for *Aerobacter aerogenes* (Miller & Stadtman,1972), 0.95 mM for soybean root nodule bacteroids (Dunn & Klucas, 1973), 0.55

mM for *Rhodopseudomonas acidophila* (Herbert *et al.*, 1978), 0.18 mM for *Bacillus megaterium*, 0.008 and 0.1 mM for *Bacillus licheniformis* (Bernlohr *et al.*, 1984), 0.077 mM for *Nocardia mediterranei* (Mei & Jiao,1988) and 0.75 mM for *Candida albicans* (Holmes *et al.*, 1989). The K_m-value determined for GOGAT in this experiment (2.5 mM) is the lowest for any of the major enzymes of the ammonia-assimilatory pathways in *B. ruminicola* BA25, suggesting that GOGAT is fairly efficient at utilising glutamine as it is produced by GS, possibly enhancing the GS reaction.

3.4.3.7 Nicotinamide Adenine Dinucleotide Specificities and Activities in *B. ruminicola* BA25

It is generally accepted that NADPH-dependent GDH activity is responsible for the biosynthesis of glutamate, whereas NADH-dependent GDH activity is assumed to be catabolic (Shapiro & Stadtman, 1970b; Brown *et al.*, 1974; Smith *et al.*, 1975; Buttery, 1976; Tyler, 1978; Glass & Hylemon, 1980; Kumar & Nicholas, 1984; Hammer & Johnson, 1988; Martinez-Bilbao *et al.*, 1988; Merrick, 1988; Mörsdorf & Kaltwasser, 1989; Vancurová *et al.*, 1989). Nevertheless, the direction in which this reaction is actually catalysed physiologically by the enzyme is a subject of controversy, even though it seems to be responsible for ammonia assimilation (i.e. metabolism) in yeasts and fungi (Martinez-Bilbao *et al.*, 1988). Assuming that this generally accepted role for GDH activity is the one operating in *B. ruminicola* BA25, it appears that when ammonia is the sole nitrogen source, metabolism to produce glutamate is the major activity of GDH in this species.

Yamamoto *et al.* (1987b) found a dual coenzyme specific GDH in members of the *Bacteroides fragilis* group (*B. fragilis, B. ovatus, B. distasonis* & *B. vulgatus*), as well as an electrophoretically distinct NADH-specific GDH in *B. fragilis* (Yamamoto *et al.*, 1987a). Although *B. fragilis* and *B. ruminicola* are not closely related, currently being classified in distinct *Bacteroides* "groups" (Paster *et al.*, 1985; Shah & Collins, 1983, 1989), it is possible that dual specificity may also be the case in *B. ruminicola* BA25. Dual nicotinamide dinucleotide activity for GDH has been reported for a number of organisms (*B. thetaiotaomicron* - Glass & Hylemon, 1980; *S. ruminantium* - Smith *et al.*, 1980; *Nitrobacter agilis* - Kumar & Nicholas, 1984; *B. fragilis, B. ovatus, B. distasonis, B. vulgatus* - Yamamoto *et al.*, 1987b; *Streptomyces fradiae* - Vancurová *et al.*, 1989), although enteric bacteria apparently only have NADPH-

dependent activity (Merrick, 1988), and most other bacteria also are considered to have one type of GDH (Vancurová *et al.*, 1989).

The characteristics of the ratio of NADH-dependent GDH activity to NADPH-dependent GDH activity for *B. ruminicola* BA25 differs markedly from those of any of the organisms listed above, being relatively constant between about 0.01 and 0.05 for all media ammonia concentrations, compared to about ten times these values for the *B. fragilis* group (0.1 - 1.0 : Yamamoto *et al.*, 1987b), *Nitrobacter agilis* (0.7 : Kumar & Nicholas, 1984), *S. ruminantium* (0.25 - 0.35 : Smith *et al.*, 1980) and about 100 times these values for *B. thetaiotaomicron* (~1 : Glass & Hylemon, 1980). Thus, GDH in *B. ruminicola* BA25 may have dual coenzyme specificity, since Smith *et al.* (1980) also found a relatively fixed ratio of NADH- to NADPH-dependent activities in *S. ruminantium* D cells grown under a variety of conditions and inferred that the enzyme had dual coenzyme specificity. Such a conclusion for *B. ruminicola* BA25 should be confirmed by further investigation so that a clear distinction can be made between the possibility of GDH activity associated with two separate enzymes, one enzyme with dual coenzyme specificity, or a combination of these possibilities.

The NADH/NADPH ratios for GOGAT were also fairly constant at all media ammonia levels (0.01 to 0.07), possibly indicating the presence of a GOGAT with dual coenzyme specificity in *B. ruminicola* BA25, as has been inferred for *S. ruminantium* D (Smith *et al.*, 1980). Catabolic activities for either NADH- or NADPH-dependent GOGAT have not been reported in the literature, since this enzyme is considered to operate unidirectionally (Brown *et al.*, 1974; Nicholas, personal communication). The NADH/NADPH ratio varied appreciably for AlaDH under various ammonia conditions (0.071 to 0.558) in *B. ruminicola* BA25.

3.4.3.8 Controls on GOGAT and GDH Activities in *B. ruminicola* BA25

GOGAT is relatively insensitive to regulation by feedback inhibition, with no simple correlation between levels of enzyme and the nitrogen status of the cell (Merrick, 1988). NADPH and glutamate may, however, be inhibitory to the enzyme (Miller & Stadtman, 1972; Mei & Jiao, 1988), and high concentrations of ammonia may compete with glutamine and inhibit the enzyme (Brown *et al.*, 1974). The level of GOGAT does, however, change in *Bacillus licheniformis* with different nitrogen sources (Bernlohr *et al.*, 1984). GOGAT

activities were not significantly influenced by the concentration of ammonia in the media for *B. ruminicola* BA25, however a trend is apparent in Figure 3.4 (Experiment 3.2).

GDH enzymes from various sources also have insensitive controls, commonly based around synthesis/degradation pathways influenced by the nitrogen source in the growth medium (Shapiro & Stadtman, 1970b; Smith *et al.*, 1975; Miflin & Lea, 1976; Bernlohr *et al.*, 1984; Vancurová *et al.*, 1989), but reversible activation/inactivation mechanisms regulated by ammonia also occur (Yamamoto *et al.*, 1987a). Although a significant effect of media ammonia concentration on GDH activity (NADH- and NADPH-dependent) was not evident in the current work, a trend is apparent in Figure 3.5 (Experiment 3.2). As was the case for GS activity (section 3.4.3.2), a significant effect may have been masked by the large variation in activity from day-to-day caused by non-synchronised cell harvest times.

The NADPH-dependent activities of GOGAT and GDH were, however, significantly lower in *B. ruminicola* BA25 cells grown in RGM than cells grown in NFMS media supplemented with various concentrations of NH_4Cl. The NADH-dependent activities of these enzymes were not significantly different when grown on either nitrogen source, and, as discussed earlier, were probably not quantitatively important in ammonia assimilation. This indicates that catabolic activity of GDH, if it is due to the NADH-dependent enzyme activity, did not increase when amino acids were present in the media as may have been expected. As was the case for the GS activity (section 3.4.3.3), GOGAT and GDH activities were lower in cells of *B. ruminicola* BA25 grown in RGM than in those grown in NFMS media supplemented with 8 mM NH_4Cl, indicating that the controlling factor is probably the nitrogen source in the media, rather than the ammonia concentration.

Comparison of the NADH/NADPH ratios for GOGAT and GDH between the nitrogen sources offered (NH_4Cl at all levels versus RGM growth) showed that for both enzymes there was about a ten fold increase in the NADH/NADPH ratio in extracts from cells grown with NH_4Cl as the sole nitrogen source, compared with cells grown in RGM (with ammonia, amino acids and peptides as nitrogen sources). These changes were largely due to a decrease in the NADPH-dependent activities of both enzymes, rather than increases in the NADH-dependent activities, and may indicate that at least two separate GDH enzymes exist in *B. ruminicola* BA25. One of these would possess dual coenzyme specificity and relatively low activity (i.e. about the level of the NADH-dependent activity), whilst the other would only have

NADPH-dependent activity which is highly dependent on the nitrogen source (i.e. very active when NH_4Cl is the sole nitrogen source, but not when amino acids and/or peptides are also available). This would be similar to the situation in *B. fragilis*, in which one GDH enzyme has only NADH-dependent activity, whilst another has dual coenzyme specificity in which the NADPH-dependent activity has a high affinity for ammonia (Yamamoto *et al.*, 1987a). It appears that there is a significant switch away from metabolism (i.e. assimilation) of ammonia by GDH to transamination or catabolism of the amino acids present in peptides, as speculated by Smith *et al.* (1981) for *S. ruminantium* D. What may be the catabolic activity of this enzyme (i.e. NADH-dependent activity), however, did not markedly increase as a result of the amino acids available as nitrogen sources.

As mentioned earlier for NFMS grown cells, the very low NADH- and NADPH-dependent AlaDH activities probably preclude this enzyme from having an important role in ammonia assimilation under the conditions of these experiments. The result was similar in RGM grown cells despite the fact that the NADH/NADPH ratio for this enzyme also markedly increased in cells grown in RGM compared to NFMS media (1.038 compared to 0.282, respectively). This increase was largely due to an enhancement in NADH-dependent activity, suggesting that catabolism of amino acids via AlaDH may occur when ammonia, amino acids and peptides are available as nitrogen sources for growth (assuming NADH-dependent activity is catabolic in this enzyme, as is apparently the case in GDH).

The residual ammonia in media of cultures harvested during experiment 3.3 further supports the premise that nitrogen assimilation is different when amino acids and peptides are present as nitrogen sources in addition to ammonia, than when NH_4Cl is the sole nitrogen source. A significant amount of ammonia was utilised for growth in NFMS media supplemented with 8 mM NH_4Cl, but not when RGM was used (except at stationary phase of cell growth). The relatively lower activities of GS, and NADPH-dependent GOGAT and GDH, in RGM cultures, and the concentration of residual ammonia in these cultures, indicate that ammonia is not used as a nitrogen source by *B. ruminicola* BA25 when amino acids and peptides are also available, supporting the observations from experiment 3.1. Ammonia-assimilating enzymes were detected in these cells, possibly indicating that they are constitutive. The data from experiment 3.1 clearly show that other nitrogen assimilating pathways, such as transamination or catabolism, may supply enough glutamate for growth of the bacteria (Smith

et al., 1981). The GS (/GOGAT) pathway of ammonia assimilation therefore may not be important in *B. ruminicola* BA25 under these conditions.

3.4.3.9 GS Activity in *S. ruminantium* MP72

The activities of GS, GOGAT, GDH and AlaDH were also determined in cultures of *S. ruminantium* MP72, but only in cell-free extracts of cells grown in NFMS media supplemented with 0.5, 8.0 or 128 mM NH_4Cl, or in RGM. This abridged form of the investigation into ammonia assimilation made in *B. ruminicola* BA25 was adopted because literature data are available for *S. ruminantium* subsp. *ruminantium* strain D (Smith *et al.*, 1980, 1981). Ammonia assimilation data has not previously been presented for *S. ruminantium* subsp. *lactilytica*. This sub-species of *Selenomonas* is very closely related to *S. ruminantium* subsp. *ruminantium*, to the extent that Kingsley & Hoeniger (1973) could find insufficient reasons to subdivide the two varieties. Nevertheless, the separate sub-species are now recognised, with *S. ruminantium* subsp. *lactilytica* strains differing from *S. ruminantium* subsp. *ruminantium* in their ability to ferment lactate and glycerol (Hungate, 1966; Hespell & Bryant, 1981; Ogimoto & Imai, 1981; Krieg & Holt, 1984).

γ-GT activity of GS was not detected in cell-free extracts of *S. ruminantium* MP72, nor has it previously been recorded in *S. ruminantium* D (Smith *et al.*, 1980, 1981; Hespell, 1984). *Lactobacillus arabinosus* (Ravel *et al.*, 1965); *Bacillus licheniformis* (Hubbard & Stadtman, 1967), *Bacillus subtilis* (Deuel & Prusiner, 1974), *Methanobacterium thermoautotrophicum* (Kenealy *et al.*, 1982) and *B. fragilis* (Southern *et al.*, 1987; Hill *et al.*, 1989) have also been shown to lack transferase activity, thus its absence in species of *Selenomonas* is not unique.

In contrast to the non-detection of GS activity in cell-free extracts of *S. ruminantium* MP72 are the results of inhibitor studies for this species (experiment 3.1), where GS was apparently vital for cell growth when NH_4Cl was the sole nitrogen source. It should be noted that the absence of γ-GT GS activity in an organism does not necessarily imply that the organism does not possess GS activity, since the γ-GT reaction is not the normal one catalysed by GS *in vivo*. For example, Smith *et al.* (1980) determined the forward activity for GS in *S. ruminantium* D despite being unable to detect γ-GT activity. Transferase activity is a

secondary reaction catalysed by GS, and is measured by adding hydroxylamine (instead of ammonia), ADP, Mn^{++} and sodium arsenate to cell-free extracts containing GS. The enzyme subsequently catalyses a transfer reaction in which the production of γ-glutamyl hydroxamate is measured (Shapiro & Stadtman, 1970a, b; Stadtman & Ginsburg, 1974). This reaction is subject to less interference by contaminating enzymes than the biosynthetic or forward assays, and enables measurement of the adenylylation status of the enzyme in the presence of Mg^{++} and Mn^{++} (Shapiro & Stadtman, 1970a, b; Stadtman *et al.*, 1970). The data from experiments 3.1 and 3.2 therefore indicate that GS activity is present in *S. ruminantium* MP72, but the transferase reaction was not detected.

3.4.3.10 GOGAT, GDH and AlaDH Activities in *S. ruminantium* MP72

As was the case for *B. ruminicola* BA25, the NADH-dependent GOGAT and GDH activities for *S. ruminantium* MP72 were very low and therefore unlikely to be quantitatively important in this species. In contrast, the NADPH-dependent activities of these enzymes were still higher than the NADH-dependent activities, but were about one tenth of those for *B. ruminicola* BA25. The NADPH-dependent AlaDH activity in *S. ruminantium* MP72 was similar to that in *B. ruminicola* BA25, however the NADH-dependent activity in *S. ruminantium* MP72 was up to four times higher than in *B. ruminicola* BA25. Nevertheless, the AlaDH activities were still very low in *S. ruminantium* MP72, but were higher than the GOGAT activities. It is not possible to speculate on the relative quantitative importance of GOGAT and GDH to the two species, since it is not clear whether both species have similar metabolic rates and turnover times, etc.

Although GOGAT has low activity in *S. ruminantium* MP72 it was still vital for growth when NH_4Cl was the sole nitrogen source (section 3.4.2.3). GOGAT may therefore metabolise glutamine produced by GS so that more ammonia can be assimilated and GS is therefore not regulated by the ammonia concentration *per se*. It is possible, however, that the results from the inhibitor experiments (3.1), which indicate the essential nature of GOGAT to cell growth when ammonia is the sole nitrogen source, may be due to other, non-specific, inhibitor effects. Although Smith *et al.* (1980) could not find a natural electron donor for GOGAT (reduced methyl viologen gave maximal activity whereas NADPH- and NADH-

dependent activities were low) the activity of this enzyme was fairly constant over a range of growth conditions. Low NADPH-dependent GOGAT activities measured in the present study, although higher than those measured by Smith *et al.* (1981), were unaffected by growth conditions, supporting the view that this enzyme is not highly regulated (Smith *et al.*, 1980). Investigations with alternative electron donors were not performed for *S. ruminantium* MP72.

GDH activities did not appear to be highly regulated in bacteria used in the present study. Smith *et al.* (1980) also found that NADPH-dependent GDH activity was not strictly regulated in *S. ruminantium* D, but that it had a low affinity for ammonia (high K_m), and its activity was highest when ammonia concentrations were not growth-limiting. Nevertheless, the enzyme was still apparently important under ammonia-limited conditions in this species. Thus, an important ammonia-assimilatory role for GDH in *S. ruminantium* MP72 under a wide range of ammonia concentrations in the environment is not precluded by the current work. This is consistent with results for *E. coli* , in which GDH can participate in glutamate synthesis even when the ammonia concentration is 75 μM, but it is less efficient than the GS/GOGAT pathway. As found in many systems, GDH may, in fact, be especially important if GS activity is regulated by the ammonia concentration in the environment. Smith *et al.* (1980) suggested that this mechanism operated in *S. ruminantium* D, and it is also implied from the inhibitor studies of experiment 3.1, where enzyme activity was vital for cell growth when media ammonia concentrations were low, and GS was not essential at very high media ammonia concentrations. GDH is the logical enzyme to assume the predominant ammonia-assimilating role under these conditions, although the low activity of this enzyme, and the presence and low activity of AlaDH, implies that neither enzyme predominates. Perhaps both enzymes are involved in ammonia assimilation in *S. ruminantium* MP72 under these conditions.

The NADH/NADPH ratios for GOGAT and GDH in *S. ruminantium* MP72 when NH_4Cl was the nitrogen source were very different to those measured in *B. ruminicola* BA25 under similar growth conditions. The values of 0.21 to 0.25 for GDH were, in fact, very similar to those determined by Smith *et al.* (1980) (0.25 to 0.35). The relatively fixed value for this ratio in *S. ruminantium* MP72 under a limited range of growth conditions is also similar to that found by Smith *et al.* (1980), who inferred that the enzyme had dual coenzyme specificity.

The activities of GOGAT, GDH and AlaDH in cell-free extracts of *S. ruminantium* MP72 grown in RGM were not significantly different from those for cells grown with NH_4Cl

as the sole nitrogen source (GS transferase activity not detected), nor were the NADH/NADPH ratios for these enzymes significantly different under these growth conditions. These enzymes therefore appear to be unaffected by the source of nitrogen available for cell growth.

Discussion of GS activities in cells grown in these media are clearly speculative, since the forward assay was not used to detect activity. Nevertheless, Smith *et al.* (1980) found the GS activity was only 60 nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$ when Casamino Acids were provided as the nitrogen source, whereas it rose to 183 nmoles γ-glutamyl hydroxamate produced $min.^{-1}$ $(mg\ protein)^{-1}$ in ammonia-limited cells. These results suggest that the GS activity in RGM grown cells for experiment 3.2 would probably be lower than that in cells grown with low concentrations of NH_4Cl as the sole source of nitrogen. Results from the inhibitor experiments discussed earlier indicated that under these growth conditions, *S. ruminantium* MP72 did not rely on GS activity for growth. Hence the activity of this enzyme would be expected to be low, as was found for *B. ruminicola* BA25. Smith *et al.* (1981) showed that NADPH-dependent GDH activity also decreased in cells grown with Casamino Acids as the nitrogen source, relative to NH_4Cl grown cells (181, 142 & 53 nmoles NADPH oxidised $min.^{-1}$ $(mg\ protein)^{-1}$ for 10 mM NH_4Cl grown cultures, 0.05 mM NH_4Cl grown cultures, and Casamino Acids grown cultures, respectively). This effect was not apparent for *S. ruminantium* MP72 in the current experiments.

3.4.3.11 GS, GOGAT, GDH and AlaDH Activities in *Butyrivibrio fibrisolvens* 49

γ-GT activity of GS was also not detected in cell-free extracts of *Butyrivibrio fibrisolvens* 49, confirming the results of the inhibitor studies (experiment 3.1), which suggested that GS was not important in nitrogen assimilation. Although these results do not prove that this strain is devoid of GS activity, they indicate that the enzyme is not important for growth.

NADH- and NADPH-dependent GOGAT, GDH and AlaDH activities were detected in cell-free extracts of *Butyrivibrio fibrisolvens* 49, however they were generally much lower than for either *B. ruminicola* BA25 or *S. ruminantium* MP72, especially the NADPH-dependent activities. These results indicate that GS, GOGAT, GDH and AlaDH may not play active roles in nitrogen assimilation (via ammonia assimilation) in this species. Clearly the GS/GOGAT pathway is not vital for cell growth, and GDH and AlaDH also may not be

important. These are not surprising findings, since *Butyrivibrio fibrisolvens* 49 did not grow with NH_4Cl only, but requires more complex nitrogen sources, such as amino acids and/or peptides. It probably derives its nitrogen requirements through transamination and catabolism of the amino acids directly, or after uptake and breakdown of peptides.

Although the results for this species are very different to those for *B. ruminicola* BA25 and *S. ruminantium* MP72, the data show that the latter are also different to each other in terms of the mechanisms whereby they assimilate ammonia.

3.4.4 NITROGEN SOURCE PREFERENCES FOR GROWTH BY *B. ruminicola* BA25 and *S. ruminantium* MP72 (Experiment 4)

3.4.4.1 Aims and Introductory Remarks

The previous experiments discussed in this chapter provide an outline of ammonia assimilation in *B. ruminicola* BA25 and *S. ruminantium* MP72, with some comparisons to *B. ruminicola* subsp. *ruminicola, Butyrivibrio fibrisolvens* and *S. ruminantium* subsp. *ruminantium*. Nevertheless, ammonia is only one of the nitrogen sources available in the rumen and, despite the fact that most rumen bacterial species are able to grow with ammonia as the sole source of nitrogen, this may not be their preferred nitrogen source (Bryant & Robinson, 1961, 1962, 1963; Allison, 1970; Hespell & Bryant, 1979; Hobson & Wallace, 1982b; Russell, 1984). The importance of nitrogen sources other than ammonia can be appreciated by the statement by Russell & Hespell (1981) that "*Although ammonia is a major source of nitrogen for bacterial growth, peptides and amino acids are also important, particularly with low quality diets (high fiber* [sic]*, low protein) where up to 40 % of the bacterial nitrogen does not come from ammonia.*" In fact, although many rumen bacteria can grow with ammonia as their sole source of nitrogen, many also have an absolute requirement for, or are stimulated by addition of, amino acids and/or peptides (Bryant & Robinson, 1963; Maeng *et al.*, 1976; Hespell & Bryant, 1979; Cotta & Russell, 1982; Hobson & Wallace, 1982b; Owens & Bergen, 1983; Russell, 1984; Cotta & Hespell, 1986a; Owens & Zinn, 1988; Wallace & Cotta, 1988).

The final experiments discussed for this thesis therefore were designed to test the hypothesis : "When provided with a variety of nitrogen sources (protein, peptides, amino acids & ammonia), *B. ruminicola* subsp. *brevis* and *S. ruminantium* subsp. *lactilytica*

preferentially utilise peptide nitrogen, but can also utilise all other nitrogen sources". These experiments, in which the potential of nitrogen sources other than ammonia to supply the growth requirements of *B. ruminicola* BA25 and *S. ruminantium* MP72 was investigated in batch cultures, should place the mechanisms of ammonia assimilation determined earlier into context in terms of what may occur within the rumen.

Casein, Tryptone, Casamino Acids and NH_4Cl were used as representatives of the nitrogen sources available in the rumen. As mentioned above, ammonia is one of the major forms of nitrogen utilised in the rumen, providing up to 80 % of the microbial nitrogen requirements (Hespell & Bryant, 1979; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Baldwin & Allison, 1983; Owens & Bergen, 1983; Leng & Nolan, 1984; Cotta & Hespell, 1986a; Mackie & Bryant; 1990). Casamino Acids, which are the products of acid hydrolysis of casein "*. . . until all the nitrogen in the casein is converted to amino acids or other compounds of relative chemical simplicity . . .*" (Difco Manual, 1971), was the source of amino acids for the current experiments, and Tryptone, a pancreatic digest of casein which results in mostly peptide nitrogen plus some free amino and similarly simple nitrogen sources (Difco Manual, 1971), was the source of peptide nitrogen. Casein was the source of protein nitrogen. Thus, the nitrogen sources were all derived from casein (i.e. casein, casein pancreatic digest, casein acid hydrolysate and ammonia) and should have the same overall composition for bacterial growth.

3.4.4.2 Nitrogen Sources as Energy Sources for Rumen Bacterial Growth?

As well as studying the nitrogen source preferences for growth by *B. ruminicola* BA25 and *S. ruminantium* MP72, two glucose concentrations (0.05 % [w/v] and 0.5 % [w/v]) in the media were superimposed over the nitrogen source treatments. This was done to determine whether these species were able to utilise the nitrogen sources, in particular amino acids or peptides, as alternative energy sources for growth when a direct energy source (glucose) was unavailable or limiting.

Growth of *B. ruminicola* BA25 and *S. ruminantium* MP72 cultures plateaued very early in log phase, for all nitrogen source treatments when 0.05 % [w/v] glucose was provided as the principal energy source. Although the level of glucose was, in fact, not measured in these cultures either before growth, or when the cells were harvested, it is clear

from the growth data (Tables 3.21 & 3.22) that a nutrient had become growth limiting. The changes in optical density of cultures which differed from the 0.05 % [w/v] glucose cultures only in their glucose concentrations (i.e. 0.5 % [w/v] glucose cultures) clearly show that the nitrogen sources were not at growth limiting levels when the cultures were harvested. Thus, the growth limiting nutrient was glucose. Casein, Tryptone, Casamino Acids and NH_4Cl therefore were not used as alternative energy sources by either of these bacterial species when the supply of the principal energy source (glucose) was exhausted by cell growth.

Most rumen bacteria are unable to grow with peptides or amino acids as the principal energy source in the absence of carbohydrates (Russell & Hespell, 1981; Russell *et al.*, 1983; Russell, 1983, 1984; Cotta & Hespell, 1986a; Russell *et al.*, 1988a; Mackie & Bryant, 1990). Argyle & Baldwin (1989), however, suggest that amino acids (and peptides?) can be fermented as energy sources by rumen bacteria, but growth of the microorganisms under these conditions is generally negligible. Nevertheless, they found that small quantities of amino acids and peptides can stimulate microbial growth when other energy sources are available, but as amino acid sources rather than as sources of energy, ammonia or volatile fatty acids (Argyle & Baldwin, 1989). In contrast, Hespell & Bryant (1979) suggest that this stimulation of growth is due to decreased energy uncoupling when amino acids are present, rather than to lower energy costs due to decreased biosynthesis of amino acids. That is, energy from amino acid/peptide fermentation is used to offset some of the maintenance energy requirements, or to enhance the survival of amino acid fermenting species (Cotta & Hespell, 1986a).

In some unique environments, such as anaerobic digesters, there exist specialized bacteria, such as the proteolytic clostridia, that can derive sufficient energy for growth from the fermentation of amino acids or peptides (Mackie & Bryant, 1990). In the unique environment of the rumen, Chen & Russell (1988), Russell *et al.* (1988a) and Chen & Russell (1989a) have found a species of *Peptostreptococcus*, a species of *Clostridium*, and two additional ammonia-producing, monensin-sensitive bacteria, which grow rapidly with amino acids or peptides as the sole energy and nitrogen source. Morphotypes of these species were present in high numbers in the rumen (> 10 % of the counts obtained with glucose, maltose and cellobiose as carbohydrate sources) and they may therefore play an important role in the fermentation of dietary amino acids, and thus ammonia production, in the rumen (Russell *et al.*, 1988a; Chen & Russell, 1989a).

In support of Argyle & Baldwin (1989), Russell (1983) suggested that the reason most rumen bacteria are not able to use peptides (or amino acids?) as energy sources was their inability to transport and ferment peptides at a fast enough rate to meet maintenance energy requirements. This view has been confirmed by the work of Chen *et al.* (1987b) with mixed rumen bacteria, in which K_m-values for the uptake and deamination of peptides were compared with those for simple sugars for some rumen bacteria. This work clearly showed that many rumen bacterial species are able to grow rapidly with sugar as an energy source, but are unable to take up peptides at a rate fast enough to meet maintenance energy requirements. The K_m-value for peptides was nearly 1,000-fold greater than that of some simple sugars.

3.4.4.3 Preferences for Nitrogen Sources for Growth by *B. ruminicola* BA25

In contrast to the abrupt cessation of growth in *B. ruminicola* BA25 cultures when only 0.05 % [w/v] glucose was provided, cultures supplemented with 0.5 % [w/v] glucose (plus various nitrogen sources) showed more traditional logarithmic growth patterns, with large changes in optical density (> 1.5 units). Thus, this species is able to utilise NH_4Cl (ammonia), Casamino Acids (amino acids), Tryptone (peptides) and casein (protein) effectively as nitrogen sources when adequate glucose is present. On the basis of the different cell densities produced in these cultures, peptides appear to be utilised most effectively, followed, in descending order, by ammonia, protein and amino acids.

Tryptone was the only nitrogen source that decreased as a result of growth of *B. ruminicola* BA25, although large changes (both increases and decreases) in the concentrations of ammonia also occurred in many cultures. An interesting feature of these data is that either the concentration of ammonia remained unchanged, or increased markedly, in cultures containing Tryptone. Apart from confirming reports by Argyle & Baldwin (1989) that ammonia is not utilised when Tryptone (or Trypticase† in the case of Argyle & Baldwin, 1989) is supplied, these results show that ammonia is produced as a consequence of peptide utilisation. Despite the consistent changes in Tryptone levels, only a small proportion of the available Tryptone was utilised during growth. Similarly, only a small amount of the available Casamino Acids

† Similar to Tryptone in that it is the result of enzymatic digestion of casein and is therefore composed of peptides and relatively few free amino acids (Chen & Russell, 1988), however Trypticase is a product of BBL Microbiology Systems (Cockeysville, U.S.A.), whereas Tryptone is a product of Difco Laboratories (Detroit, U.S.A.).

and casein were utilised during growth in these cultures. In contrast, at least 45 % of the available ammonia was used in cultures supplemented with NH_4Cl, Casamino Acids, casein, and NH_4Cl plus casein. Russell (1983) and Chen & Russell (1988) also found that only a low percentage of Trypticase or Casamino Acids was degraded when these compounds were used as sole nitrogen sources as well as energy sources. Russell *et al.* (1983) found that casein was not completely metabolised.

On the basis of the availability of the four nitrogen sources, peptides and ammonia appear to be readily used for growth by *B. ruminicola* BA25, whereas amino acids and protein are not. Similarly, the maximum growth rate of *B. ruminicola* BA25 cultures was achieved with Tryptone as the sole nitrogen source, followed in descending order by NH_4Cl, casein and Casamino Acids. In addition, the lag time, and the time from inoculation to stationary phase of culture growth, were shorter when Tryptone was the sole nitrogen source than with casein, NH_4Cl or Casamino Acids (in that order). Cultures in which combinations of these nitrogen sources were present resulted in growth parameters (i.e. maximum growth rate, change in cell density, lag time and time from inoculation to stationary phase of culture growth) intermediate between those determined when the sources were present singly. The exception was the culture supplemented with NH_4Cl plus Tryptone, where growth parameters exceeded those of either source individually. Thus, peptides were the most effective nitrogen source for *B. ruminicola* BA25, followed in descending order by ammonia, protein and free amino acids.

As expected, growth of this species in RGM, in which ammonia, amino acids and peptides were available as nitrogen sources, was very similar to growth in the NFMS cultures in which Tryptone was one of the nitrogen sources. The RGM cultures, however, had a shorter lag time, and did not reach the same maximum optical density, as NFMS cultures containing Tryptone. This is probably associated with an inadequate energy supply in RGM (0.2 % [w/v]) and is further evidence that *B. ruminicola* BA25 cannot ferment complex nitrogen sources for an adequate energy supply (section 3.4.4.2).

The low percentage of Tryptone, Casamino Acids and casein degraded in these experiments suggests that a high concentration of amino acids, peptides or protein may be necessary for uptake and hydrolysis of these compounds by *B. ruminicola* BA25 cells. Transport systems for amino acids and peptides, however, have been detected in rumen bacteria and are present in most bacteria (discussed later). Cotta & Russell (1982) also found

that utilisation of amino acids is rarely complete in pure cultures, and speculate that this may be because not all of the peptides in Trypticase or Tryptone can be transported into rumen bacteria (Rice *et al.*, 1978). Thus, there may be a synergism amongst rumen bacteria that allows the use of most of the peptides *in vivo*, but in pure cultures they are not fully utilised. A similar effect may also operate for free amino acids, especially since amino acid transport systems specific for each of the amino acid structural groups have been reported (Stevenson, 1979; Anraku, 1980; Eddy, 1980; Wolfinbarger, 1980a; Strobel *et al.*, 1989).

Although this study is apparently the first to investigate the utilisation of four nitrogen compounds (ammonia, amino acids, peptides and protein) in the same rumen bacterium, some preferences of various rumen species have previously been recorded. For example, Bryant & Robinson (1961, 1963) showed that although ammonia is generally preferred to amino acids as the nitrogen source by cellulolytic bacteria, many species and strains grow with ammonia as the sole nitrogen source, and some have an absolute growth requirement for ammonia. *B. ruminicola* is able to grow with ammonia as the sole source of nitrogen, however some peptides can replace ammonia, whereas small peptides of less than four residues, free amino acids and some other low molecular weight compounds can not (Pittman & Bryant, 1964). Wallace & Cotta (1988) therefore suggest that this species utilises mainly peptides and ammonia *in vivo*, whilst the current work supports these findings. Although *Streptococcus bovis* can grow with ammonia as the sole nitrogen source, this species grows twice as fast with amino acids and peptides (Russell *et al.*, 1988b; Chen & Russell, 1989b), and *Ruminobacter (Bacteroides) amylophilus* has a higher growth rate, higher yield and diminished lag period when Tryptose[§] is included in the basal media with ammonium sulphate ($(NH_4)_2SO_4$) as the sole nitrogen source, even though this bacterium still has an absolute requirement for ammonia (Hullah & Blackburn, 1971).

In mixed rumen bacterial cultures, amino acids and peptides often stimulate growth rates and yields, even when ammonia and volatile fatty acids are available (Baldwin & Allison, 1983; Russell, 1984; Broderick & Wallace, 1988; Argyle & Baldwin, 1989), and many species have absolute requirements for, or are stimulated by addition of, amino acids or peptides (Hungate, 1966; Pittman *et al.*, 1967; Wright, 1967; Cotta & Russell, 1982; Russell, 1984; Cotta & Hespell, 1986a; Owens & Zinn, 1988; Wallace & Cotta, 1988; Argyle &

[§] Very similar to Tryptone; also a product of Difco Laboratories (Detroit, U.S.A.).

Baldwin, 1989; Mackie & White, 1990). Argyle & Baldwin (1989) suggest that growth stimulation of mixed rumen bacterial cultures by amino acids and peptides is due to the number of amino acids provided, rather than the supply of specific growth-limiting amino acids. Peptides are more effective nitrogen sources than free amino acids for rumen bacteria (Pittman & Bryant, 1964; Hungate, 1966; Pittman *et al.*, 1967; Wright, 1967; Russell, 1984; Cotta & Hespell, 1986a; Chen *et al.*, 1987b; Hino & Russell, 1987; Broderick & Wallace, 1988; Broderick *et al.*, 1988; Russell *et al.*, 1988b; Wallace & Cotta, 1988; Argyle & Baldwin, 1989; Mackie & White, 1990). Russell (1983) is the only worker hitherto to report a preference by rumen bacteria for ammonia nitrogen over amino nitrogen, and Chen & Russell (1988) showed that Casamino Acids was a more effective nitrogen source than Trypticase in "*. . . a monensin-sensitive ruminal peptostreptococcus.*"

Differences in lag time from inoculation to log phase of culture growth is often related to the availability of energy, however all cultures in the current experiment were supplied with the same concentration of glucose (0.5 % [w/v]). The shorter lag time for Tryptone cultures than for NH_4Cl, casein and Casamino Acids cultures, respectively, therefore may indicate uptake, hydrolysis and utilisation differences between the nitrogen sources. Lag time differences may, however, have been influenced by other factors, for example traces of oxygen in some cultures or the extent of cell death in various inoculums.

Peptide uptake and hydrolysis is fairly well understood in many organisms (Rice *et al.*, 1978; Becker & Naider, 1980; Higgins & Payne, 1980; Payne, 1980a, b, c; Snell, 1980; Wolfinbarger, 1980b), however it has not been well studied in rumen microorganisms (Hobson & Wallace, 1982b; Cotta & Hespell, 1986a; Broderick *et al.*, 1988; Mackie & White, 1990; Russell *et al.*, 1990). Different transport systems for peptides and amino acids, which usually involve active processes that require energy, have been found in a number of other bacteria (Rice *et al.*, 1978; Anraku, 1980; Payne, 1980a, c; Snell, 1980; Chen *et al.*, 1987b; Russell *et al.*, 1988b), hence it may be reasonable to assume that similar systems exist in rumen bacteria. In fact, Pittman *et al.* (1967), Wright (1967), Stevenson (1979), Chen *et al.* (1987b), Hino & Russell (1987), Broderick *et al.* (1988), Chen & Russell (1988) and Russell *et al.* (1988b) have shown amino acid and/or peptide uptake systems in *B. ruminicola*, *Streptococcus bovis*, species of *Peptostreptococcus* and mixed rumen microorganisms, and peptide uptake appears to be the rate limiting step in peptide utilization in artificial media (Chen & Russell, 1988). More

importantly it appears to be the rate-limiting step of protein utilization in the rumen (Russell *et al.*, 1983; Chen *et al.*, 1987a; Hino & Russell, 1987), however Broderick *et al.* (1988) suggest that peptide hydrolysis may be the rate-limiting step in the conversion of protein to ammonia, at least for rapidly degraded proteins. Interestingly, Chen *et al.* (1987b) have shown that hydrophilic peptides are catabolised twice as fast as hydrophobic peptides by mixed rumen bacteria, and Hobson & Wallace (1982b) suggest that the rather unusual property of preference for peptides over amino acids may be a more general one for rumen microorganisms.

Following transport into the bacterial cell, peptides are usually hydrolysed intracellularly prior to utilisation of the amino acids either directly for protein synthesis or, after further breakdown, as a source of nitrogen, carbon or sulphur (Payne, 1980a; Snell, 1980). Apart from being an experimental fact, this is also intuitively correct, since the peptide-bound amino acids would rarely occur in the correct sequence to be of use to the bacteria. Thus, the apparent preference of *B. ruminicola* BA25 for Tryptone as a nitrogen source can be justified in the rumen, since the ability of a bacterium to transport peptides before they are further degraded to amino acids, and ultimately ammonia, protects the constituent amino acids from catabolism and is advantageous because of the intense competition for nutrients which occurs in this environment (Wright, 1967; Payne, 1980a; Snell, 1980). This premise is indirectly supported by Hobson & Wallace (1982b) and Wallace & Cotta (1988), who state that the fate of free amino acids in the rumen is to be degraded rather than incorporated intact into microbial protein.

The improved growth performance of cells supplied with peptide nitrogen rather than free amino acid nitrogen may also reflect a more effective and energetically efficient means of uptake of the peptide-bound amino acids compared to the component free amino acids (Wallace & Cotta, 1988), since some incorporation of amino acids into cell material does occur (Hobson & Wallace, 1982b). This idea is supported by Wright (1967), who suggested that peptide uptake (in mixed rumen bacterial samples) may stimulate growth by providing amino acids which are in limited supply to the cells because of inadequate uptake or rapid metabolism. Similarly, Snell (1980) indicates that, since peptides and free amino acids commonly enter bacteria by different transport systems, the constituent amino acids of these peptides may bypass transport systems for free amino acids that are not fully operative. Peptides may therefore maintain a high level of essential (and non-essential) amino acids within the cells (Rice *et al.*, 1978), thus enhancing growth over that achieved with free amino acids.

Chen *et al.* (1987b) consider that the low concentration of amino acids in the rumen may indicate that peptides, not amino acids as previously thought, are the end products of protein degradation. Nevertheless, amino acid transport in *B. ruminicola* BA25 (and other species), which clearly is not as effective as peptide uptake, may provide a means of scavenging residual amino nitrogen, as suggested for *Streptococcus bovis* by Russell *et al.* (1988b). The sodium-dependent transport of branched-chain amino acids by a ruminal peptostreptococcus may be an example (Chen & Russell, 1989c). It is often suggested that the fate of amino acids in the rumen is predominantly catabolic, rather than to be assimilated directly into microbial protein (Hobson & Wallace, 1982b; Wallace & Cotta, 1988; Mackie & White, 1990), and it is feasible that this also occurs to peptide-bound amino acids after they enter the cell. Thus, although both uptake systems may provide an auxiliary supply of the amino acids for immediate use in biosynthesis (Stevenson, 1979), they may simply provide a source of nitrogen to the cell via amino acid catabolism.

The utilisation of exogenous proteins by bacteria initially requires the degradation of these to peptides and amino acids prior to utilisation by the cells, since direct uptake of utilisable proteins generally does not occur in bacteria (Law, 1980; Wallace & Cotta, 1988). In the current experiments it is likely that casein was degraded to its constituent peptides and amino acids by a highly efficient extracellular peptidase produced by *B. ruminicola* BA25, which would have subsequently transported the peptides and amino acids into the cells. Clearly, peptides would be the products of this degradation and would be utilised most efficiently for growth, however ammonia uptake in *B. ruminicola* BA25, by active or passive means (Mackie & White, 1990; Russell *et al.*, 1990), was also faster or more effective than amino acid uptake and metabolism. Ammonia may also be an important nitrogen source for growth of this species on casein, assuming that proteolysis resulted in production of ammonia, in addition to peptides and amino acids. Ammonia may also be important as a nitrogen source in conjunction with amino acids, but it does not appear to be transported when peptides are present in the media. Despite these possibilities, data from the enzyme experiments (section 3.4.3) for cells grown in RGM, in which peptides were present, indicated that catabolism of the amino acids and ammonia derived from peptides is not a feature of this species, since only very low GS, GDH, GOGAT and AlaDH activities were found. Thus, peptides are likely to have been the major form of nitrogen taken up by the bacterial cells when grown with casein.

3.4.4.4 Nitrogen Source Preferences for Growth by *S. ruminantium* MP72

As was the case for *B. ruminicola* BA25 cultures, *S. ruminantium* MP72 cultures supplemented with NH_4Cl, Tryptone or casein as sole nitrogen sources, and 0.5 % [w/v] glucose, resulted in more traditional logarithmic growth patterns and larger changes in optical density (> 1.1 units) than were evident when only 0.05 % [w/v] glucose was supplied in similar cultures. In contrast, cultures supplemented with Casamino Acids as the sole nitrogen source, and 0.5 % [w/v] glucose, did not grow effectively (change in optical density of only 0.4 units). Thus, this species is able to utilise ammonia, peptides and protein, but not amino acids, as nitrogen sources when adequate glucose is present. The different cell densities and maximum growth rates achieved in these cultures (Table 3.22) indicate that Tryptone (peptides) was the most effective nitrogen source for growth of *S. ruminantium* MP72, followed in descending order by NH_4Cl (ammonia) and casein (protein). Casamino Acids (amino acids) were a very poor nitrogen source for this species. Thus, peptide uptake and utilisation is more effective than ammonia, protein or amino acid uptake and utilisation in both of these species.

In contrast to growth by *B. ruminicola* BA25, lag times and the times from inoculation to stationary phase of growth of *S. ruminantium* MP72 cultures were about the same when Tryptone was the sole nitrogen source as when either NH_4Cl or casein were the sole sources. Thus, the breakdown/transport systems for peptides may be similar for ammonia and protein in *S. ruminantium* MP72, in contrast to *B. ruminicola* BA25 where differences between the nitrogen sources were evident. The lag time and time from inoculation to stationary phase of culture growth values were inconclusive for cultures supplemented with Casamino Acids as the sole source of nitrogen, due to the very poor growth *per se*. Growth parameters for RGM cultures of *S. ruminantium* MP72 were similar to those for Tryptone based cultures, except that maximum cell density in the RGM cultures was less.

Although the length of the lag until log phase of culture growth was shorter for *B. ruminicola* BA25 cultures than *S. ruminantium* MP72 cultures, the doubling time of the respective cultures, and the maximum optical densities for *B. ruminicola* BA25 cultures tended to exceed those of the corresponding *S. ruminantium* MP72 cultures. Interpretation of the relative importance of the maximum optical density values is difficult for the two species, since cell sizes must influence the cell densities. Nevertheless, it appears that *B. ruminicola* BA25 cultures generally grew more vigorously than those of *S. ruminantium* MP72, indicating that

the former may have more effective and efficient hydrolysis and uptake systems for protein and its breakdown products (peptides, amino acids, ammonia). In particular, *B. ruminicola* BA25 clearly has more effective and efficient uptake and utilisation systems for amino acids than does *S. ruminantium* MP72.

Combinations of the nitrogen sources resulted in growth of *S. ruminantium* MP72 cultures which was intermediate between that achieved when either nitrogen source was present singly. This was also observed for *B. ruminicola* BA25 cultures. Thus, a nitrogen source which produced maximum growth determined the growth pattern of the cultures when less effective nitrogen sources were added. This is not surprising, but is an important observation because it also occurred in cultures in which Casamino Acids were supplied as one of the nitrogen sources. This implies that other nitrogen components were not inhibitory to cell growth, as might be suggested from the poor growth performance of this species when free amino acids were the sole nitrogen source. The two exceptions to this conclusion were when NH_4Cl plus Tryptone and NH_4Cl plus casein were the nitrogen sources. Under these conditions growth was superior to that observed when Tryptone was present alone. The very poor growth of *S. ruminantium* MP72 with Casamino Acids as the sole nitrogen source therefore indicates that this species may lack, or at least have ineffective, uptake mechanisms for free amino acids. Rice *et al.* (1978) suggest that uptake of some amino acids can occur by passive diffusion, but Russell *et al.* (1990) point out that hydrophilic compounds such as amino acids do not easily pass through lipid bilayers and must be transported across cell membranes on carrier proteins. Neither of these possibilities explain the poor growth of *S. ruminantium* MP72 on free amino acids. Amino acid uptake mechanisms have not been studied in species of *Selenomonas*.

S. ruminantium MP72 cultures supplemented with NH_4Cl or NH_4Cl plus casein were the only ones in which a large proportion of ammonia (> 45 %) was utilised. The NH_4Cl plus Casamino Acids culture did not significantly differ in ammonia concentration prior to growth and after cell harvest, however growth of all other cultures resulted in significantly increased ammonia concentrations at harvest compared to the levels prior to growth. These increases tended to be greater than those for *B. ruminicola* BA25 cultures, indicating that *B. ruminicola* BA25 is efficient in utilising the amino acids derived from protein and peptide

breakdown. In contrast, *S. ruminantium* MP72 may not use such a large quantity of these products, but instead proteins, peptides and amino acids are converted to ammonia.

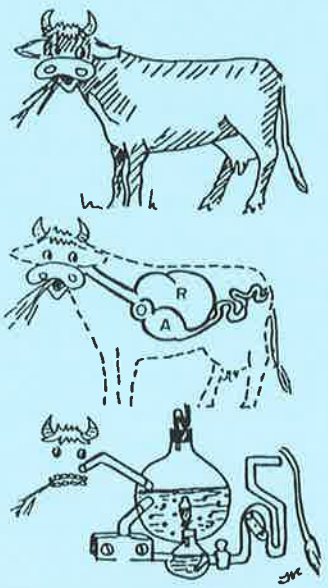
The variety of nitrogen source preferences evident in this very small sub-sample of rumen species indicates that a full range of nitrogen source preferences may exist within the large number of bacterial species in the rumen. For example, ammonia is the major and preferred nitrogen source in species of *Ruminococcus*, but does not stimulate growth in the *Lactobacilli* or some strains of *Bacteroides ruminicola*, whereas utilisation of amino acids is good by *Peptostrptococcus elsdenii*, but poor by *Ruminococcus flavefaciens* (Bryant & Robinson, 1961; Hungate, 1966; Krieg & Holt, 1984; Russell, 1984). Investigation of ammonia assimilation in rumen bacteria with all types of nitrogen source preferences is necessary before accurate predicitions of ammonia assimilation *in vivo* can be made.

CHAPTER 4

Final Discussion and Conclusions

"Ruminants eat intermittently but metabolise continuously "

(Baldwin, 1968)



Development of the rumen (2)

Czerkawski (1986)

Chapter 4

Conclusions and General Discussion

4.1 CONCLUSIONS FROM EXPERIMENTS 1 and 2 (Chapter 2 : Activity of Ammonia-Assimilating Enzymes in Rumen Bacteria *in vivo*)

4.1.1 AIMS AND SUMMARY OF RESULTS

As discussed in the Introduction to Chapter 2, the aims of the *in vivo* experiments (1 & 2) were firstly to determine which, if any, of the rumen fluid parameters (i.e. pH, ammonia, carbohydrates or free protein) affect the daily activity of the ammonia-assimilating enzymes; secondly, to collate data on the activities of the ammonia-assimilatory enzymes under a variety of *in vivo* conditions for comparison with pure culture activity data; and thirdly, to investigate the short-term regulation of these enzymes *in vivo* under conditions where day-to-day variation in rumen conditions was minimised.

Hespell (1984) suggested that, for nitrogen-limited growth, or when environmental ammonia levels are less than 1 mM, the GS pathway of ammonia assimilation predominates in the rumen, whereas when ammonia levels are higher, the GDH pathway is more important. The data presented in Chapter 2 support this hypothesis and provide evidence that GOGAT is also an important enzyme. These data therefore support Hespell's (1984) view that one pathway does not predominate in overall ammonia assimilation in the rumen. Nevertheless, they do indicate that the GS pathway may predominate, at least in some bacteria, at very low ammonia concentrations, probably as the GS/GOGAT pathway. This has also been found in microorganisms from other environments (*Rhodobacter (Rhodopseudomonas) capsulata* (Moreno-Vivián *et al.*, 1983); *Beggiatoa alba, Xanthobacter* sp., *Anabaena* sp., *Rhodopseudomonas acidophila* (Vargas & Strohl, 1985); *Sclerotinia sclerotiorum* (Rachim & Nicholas, 1985); *Azospirillum brasilense* (Westby *et al.*, 1987); and in soybean nodules and most plants (Pate, 1989)). The GS/GOGAT pathway, however, declines in activity and importance as ammonia concentrations rise above about 1 mM (0.5 mM according to Erfle *et al.* (1977)). GDH activity is fairly constant at low rumen ammonia concentrations (between 1 and 5 mM), and increases at higher concentrations (mainly through increases in NADH-dependent

activity), thereby becoming the predominant assimilatory enzyme. GOGAT activity, clearly important in this scheme at low rumen ammonia concentrations, appears to be constitutively present at all levels of rumen ammonia, however its role when the value exceeds 1 mM (i.e. when GS activity is low) is not clear. AlaDH and AsS, in contrast, do not appear to play significant roles in ammonia assimilation *in vivo*, at least in the diets studied.

4.1.2 FACTORS INFLUENCING *in vivo* ENZYME ACTIVITY

This summary of ammonia assimilation in the rumen, based on the experiments outlined in Chapter 2, largely confirms the expectations and observations of other investigators in this field (Chalupa, 1972; Erfle *et al.*, 1977; Baldwin & Denham, 1979; Hobson & Wallace, 1982b; Hespell, 1984; Mackie & White, 1990) and fulfils the second of the aims listed above.

Results from experiments 1 and 2 (Chapter 2) also support the view that, although the ammonia concentration in the rumen apparently has some effect on the activity of at least some of the ammonia-assimilating enzymes, this parameter cannot explain all of the changes in enzyme activity. It is also likely that parameters such as pH, free protein and carbohydrates are not the only others influencing the activity of ammonia-assimilating enzymes. At least two interdependent "factors" may therefore affect ammonia assimilation in the rumen. As a consequence of diet, rumen conditions such as pH, nitrogen, energy and carbon source largely determine the proportions of various rumen microbial species. Differing proportions of these species in turn affect the total activities of the enzymes involved in ammonia assimilation. General rumen conditions, especially the ammonia content, clearly have an important influence upon the basal GS activity in the rumen population for any particular diet. Day-to-day variations under these conditions, however, appear to have limited effects upon the overall activities of the ammonia-assimilating enzymes.

The pH, ammonia and free protein values in experiments 1 and 2 were measured in small sub-samples of rumen fluid and assumed to be representative of the values in the whole rumen. Nevertheless, an important consideration when sampling from the rumen is that microorganisms are located in a wide range of different microenvironments (e.g. different plant materials, clustered around starch, free in the fluid, etc.), each of which has unique pH, ammonia, free and bound protein and carbohydrates contents (Hobson & Wallace, 1982b; Odle

& Schaefer, 1987; Owens & Zinn, 1988; Wallace & Cotta, 1988). The effects of nitrogen (ammonia), energy and carbon source on enzyme activities within these microenvironments are likely to be very different to averaged effects over the whole rumen, which is effectively what is measured using sub-samples of rumen fluid. In fact, individual microenvironments are likely to be affected in different ways depending upon the species composition of the microenvironment, growth phase and cell growth rates of the microorganisms, as well as substrate availability. Thus, a high concentration of ammonia in the rumen may be needed to penetrate microenvironments and therefore reach microbes in isolated niches (Owens & Bergen, 1983). The statement by Hespell (1984) that ". . . *as found for other bacterial species, the pathways of ammonia assimilation in ruminal bacteria are influenced by the levels of ammonia available to the cells* . . ." may therefore be true, but there is no information about what these levels are *in vivo*.

Another confounding factor in interpreting rumen data is the lack of information about the parameters actually encountered by the enzymes and about the uptake mechanisms for ammonia by microorganisms in the rumen. Uptake mechanisms could be different for various species and may be modified by environmental conditions (Hespell, 1984; Russell & Strobel, 1987). Thus, the factors which affect intra-cellular levels of ammonia and other enzyme substrates are clearly of great significance in determining the mechanisms of ammonia assimilation and cannot be determined from *in vivo* studies.

4.1.3 NICOTINAMIDE ADENINE DINUCLEOTIDE SPECIFICITIES FOR GOGAT, GDH AND AlaDH IN THE RUMEN

NADH-dependent activities of GOGAT, GDH and AlaDH predominated over NADPH-dependent activities in bacterial samples for the diets offered in experiments 1 and 2 (Chapter 2). Although Chalupa *et al.* (1970), Wallace (1979) and Lenártová *et al.* (1985) have shown similar effects for these enzymes from rumen bacteria *in vivo*, the significance of this observation is not clear. In contrast, Erfle *et al.* (1977) found the opposite trend for GDH in mixed rumen bacterial cultures. It can also be concluded from experiments 1 and 2 that NADPH-dependent activities of GOGAT, GDH and AlaDH are relatively unaffected by rumen conditions and the enzymes may be constitutive, whereas the NADH-dependent activities of these enzymes fluctuate in response to undetermined factors.

The presence of both NADH- and NADPH-specificities for GOGAT, GDH and AlaDH in the rumen indicates that a range of iso-enzymes, with various cofactor requirements, occur within the rumen bacterial population for each enzyme activity. Previous reports for non-rumen microorganisms indicate that the NADPH-dependent activity of GDH is involved in the biosynthesis of glutamate, whilst NADH-dependent activities have a catabolic function under low ammonia and/or high amino acid conditions (Brown *et al.*, 1974; Smith *et al.*, 1975; Tyler, 1978; Martinez-Bilbao *et al.*, 1988). In contrast, the predominance of NADH-dependent GDH activity in the rumen when the ammonia concentration was relatively high in experiments 1 and 2 indicates that the NADH-dependent activity may be biosynthetic in the rumen (assimilating ammonia-nitrogen to amino-nitrogen) under these conditions. An alternative interpretation is that the amino acids available as a consequence of the digestion of feedstuffs in the rumen "drive" the catabolic reaction, whilst the low activity of NADPH-dependent GDH assimilates ammonia-nitrogen. Although the GOGAT reaction is apparently unidirectional (Brown *et al.*, 1974), it is interesting to speculate that the NADH-dependent activities of GOGAT and AlaDH may also be catabolic when amino acids are available, especially since the GOGAT-catalysed reaction would utilise two moles of glutamate. Conversely, the GOGAT activity may be biosynthetic, utilising the 2-oxoglutarate formed by the catabolic activity of GDH, plus glutamine (i.e. glutamine + 2-oxoglutarate $\rightarrow$ 2 glutamate). If the concept of catabolic NADH-dependent activity for these enzymes is correct it is relevant that the lower, and fairly constant, NADPH-dependent activities are still present and operating biosynthetically, apparently constitutively.

Irrespective of the NADH/NADPH-specificities for GOGAT, GDH and AlaDH, the very low activities of AlaDH, as well as AsS, indicate that these two enzymes play insignificant roles in ammonia assimilation in the rumen, and, in fact, may be non-important alternatives to the major pathways of GS/GOGAT and GDH. Nevertheless, this is the first report of GS, AsS, and NADH- and NADPH-dependent GOGAT, GDH and AlaDH activities present concurrently in the rumen microflora, confirming that the same ammonia-assimilating enzymes occur in this ecosystem as are present in other (non-rumen) environments.

4.1.4 REGULATION OF THE AMMONIA-ASSIMILATING ENZYMES *in vivo*

In experiment 2 (Chapter 2), boluses of NH_4Cl, glucose, or NH_4Cl plus glucose, were added to the rumen of animals on two extreme diets and the effects on the activities of the ammonia-assimilatory enzymes monitored. From this experiment it was concluded that GS is sensitive to rapid increases in rumen ammonia concentration when its activity is high, usually as a result of a very low rumen ammonia concentration (i.e. below 1 mM). The regulation (dose-dependent decrease) of GS activity is rapid, occurring within 30 mins of the bolus addition to the rumen. In contrast, GS activity was relatively insensitive to similar increases when GS activity was low (i.e. at rumen ammonia concentrations above 1 mM). The GOGAT, GDH, AlaDH and AsS activities were also insensitive to the bolus additions, irrespective of their activity values.

Thus, there appears to be a low basal GS activity for the rumen flora which can increase in response to rumen conditions (i.e. ammonia concentration below 1 mM), but it did not decrease further. When GS activity is at this very low level, the rumen flora probably assimilate sufficient nitrogen for their needs via other assimilatory pathways. The other ammonia-assimilating enzymes studied did not appear to respond to increases in ammonia and/or glucose contents in the rumen, hence their control mechanisms in response to ammonia concentration are not as effective as those for GS.

In section 4.1.2 it was suggested that the levels of rumen ammonia, pH, free protein and carbohydrates could not account for the changes in enzyme activity in rumen bacteria *in vivo*. In contrast, the results discussed above indicate that rumen factors, in particular ammonia concentration, can have direct effects upon GS enzymes in the rumen microbial population. Nevertheless, the fact remains that enzyme activities fluctuate independently of the day-to-day changes in these parameters. An explanation for these contrasting observations may be that averaged concentrations of substrates, such as those measured in sub-samples of rumen fluid from day-to-day, do not affect the rumen flora due to the existence of microenvironments within the rumen. If the concentrations of nutrients and substrates are changed, either rapidly via a bolus of one or more compounds, or more gradually by changing the animal's diet, conditions in the microenvironments may also be affected. Under these conditions the enzyme activities may be affected. GS activity clearly responds to such changes in environmental ammonia concentration, whereas the other enzymes studied were not affected.

4.2 CONCLUSIONS FROM EXPERIMENTS 3.1 TO 3.4

(Chapter 3 : Ammonia Assimilation in Selected Species of Rumen Bacteria)

4.2.1 INTRODUCTION AND AIMS

In vivo experiments, such as those described in Chapter 2, have serious limitations, for example difficulty in obtaining a bacterial sample which is truly representative of the population, influences the animal can have upon the microbial population and the inconsistency of methods for separating bacteria from other rumen components (see Literature Review; section 1.6.1). Many of these problems are highlighted in Chapter 2 by the difficulty encountered in interpreting the results, and, as a consequence, experiments involving pure cultures of some species of rumen bacteria were planned as the second stage of the investigation into the assimilation and metabolism of nitrogen by rumen bacteria. Such cultures should have overcome many of the problems associated with *in vivo* experiments, in particular the interpretation of results from experiments involving mixed cultures of rumen bacteria. Nevertheless, pure culture investigations also have their own limitations.

The most striking limitation of pure culture studies of rumen bacteria is that the data may not be relevant to the natural system. Nevertheless, bacterial growth conditions can be manipulated in batch cultures so that, for example, experiments can be performed to determine what enzymes are present in the species, whether the enzymes are important for growth under certain conditions, and how the enzymes are regulated. It is clearly not possible to draw conclusions about ammonia assimilation in the rumen from such experiments, but with data from many rumen bacterial species it may be possible to accurately predict these processes. The major findings from these experiments presented in Chapter 3 are summarised in Table 4.1.

4.2.2 AMMONIA ASSIMILATION IN *Bacteroides ruminicola* subsp. *brevis*

4.2.2.1 Growth Details of *B. ruminicola*

B. ruminicola BA25 is a locally isolated strain of the numerically important species *B. ruminicola*. This species is able to utilise a wide range of carbon and nitrogen sources, is proteolytic, can represent 6 to 19 % of the carbohydrate fermenting bacteria present in the rumen, and is maintained at relatively high levels under a wide variety of feeding

Table 4.1 : Summary of the major findings from Chapter 3.

"X" in a cell indicates that inhibition or enzyme activity was not evident, or that the species did not grow in the particular nitrogen source.
"√" for experiments 3.2 & 3.3 indicates that the activity was detected, or that the species grew in that nitrogen source.
"-" for experiments 3.2 & 3.3 indicates that the activity was not measured; for experiment 4 this indicates that growth was not tested in the particular nitrogen source.

† GS K_m-value is expressed as mM hydroxylamine; GOGAT K_m-value is for the NADPH-dependent activity, and is expressed as mM L-glutamine; GDH (NADPH & NADH) K_m-values are expressed as mM NH_4Cl.
£ GS V_{max}-value is expressed as µmoles γ-glutamyl hydroxamate produced $min.^{-1}$; GOGAT and GDH V_{max}-values are expressed as nmoles NAD(P)H oxidised min^{-1}.
§ The greater the number of ticks for a particular nitrogen source the greater the preference by that species for the particular nitrogen source.

SPECIES	Experiment 3.1 INHIBITION BY		Experiments 3.1 and 3.2 ACTIVITY OF							Experiment 3.4 ENZYME KINETICS		Experiment 4 NITROGEN SOURCE PREFERENCES§				
			GS	GOGAT		GDH		AlaDH								
	MSX	Aza.	γ-GT	NADH	NADPH	NADH	NADPH	NADH	NADPH	K_m †	V_{max}£	As an Energy Source	NH_4Cl	Amino Acids	Peptides	Protein
B. ruminicola subsp. *brevis* strain BA25	2-128 mM NFMS cultures (not RGM)	0.25-128 mM NFMS cultures (not RGM)	√	low	√√ (10x NADH)	low	√√ (100x NADH)	low	√√ (10x NADH)	GS = 4.5 GOGAT = 2.5 GDH : NADPH = 8.1 NADH = 24.3	0.06 7.05 8.12 0.98	X	√√√	√	√√√√	√√
B. ruminicola subsp. *ruminicola*	30-50 % in NFMS (25-30% in RGM)	30-50 % in NFMS (25-30% in RGM)	-	-	-	-	-	-	-	-	-	-	X	(√)		-
S. ruminantium subsp. *ruminantium* strain MP72	0.25-32 mM NFMS cultures (not RGM)	0.25-128 mM NFMS cultures (not RGM)	X	low	√√	low	√√	√	√	-	-	X	√√	X	√√√	√
S. ruminantium subsp. *ruminantium* strain ME13	0.25-32 mM NFMS cultures (not RGM)	0.25-128 mM NFMS cultures (not RGM)	-	-	-	-	-	-	-	-	-	-	√	(√) RGM		-
Butyrivibrio fibrisolvens strain 49	X	20 % in NFMS cultures	X	√	√	√	√	√	√	-	-	-	X	(√)		-

conditions (Bryant, 1959; Hungate *et al.*, 1964; Hungate, 1966; Joyner & Baldwin, 1966; Howlett *et al.*, 1976; Ogimoto & Imai, 1981; Russell & Hespell, 1981; Russell, 1984). Although it is therefore very important in the rumen, little is known about its ammonia assimilation.

Growth of *B. ruminicola* BA25 in defined media at a wide range of NH_4Cl concentrations (0 to 128 mM) indicated that, although approximately 8 mM NH_4Cl was optimal for growth when this was the sole nitrogen source, this species could grow at ammonia concentrations below 1 mM (down to approx. 0.1 mM). Further study is clearly necessary to establish whether this is due to an active ammonia uptake mechanism in the species. In addition, although this species could grow with ammonia as the sole nitrogen source, peptides may be preferred as a nitrogen source since its growth was faster and more vigorous when peptides were at least one of the nitrogen sources.

4.2.2.2 GS in *B. ruminicola* BA25

Results from the inhibitor experiments in Chapter 3 (experiment 3.1) indicate that ammonia assimilation in *B. ruminicola* BA25 does not fit Hespell's (1984) model. In particular, GS did not appear to be the principal assimilatory enzyme when less than 1 mM NH_4Cl was the sole nitrogen source, however this enzyme was important for growth at NH_4Cl concentrations between 1 and 128 mM. These results indicate that other enzymes, possibly including GS, assimilate ammonia in *B. ruminicola* BA25 under very low ammonia concentrations, as has been shown for *Rhodobacter (Rhodopseudomonas) capsulata* strain E1F1 by Moreno-Vivián *et al.* (1983) (AlaDH, coupled with alanine : 2-oxoglutarate aminotransferase activity, were active when GS was inhibited with MSX).

It is possible, however, that the results from experiment 3.1 may be an artifact brought about by transport effects associated with MSX treatment (see section 3.4.2.2). This possibility is further supported by the results of enzyme experiments with *B. ruminicola* BA25 (experiment 3.2) in which GS activity was found to be inversely related to the media ammonia concentration. It is not clear whether the GS activity is induced by a low ammonia concentration or repressed by a high ammonia concentration, although the relationship between GS activity and media ammonia concentration is an exponential one in which media ammonia

concentration may act directly as the main inhibitor of GS. End products of glutamine metabolism may also be inhibitory (cumulative feedback inhibition). Nevertheless, the high GS activity observed at the very low ammonia concentrations at which MSX did not inhibit cell growth (i.e. < 1 mM) appear to support the view that MSX transport into the cell may be affected at these ammonia concentrations. This is clearly an area where further work is needed.

Further evidence for *B. ruminicola* BA25 not fitting the ideal ammonia-assimilatory model suggested by Hespell (1984) is that the K_m-value for hydroxylamine was 4.5 mM for GS, indicating that the enzyme may not be a nitrogen scavenger, as was suggested by Stadtman (1973), Brown *et al.* (1974) and Brown (1980). The enzyme has a relatively low affinity for ammonia despite its very high activity at low ammonia concentrations, which may be a further indication of active ammonia transport occurring in this species.

The inverse relationship between GS activity and media ammonia concentration also shows that, quantitatively, the GS activity appeared to be unimportant for ammonia assimilation in *B. ruminicola* BA25 at very high media ammonia concentrations (e.g. 16 - 128 mM). Nevertheless, MSX inhibited growth at these ammonia concentrations, indicating that the cells may have a requirement for at least a low level of glutamine from GS at all times when NH_4Cl is the sole nitrogen source. Although this conclusion also requires confirmation, it is not surprising given the central role of glutamine and glutamate in nitrogen metabolism (Literature Review; section 1.9.1).

It is significant that GS activity was lower when *B. ruminicola* BA25 was grown in RGM than in NFMS media supplemented with NH_4Cl, and that cell growth was unaffected by MSX in RGM cultures. These results indicate that GS was not essential for cell growth when more complex nitrogen sources were available for growth (e.g. amino acids and/or peptides), and that GS activity may have been repressed by these more complex nitrogen sources. Moreover, these results, in conjunction with the low enzyme activity when 128 mM NH_4Cl was provided, indicate that GS is constitutively present in *B. ruminicola* BA25.

4.2.2.3 GOGAT, GDH and AlaDH in *B. ruminicola* BA25

In contrast to GS, GOGAT was apparently vital for growth by *B. ruminicola* BA25 at all media ammonia concentrations studied (i.e. 0 to 128 mM), as determined by

azaserine inhibition of growth (experiment 3.1). Nevertheless, further studies are required to confirm this conclusion because the inhibitor may not be specific for GOGAT (Miflin & Lea, 1976; Fentem *et al.*, 1983). GOGAT, GDH and AlaDH activities were much higher with NADPH as the electron donor than with NADH when NH_4Cl was the sole nitrogen source, in contrast to the *in vivo* data (Chapter 2). Although NADH- and NADPH-dependent activities were detected for each of these enzymes, the NADPH-dependent GOGAT and GDH activities appear to be quantitatively important in this species. In contrast to the activity of GS, the NADPH-dependent activity of GOGAT and GDH were not greatly influenced by ammonia concentration in the media.

Thus, the products of the GS/GOGAT pathway of ammonia assimilation, namely glutamine and glutamate, are clearly vital for the nitrogen metabolism of *B. ruminicola* BA25 when more than 1 mM NH_4Cl is available as the sole nitrogen source in the medium. Further study is required to clarify the interactions of MSX and GS occurring at ammonia concentrations below 1 mM so that this pathway is confirmed to be important at very low ammonia concentrations. Although GS activity declines as the media ammonia concentration increases, the NADPH-dependent activity of GOGAT remained relatively unchanged. This enzyme has a K_m-value for L-glutamine of 2.5 mM, indicating that it is fairly efficient at utilising the glutamine produced by GS.

Although the NADPH-dependent GDH activity detected in *B. ruminicola* BA25 was relatively high at all media ammonia concentrations, and therefore appeared to be quantitatively important, this enzyme alone may be unable to supply sufficient glutamate for cell growth. This is apparent since GS and GOGAT were still vital for growth by *B. ruminicola* BA25 at very high media ammonia concentrations (i.e. 128 mM), even though GDH is normally expected to be the predominant ammonia-assimilatory pathway under these conditions (Stadtman, 1973; Brown *et al.*, 1974; Tyler, 1978; Brown, 1980; Hespell, 1984). The K_m-value of 8.1 mM for NH_4Cl by this enzyme (NADPH-dependent) supports the widely held belief that the enzyme is an important assimilator of ammonia when it is present at high concentrations, but has a negligible role when the ammonia concentration is low (Stadtman, 1973; Brown *et al.*, 1974; Tyler, 1978; Brown, 1980; Hespell, 1984). Nevertheless, activity of an enzyme does not necessarily mean that it is important biosynthetically (Vairinhos *et al.*,

1983; Mackie & White, 1990). This applies to GDH in this species because of the vital role of the GS/GOGAT pathway.

Assuming that the NADPH-dependent GDH activity has a biosynthetic role in *B. ruminicola* BA25, then this is the major function of the enzyme when NH_4Cl is the sole nitrogen source. The relatively fixed ratio of NADH- to NADPH-dependent GDH activities under these growth conditions, plus the 10-fold increase in the ratio (due to lower NADPH-dependent activity) when the cells were grown in RGM, may indicate that at least two separate GDH enzymes exist in *B. ruminicola* BA25. One of these enzymes would possess dual coenzyme specificity and relatively low activity, whilst the other would only have NADPH-dependent activity which is highly controlled by the nitrogen source in the medium. The latter activity is high when NH_4Cl is the sole nitrogen source, but is low when amino acids and/or peptides are present. Interestingly, the ammonia-dependent activity does not appear to be effectively controlled by varying ammonia concentrations. Thus, ammonia assimilation occurring when NH_4Cl is the sole nitrogen source is largely switched off when amino acids and/or peptides are available, and the catabolic activity of GDH (NADH-dependent activity) did not increase. Instead the nitrogen may be assimilated by transamination and catabolism of the amino acids and peptides via undetermined pathways.

In contrast to GDH, GOGAT apparently catalyses a unidirectional reaction (Brown *et al.*, 1974), although this designation may only apply because catabolic activities have not been discovered. The relatively fixed NADH/NADPH ratio for this enzyme with NH_4Cl as the sole nitrogen source, plus the large increase in the ratio (due to decreased NADPH-dependent activity) when the cells were grown in RGM, virtually parallels that for GDH. Thus, GOGAT activity also appears to be shared between two enzymes, one with dual coenzyme specificity and one which is NADPH-dependent only. These activities also were relatively insensitive to ammonia level in the medium.

4.2.2.4 A Scheme for Ammonia Assimilation in *B. ruminicola* BA25

Although these studies highlighted some of the deficiencies of inhibitor studies, they also indicated that *B. ruminicola* BA25 does not rigidly follow the ammonia-assimilatory pathways described in other microorganisms (Stadtman, 1973; Brown *et al.*, 1974; Senior,

1975; Conn & Stumpf, 1976; Tyler, 1978; Brown, 1980; Stryer, 1981) and reported for some rumen bacteria (Hespell, 1984). In particular, the GS/GOGAT pathway is vital for growth of this species when NH_4Cl is the sole nitrogen source, irrespective of the ammonia concentration in the medium. Thus, the enzyme is essential for growth even when its activity is low as a consequence of very high ammonia concentrations. In contrast, the other major ammonia-assimilating pathway, GDH, appears to be quantitatively important to *B. ruminicola* BA25 when NH_4Cl is the sole nitrogen source, however this enzyme is unable to provide adequate glutamate even at very high ammonia concentrations. It is not known whether *B. ruminicola* BA25 is able to assimilate ammonia in the absence of GDH activity, however it appears that this is just one of the enzymes necessary for ammonia assimilation at high concentrations of the substrate. The other ammonia-assimilatory enzyme studied, AlaDH, was not important in this species.

B. ruminicola BA25 therefore appears to rely on the GS/GOGAT pathway of ammonia assimilation when ammonia is the sole source of nitrogen, at least when the ammonia concentration is between 0 and 128 mM. In addition, it appears likely that the GDH pathway is also important, at least at ammonia concentrations above about 8 mM, however it may also be important at much lower ammonia concentrations. In contrast, when amino acids and/or peptides are available as nitrogen sources for growth of *B. ruminicola* BA25, in addition to ammonia, organic nitrogen compounds are not derived from ammonia assimilation. Instead transamination and catabolism of the amino acids and/or peptides may be more important reactions. Other ammonia-assimilatory enzymes, such as AlaDH, are also present in *B. ruminicola* BA25, but may only occur as auxiliary enzymes to the major pathways of GS/GOGAT and GDH.

An interesting feature of the enzyme activity data from experiment 3.1 is that the GS activities measured in *B. ruminicola* BA25 *in vitro* were much higher than those in mixed bacterial samples at similar ammonia concentrations (experiments 1 and 2, Chapter 2). This may be a further indication of microenvironment effects occurring in the rumen. Although the ammonia concentration may have been similar in both studies, the actual concentration encountered by the rumen flora *in vivo* may have been higher than the value reported due to the microenvironment shielding the bacteria from the general concentration. The ammonia

concentration in these microenvironments is more relevant than the general rumen fluid values (Hobson & Wallace, 1982b).

4.2.3 AMMONIA ASSIMILATION IN *Bacteroides ruminicola* subsp. *ruminicola*

Although Krieg & Holt (1984) report that this sub-species is able to utilise ammonia as a nitrogen source, strain variations occur. The strain used in this thesis was unable to grow with NH_4Cl as the sole nitrogen source. Such variation in nitrogen metabolism within the broad classification which encompasses *B. ruminicola* (Ogimoto & Imai, 1981; Krieg & Holt, 1984; Paster *et al.*, 1985) indicates the importance of diverse species such as this in the rumen.

B. ruminicola subsp. *ruminicola* required amino acids and/or peptides for growth. Nevertheless, the glutamate and glutamine analogues, MSX and azaserine (respectively), inhibited growth in this sub-species by up to 50 %. Although these results may indicate that *B. ruminicola* subsp. *ruminicola* obtains some of its nitrogen requirement via the GS/GOGAT pathway of ammonia assimilation, despite the fact that it cannot grow with ammonia as the sole nitrogen source, two alternative explanations for those results are presented in Chapter 3 (section 3.4.2.4). Further study is necessary to confirm this hypothesis.

4.2.4 AMMONIA ASSIMILATION IN *Selenomonas ruminantium*

4.2.4.1 General Details of *S. ruminantium*

As is the case for *B. ruminicola*, *S. ruminantium* is able to utilise a wide range of carbon and nitrogen sources for growth, is proteolytic and is present at relatively high levels under a wide range of feeding conditions (Bryant, 1959; Hungate *et al.*, 1964; Hungate, 1966; Joyner & Baldwin, 1966; Hespell & Bryant, 1981; Ogimoto & Imai, 1981; Russell & Hespell, 1981; Russell, 1984). Hungate (1966) reports that 40 % of the total colony count from the rumen of steers fed cracked corn and urea was *S. ruminantium*. This species can be divided into two sub-species, namely *S. ruminantium* subsp. *ruminantium* and *S. ruminantium* subsp. *lactilytica*, which are distinguished by the latter's ability to ferment lactate (Kingsley & Hoeniger, 1973; Hespell & Bryant, 1981; Ogimoto & Imai, 1981; Krieg & Holt, 1984). Both sub-species were studied in this thesis.

S. ruminantium subsp. *ruminantium* has been the focus of extensive studies by Smith *et al.* (1980, 1981), and the current work extends these studies to the lactate-fermenting sub-species. Both strains studied were able to grow in batch culture with NH_4Cl as the sole source of nitrogen. As was the case for *B. ruminicola* BA25, growth was optimal in these strains when approximately 8 mM NH_4Cl was provided, but was also supported at much lower ammonia concentrations (down to approx. 0.1 mM). These sub-species also grew faster and more vigorously when peptides were supplied, indicating that this more complex nitrogen source was preferred for growth.

4.2.4.2 GS in *S. ruminantium*

Results from the inhibitor experiments of Chapter 3 (experiment 3.1) indicated that GS was essential for growth by *S. ruminantium* subsp. *ruminantium* strain ME13 and *S. ruminantium* subsp. *lactilytica* strain MP72 at ammonia concentrations from 0 to at least 32 mM (when NH_4Cl was the sole nitrogen source), however at very high ammonia concentrations (e.g. 128 mM) it was not vital for growth in either sub-species. Despite these results no γ-GT activity for GS was detected in *S. ruminantium* MP72 (experiment 3.2), confirming the results of Smith *et al.* (1980) with *S. ruminantium* subsp. *ruminantium* strain D. Thus, *S. ruminantium* appears to have GS activity which is essential for the assimilation of nitrogen compounds when ammonia is the sole nitrogen source, but GS does not catalyse the transferase reaction for this enzyme.

As for *B. ruminicola* BA25, GS was not essential for growth by either sub-species of *S. ruminantium*, and nor did they assimilate ammonia, when amino acids and/or peptides were available as nitrogen sources. These results confirm that peptides may be preferred over ammonia for growth of this species.

4.2.4.3 GOGAT, GDH and AlaDH in *S. ruminantium*

GOGAT was required for growth by *S. ruminantium* ME13 and *S. ruminantium* MP72 when NH_4Cl was the sole nitrogen source (0 - 128 mM; experiment 3.1). The NADPH-dependent activity of this enzyme was relatively low in *S. ruminantium* MP72, especially

when compared with that in *B. ruminicola* BA25. The NADH-dependent activities of GOGAT, GDH and AlaDH, however, were even lower and probably unimportant.

The NADPH-dependent GDH activity appeared to be quantitatively important, although it was not affected by the ammonia concentration in the medium. GDH may be especially important at high ammonia concentrations when GS was not essential for growth. AlaDH activity was also detected in *S. ruminantium* MP72 and may contribute to ammonia assimilation at high media ammonia concentrations.

4.2.4.4 Ammonia Assimilation in *S. ruminantium*

The results for *S. ruminantium* MP72 and *S. ruminantium* ME13 are similar to those of Smith *et al.* (1980) for *S. ruminantium* subsp. *ruminantium* strain D. They indicate that *S. ruminantium* MP72 (and probably *S. ruminantium* ME13) relies upon the GS/GOGAT pathway to assimilate ammonia when it is the sole source of nitrogen at concentrations below 32 mM. NADPH-dependent GDH activity was also detected at these low ammonia concentrations, but it may only be important for the assimilation of ammonia at higher concentrations. AlaDH may also contribute to ammonia assimilation, at least at high concentrations of ammonia.

4.2.5 AMMONIA ASSIMILATION IN *Butyrivibrio fibrisolvens*

Butyrivibrio fibrisolvens is also one of the most numerous and versatile rumen bacteria (Hungate *et al.*, 1964), fermenting a wide range of carbohydrates (Bryant, 1959; Hungate, 1966; Bryant, 1973; Ogimoto & Imai, 1981; Russell, 1984) and is well known as a starch digester. Hespell & Bryant (1981) report that most strains of *Butyrivibrio* utilise ammonia as the nitrogen source, but many strains also utilise mixtures of amino acids or complex nitrogen sources, such as casein hydrolysate or peptone. Despite a report by Cotta & Hespell (1986b) that *Butyrivibrio fibrisolvens* 49 was able to grow with NH_4Cl as the sole nitrogen source, in this thesis it did not grow in culture unless amino acids and peptides were included in the media.

Addition of MSX to cultures of *Butyrivibrio fibrisolvens* 49 did not inhibit their growth, whereas azaserine addition resulted in a 20 % inhibition. This species did not possess

γ-GT activity for GS, and GOGAT, GDH and AlaDH (NADH- and NADPH-dependent) activities were not important for nitrogen assimilation. Since azaserine is known to inhibit glutamine amide transfer reactions, including GOGAT (Miflin & Lea, 1976; Fentem *et al.*, 1983), these results indicate that ammonia assimilation is probably not important in this strain of *Butyrivibrio*. It is likely that *Butyrivibrio fibrisolvens* 49 derives its nitrogen requirements via transamination and catabolism reactions involving amino acids and peptides.

4.2.6 NITROGEN ASSIMILATION IN RUMEN BACTERIA

As a general rule for all of the species studied, transamination and catabolism reactions appear to be the major nitrogen-assimilatory mechanism in those which require amino acids and/or peptides for growth (*B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens*), as well as for the other species when amino acids and peptides were supplied as nitrogen sources in addition to ammonia (i.e. *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13).

4.3 CONCLUSIONS FROM EXPERIMENT 4

(Chapter 3 : Nitrogen Source Preferences for Growth)

The results of the nitrogen source preference experiments for *B. ruminicola* BA25 and *S. ruminantium* MP72 indicate that, even though ammonia is assimilated by fairly conventional enzyme systems when it is the only nitrogen source (section 4.2), these species are able to grow more readily in the presence of peptides as complex nitrogen sources. Free amino acids are not effective as nitrogen sources for *S. ruminantium* MP72, but can be utilised by *B. ruminicola* BA25, albeit less effectively than other nitrogen sources. Both species are able to hydrolyse protein (casein) and utilise the products. Nevertheless, growth with protein as the nitrogen source is not as effective as that with ammonia or peptides as the sole nitrogen source.

Thus, the hypothesis that peptide nitrogen is used as a nitrogen source by these species in preference to ammonia, free amino acids and proteins appears to be quite sound. In addition, ammonia and protein can be used as nitrogen sources by both species, but amino acids were not effectively utilised by *S. ruminantium* MP72. These nitrogen sources were not, however, used

as the sole energy source by *B. ruminicola* BA25 and *S. ruminantium* MP72. Despite the poor uptake rates of nitrogen sources relative to the uptake rate for glucose (Chen *et al.*, 1987b), peptides, ammonia and free amino acids (except in *S. ruminantium* MP72) are able to enter the cells at sufficient rates to supply the nitrogen source requirements of fast growing cultures.

These nitrogen source preference results do not detract from the importance of the ammonia assimilation data of experiments 3.1 to 3.4 (summarised in section 4.2). Although it is clear that *B. ruminicola* BA25 and *S. ruminantium* MP72 initially utilise peptide nitrogen when present in a microenvironment, they can also utilise ammonia, protein and free amino acid nitrogen. Thus, these species may utilise the nitrogen sources available in progression from peptides through to free amino acids, as they become available in the rumen microenvironments.

In contrast, *Butyrivibrio fibrisolvens* 49 did not grow with NH_4Cl as the sole nitrogen source (contrary to the results of Cotta & Hespell (1986b)), but grew well when amino acids and peptides were nitrogen sources. Like *B. ruminicola* BA25 and *S. ruminantium* MP72, *Butyrivibrio fibrisolvens* 49 is proteolytic, however results reported in this thesis indicate that this strain can utilise peptides and amino acids from proteolysis, rather than ammonia which is also produced. This species therefore could not survive in as many microenvironments in the rumen as could *B. ruminicola* BA25 and *S. ruminantium* MP72, which can utilise a wider range of nitrogen sources.

4.4 GENERAL DISCUSSION

4.4.1 AMMONIA ASSIMILATION IN THE RUMEN

The results from these experiments confirm that ammonia is assimilated by the bacterial population in the rumen by the same enzyme pathways as found for many other bacterial systems (Hespell, 1984). In particular, GS/GOGAT and GDH are the major assimilatory pathways *in vivo*, with the former predominating at ammonia concentrations below 1 mM, but declining in importance above this value. Conversely, GDH increases in importance as the ammonia concentration in the rumen increases. AlaDH and AsS were also detected in the rumen population, but only appear to be alternatives to the two major

assimilatory pathways. In addition, the results indicate that the GS activity detected in mixed rumen bacterial samples is sensitive to rumen ammonia when its activity is high, in response to very low ammonia concentrations.

Although these conclusions appear to be clear cut, they are partly based on results from largely artificial diets and their validity may therefore be questioned. Nevertheless, it is likely that conditions exist within the many microenvironments in the rumen which mirror these artificial diets. The GS/GOGAT and GDH pathways of ammonia assimilation were also important in pure batch cultures of *B. ruminicola* BA25, *S. ruminantium* MP72 and *S. ruminantium* ME13 when NH_4Cl was the sole nitrogen source. AlaDH was again only of minor importance. When amino acids and peptides were also available as nitrogen sources for these species, as well as in *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49, these ammonia-assimilatory enzymes were not essential for growth. In fact, under these conditions ammonia did not appear to be assimilated, since the bacteria probably obtained their nitrogen requirements by transamination and catabolism of the free amino acids and peptides.

The various mechanisms for nitrogen uptake and metabolism suggested by these data, in conjunction with the nitrogen source preference information which indicated that *B. ruminicola* BA25 and *S. ruminantium* MP72 may utilise nitrogen sources in progression from peptides through to free amino acids as they become available in the rumen, probably reflect different environmental niches that the microorganisms occupy (Wallace & Cotta, 1988). These data highlight the importance of understanding the microenvironments which occur within the rumen, in particular how nitrogen is utilised within such microenvironments. For example, do the variety of rumen bacterial species within a microenvironment utilise a progression of nitrogen sources, or do the various strains and species utilise a nitrogen source each and then share the products (i.e. synergism) ? These and other questions can only be resolved by further study of pure rumen bacterial species, as well as mixed cultures of such species (i.e. co-culture in defined media), since studying actual microenvironments *in vivo* is technically difficult.

Another area of importance relating to nitrogen assimilation and metabolism within the rumen is the uptake mechanisms for the various nitrogen sources. Little is presently known about uptake of ammonia, such as whether it is active or passive, at what concentrations it occurs, and whether it occurs when other nitrogen sources are also available (Smith, 1979; Russell & Hespell, 1981; Hobson & Wallace, 1982b; Hespell, 1984; Mackie & White, 1990;

Russell *et al.*, 1990), nor is much known of the mechanisms of amino acid and peptide uptake and utilisation (Pittman *et al.*, 1967; Allison, 1970; Hullah & Blackburn, 1971; Mercer & Annison, 1976; Stevenson, 1979; Cotta & Russell, 1982; Hobson & Wallace, 1982b; Mackie & White, 1990; Russell *et al.*, 1990).

4.4.2 POTENTIAL GENETIC MANIPULATIONS OF NITROGEN METABOLISM IN RUMINANTS

The stated aim of this thesis (Literature Review : section 1 11) is to understand more fully the processes of nitrogen metabolism and assimilation, especially those associated with ammonia. It was also stated that this project is part of a larger gene manipulation program, aimed at modifying nitrogen metabolism in ruminants. Thus, it is appropriate that, in conclusion, the results of this work be discussed in terms of what manipulations may increase the efficiency of nitrogen utilisation in ruminants.

High ammonia concentrations in the rumen, brought about by supplementary nitrogen programs or the utilisation of high-quality pasture protein by the animals, generally result in the bulk of the nitrogen being ineffectively utilised by either the rumen microorganisms or the animal (Chalupa, 1972; Johnson, 1976; Bartley & Deyoe, 1977; Nikolic *et al.*, 1980; Allden, 1981; Coombe, 1981; Huber & Kung, 1981; Simpson & Stobbs, 1981; Thomas, 1985). In microorganisms it has been assumed that the main limitation is the rate at which the bacterial enzymes can metabolise large amounts of ammonia, since high levels of ammonia apparently are not harmful to the bacteria (Smith, 1975; Bartley & Deyoe, 1977; Buttery, 1977; Harrison & McAllan, 1980).

The work reported in experiment 2 of this thesis (Chapter 2) supports this assumption, since the addition of a large bolus of ammonia to the rumen of an animal with a very low ammonia concentration (and consequently high GS activity) resulted in a decrease in activity of this major ammonia-assimilatory enzyme in the microbial population. At the same time the other major ammonia-assimilatory pathway (i.e. GDH) was not markedly increased in activity and the ammonia concentration in the rumen was maintained at a high level for a considerable time. These results, as well as those from the pure culture studies of *B. ruminicola* BA25, *B. ruminicola* subsp. *ruminicola*, *S. ruminantium* ME13, *S. ruminantium* MP72 and *Butyrivibrio fibrisolvens* 49, indicate that GS is the most appropriate enzyme to target for

genetic manipulation in rumen bacteria. Manipulation of its regulation could focus on reducing the inhibitory effects of ammonia concentration on its activity so that ammonia assimilation could be maintained at higher levels in rumen bacteria irrespective of ammonia concentration. The success of this manipulation may ultimately be restricted by the energy (ATP) requirements of this enzyme. Manipulation of GDH and AlaDH may also have beneficial effects under such conditions so that ammonia assimilation is enhanced.

Such manipulations are not feasible at the present time, since additional information on the metabolism of nitrogen compounds is required in rumen bacteria. The major rumen species involved in ammonia assimilation *in vivo* must be identified. Presently it is assumed that the most abundant and common rumen species, such as *B. ruminicola, S. ruminantium* and *Butyrivibrio fibrisolvens*, are also the most important species for ammonia assimilation, however this may not be the case. Further studies are also required on the uptake of ammonia and other nitrogen sources by these species, and the control and regulatory mechanisms for these enzymes (i.e. GS and GDH) must be clearly established. In particular, are these controls transcription or activity related and are phosphorylation and adenylylation involved? It may then be feasible to begin a search for genes involved in the formation and control of these key enzymes and pathways.

CHAPTER 5

Appendices

APPENDIX 1

(Chapter 2)

Figure A1.1: pH values, and free protein and ammonia concentrations, of rumen samples from diets A, B and C (experiment 1).

Rumen fluid samples (125 ml) from each animal were taken at 1130 hours on 5 days spread over a 22 day period. pH was determined immediately, and the sample divided into one 25 ml and two 50 ml aliquots. One of the 50 ml aliquots of each sample was processed into a cell-free bacterial extractfor GS, GOGAT, GDH and AlaDH assays. The other 50 ml aliquot was processed into a cell-free bacterial extract for measurement of the intracellular levels of glutamic acid, glutamine, aspartic acid and alanine. The remaining 25 ml aliquot was processed for storage and subsequent measurement of ammonia and free protein levels. Full details are in section 2.2.2.6.

pH values are single measurements; ammonia and free protein values are the mean ± the standard error of the mean from triplicate assays performed on stored samples.

(a) pH values (■) and free protein (□) levels for diet A.

(b) Ammonia concentrations (•) for diet A.

(c) pH values (■) and free protein (□) levels for diet B.

(d) Ammonia concentrations (•) for diet B.

(e) pH values (■) and free protein (□) levels for diet C.

(f) Ammonia concentrations (•) for diet C.

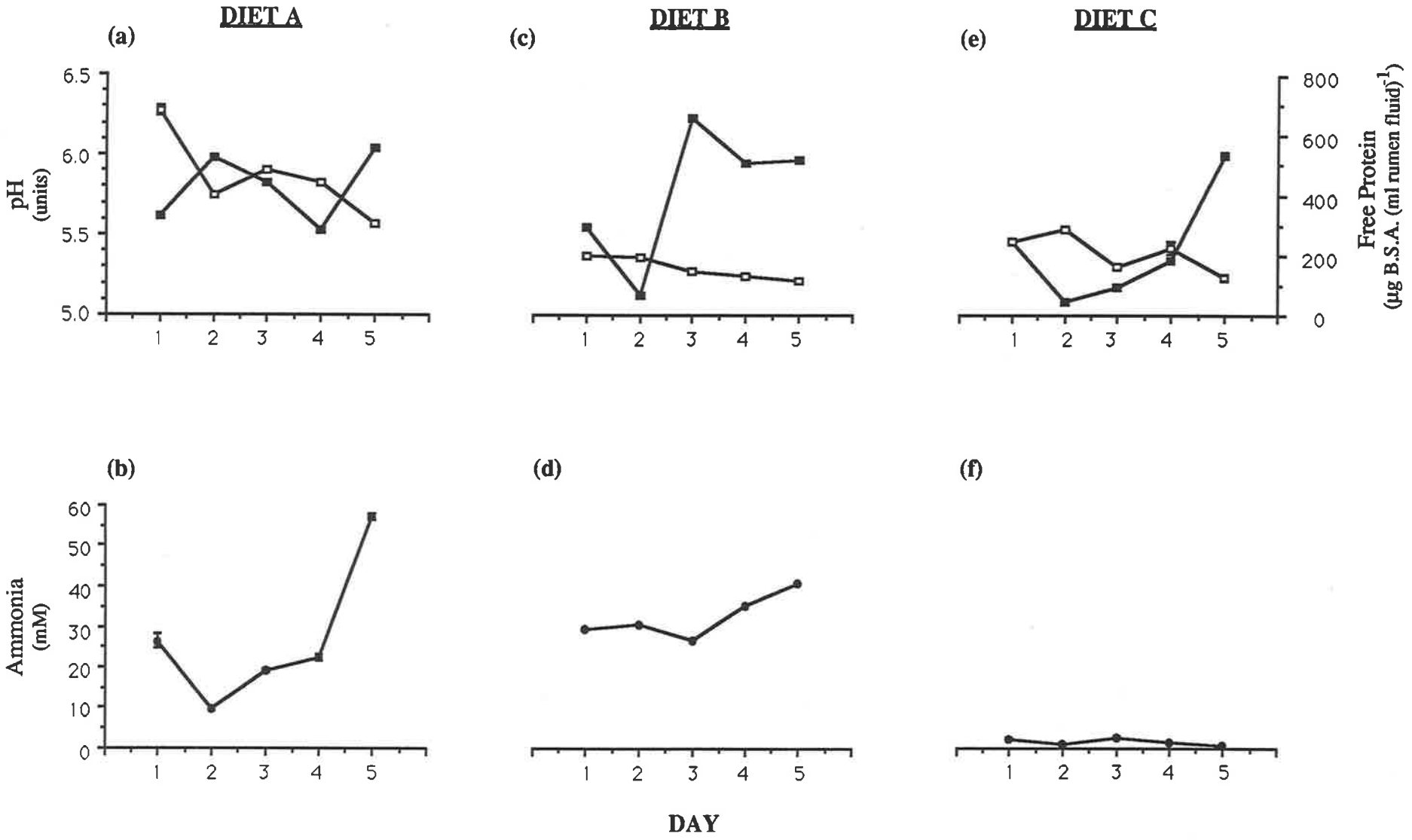

DIET A
DIET B
DIET C
(a)
(b)
(c)
(d)
(e)
(f)
pH (units)
Ammonia (mM)
Free Protein (µg B.S.A. (ml rumen fluid)$^{-1}$)
DAY
6.5
6.0
5.5
5.0
60
50
40
30
20
10
0
800
600
400
200
0
1
2
3
4
5

Figure A1.2 : NADH- and NADPH-dependent GOGAT, GDH and AlaDH specific activities, and levels of glutamic acid, glutamine, aspartic acid and alanine, in cell-free extracts prepared from rumen bacterial samples from diets A, B and C (experiment 1).

Rumen fluid sample (taken at 1130 hours on five days spread over a 22 day period) and sampling details are as for Figure A1.1.
Full enzyme assay details are in section 2.2.2.7(2, 3 & 4).
Amino acid measurement details are in section 2.2.2.8.

Enzyme specific activities (expressed as in section 2.3.2) are the mean ± the standard error of the mean from triplicate assays; amino acid values are means of duplicates which were within 10 % of each other.

* indicates undetected activity or amino acid.

(a) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for diet A.

(b) GDH NADH- (■) and NADPH-dependent (▫) specific activities for diet A.

(c) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for diet A.

(d) Levels of glutamic acid (•), glutamine (○), aspartic acid (▲) and alanine (△) for diet A.

(e) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for diet B.

(f) GDH NADH- (■) and NADPH-dependent (▫) specific activities for diet B.

(g) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for diet B.

(h) Levels of glutamic acid (•), glutamine (○), aspartic acid (▲) and alanine (△) for diet B.

(i) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for diet C.

(j) GDH NADH- (■) and NADPH-dependent (▫) specific activities for diet C.

(k) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for diet C.

(l) Levels of glutamic acid (•), glutamine (○), aspartic acid (▲) and alanine (△) for diet C.

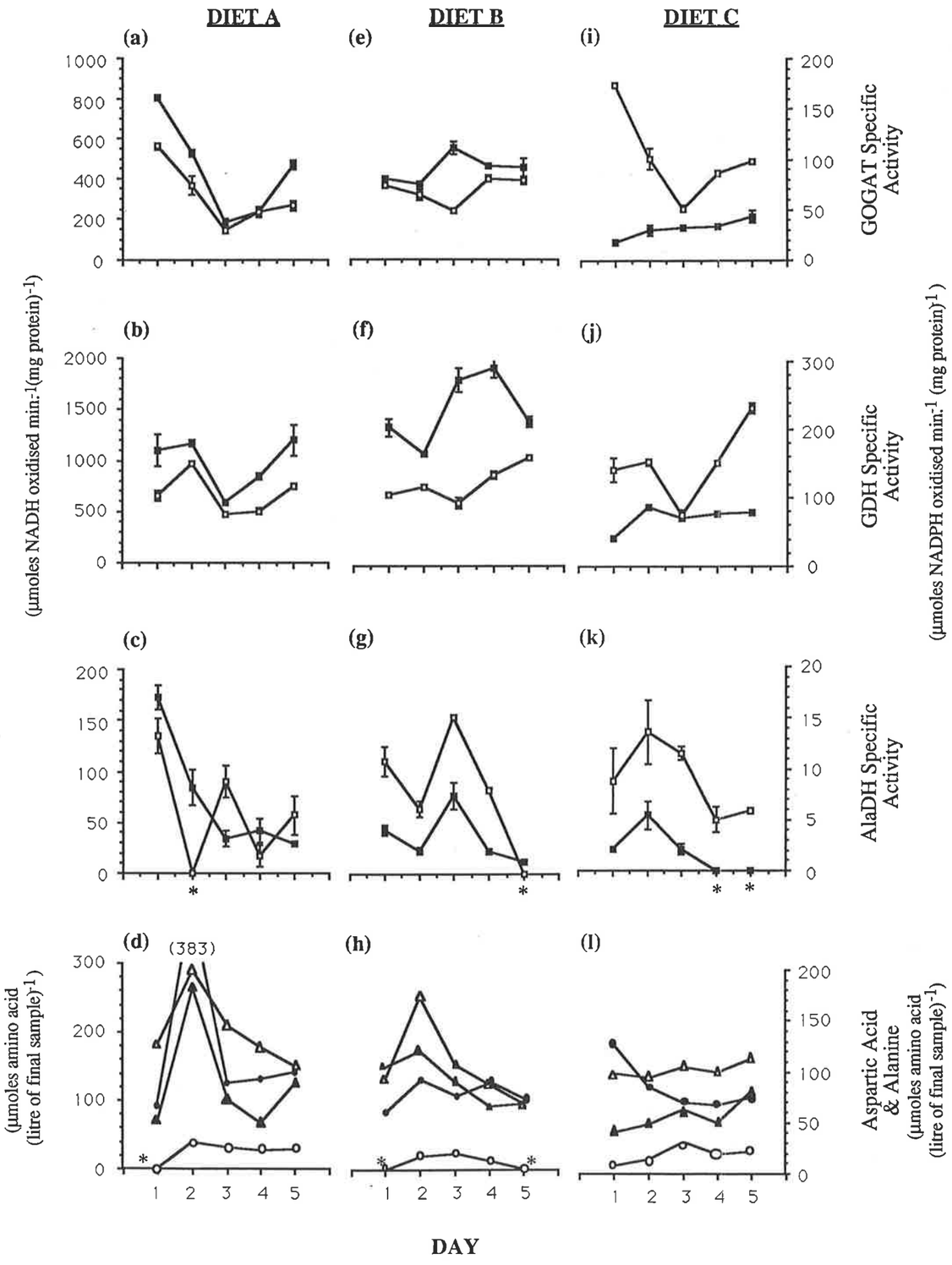

DIET A
DIET B
DIET C
(a)
(e)
(i)
(b)
(f)
(j)
(c)
(g)
(k)
(d)
(383)
(h)
(l)
(µmoles NADH oxidised min:-1(mg protein)-1)
GOGAT Specific Activity
(µmoles NADPH oxidised min.-1 (mg protein)-1)
GDH Specific Activity
AlaDH Specific Activity
(µmoles amino acid (litre of final sample)-1)
Aspartic Acid & Alanine
(µmoles amino acid (litre of final sample)-1)
DAY

Figure A1.3 : Diurnal levels of rumen fluid pH, ammonia, free protein and carbohydrates (as glucose) for the low nitrogen/low energy (D: ■) and high nitrogen/high energy (E: ▫) diets of experiment 2.

Rumen fluid (50 ml) was sampled at 2 hr intervals. pH was measured immediately and the sample subdivided and processed according to the scheme in Figure 2.3 for subsequent measurement of ammonia, free protein and carbohydrates levels, as outlined in section 2.2.2.6.

Sampling and measurement protocols were repeated and the data pooled. Data is therefore the mean ± the standard error of the mean of data from three individual days of measurements, except for asterisked (*) points which were from two data days only. Measurements within each day were in triplicate, except pH values for which only single measurements were recorded.

N.B. Arrows (↑) indicate feeding times (i.e. twice per day).

(a) Diurnal rumen pH values for diet D.

(b) Diurnal rumen ammonia concentrations for diet D.

(c) Diurnal rumen free protein levels for diet D.

(d) Diurnal rumen carbohydrates contents for diet D.

(e) Diurnal rumen pH values for diet E.

(f) Diurnal rumen ammonia concentrations for diet E.

(g) Diurnal rumen free protein levels for diet E.

(h) Diurnal rumen carbohydrates contents for diet E.

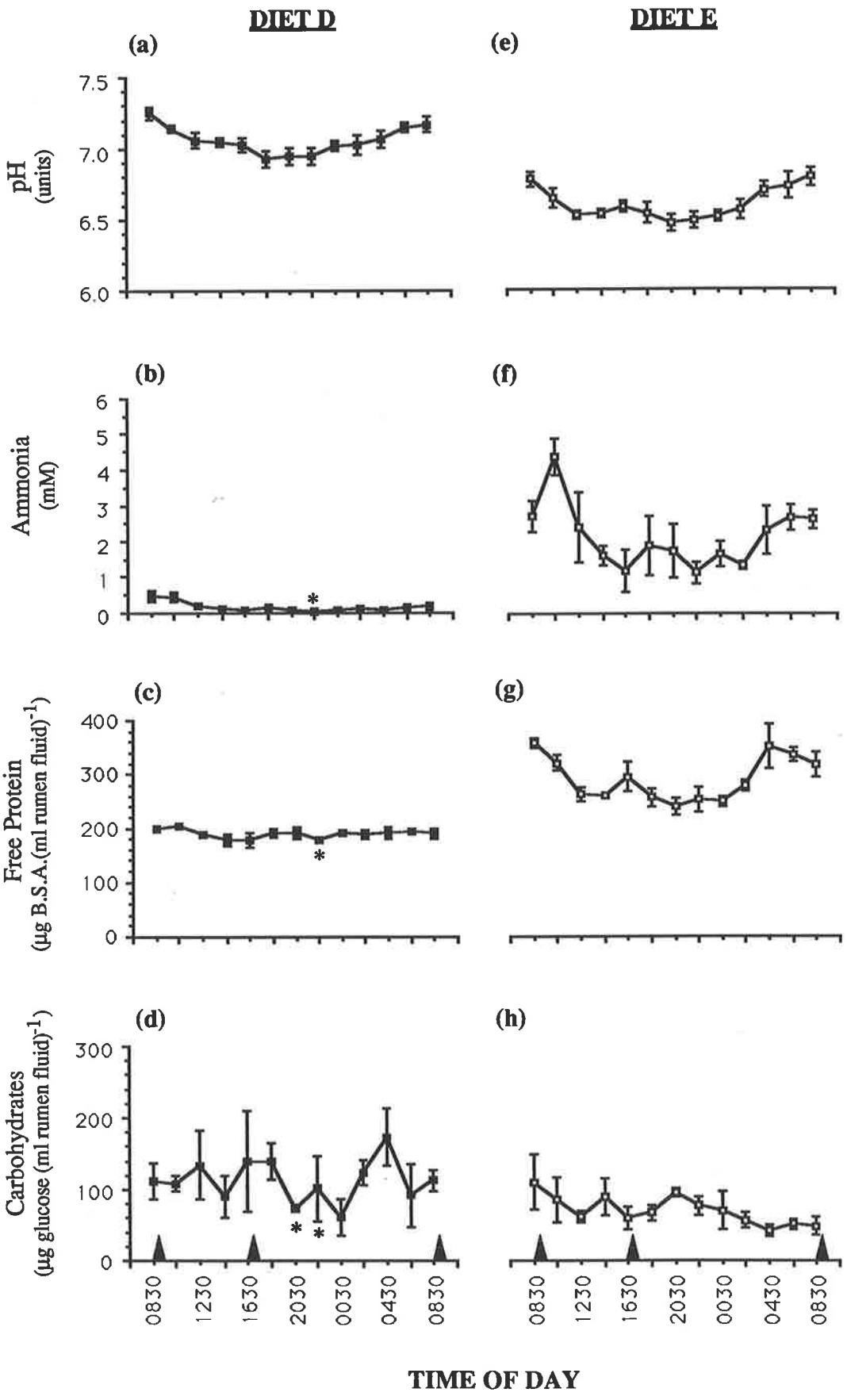

DIET D
DIET E
(a)
(e)
pH (units)
7.5
7.0
6.5
6.0
(b)
(f)
Ammonia (mM)
6
5
4
3
2
1
0
(c)
(g)
Free Protein (µg B.S.A.(ml rumen fluid)-1)
400
300
200
100
0
(d)
(h)
Carbohydrates (µg glucose (ml rumen fluid)-1)
300
200
100
0
0830
1230
1630
2030
0030
0430
0830
TIME OF DAY

Figure A1.4 : Levels of rumen fluid pH, free protein, ammonia and carbohydrates (as glucose) before adding a bolus of ammonia (low and high for diet D), glucose, or ammonia plus glucose, to the rumen of diet D and E (experiment 2) i.e equivalent to the daily data for experiment 1 in Figure A1.1.

Rumen fluid samples (150 ml) were taken from each animal prior to the addition of the respective boluses. pH was determined immediately, and each sample divided into three 50 ml aliquots. Two aliquots were processed into cell-free bacterial extracts for assays of GS, AsS, GOGAT, GDH and AlaDH activity and measurement of the levels of glutamic acid, glutamine, aspartic acid and alanine, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8, respectively, for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels. Full details are in section 2.2.2.56.

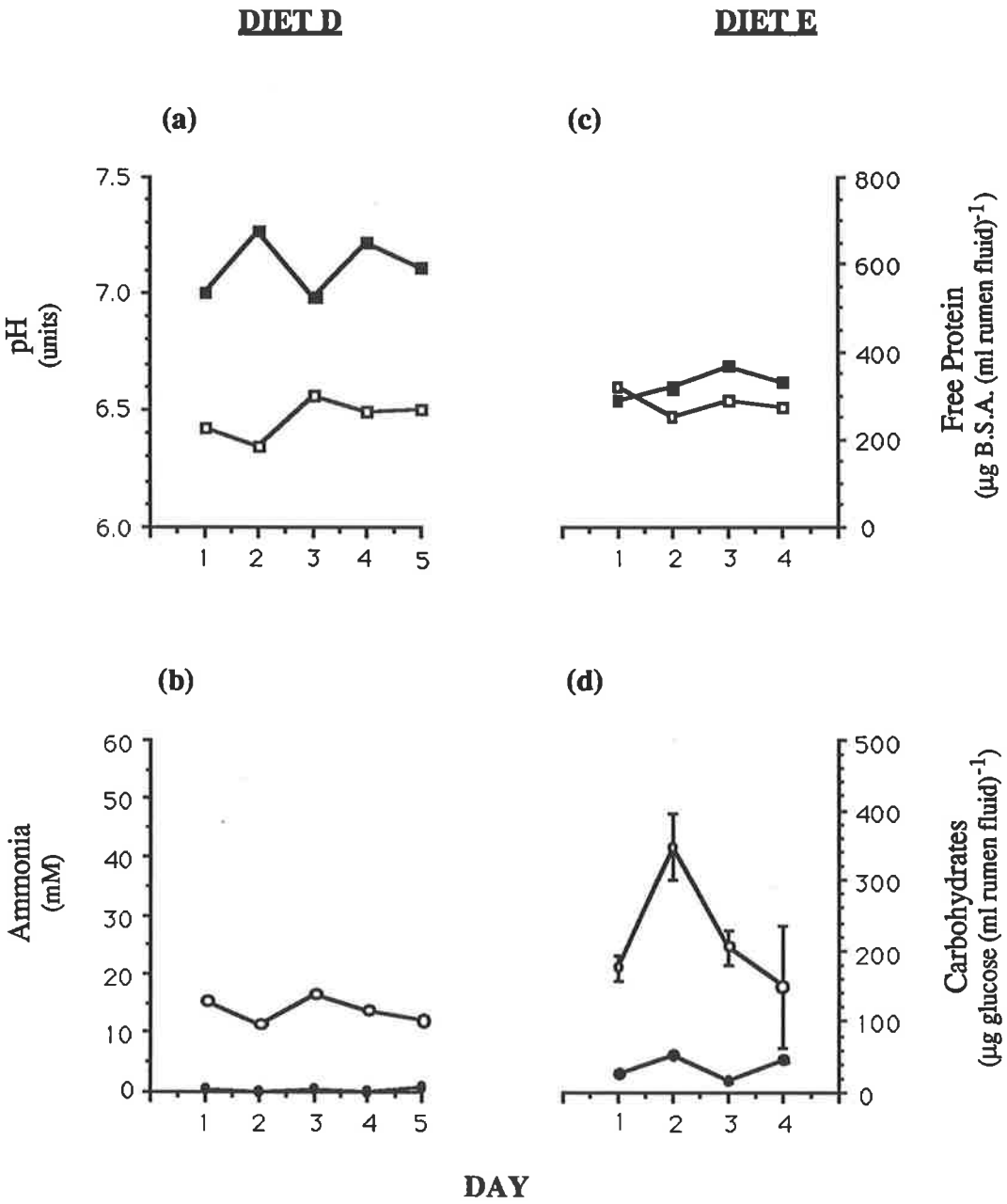
pH values are from single measurements; ammonia, free protein and carbohydrates values are the mean ± the standard error of the mean from triplicate assays on stored samples

(a) pH values (■) and free protein levels (□) for diet D.

(b) Ammonia (•) and carbohydrates (○) concentrations for diet D.

(c) pH values (■) and free protein levels (□) for diet E.

(d) Ammonia (•) and carbohydrates (○) concentrations for diet E.



(Ammonia values : 0.198, 0.120, 0.343, 0.081, 0.727 mM)

Figure A1.5 : Specific activities of GS, AsS, GOGAT, GDH and AlaDH, and levels of glutamic acid, aspartic acid and alanine, in cell-free bacterial extracts prepared from the rumen bacterial samples taken before the bolus addition of ammonia, glucose, or ammonia plus glucose, to diets D and E (experiment 2). i.e. equivalent to the daily data for experiment 1 in Figure A1.2.

Rumen fluid sample and sampling details are as for Figure A1.4.
Full enzyme assay details are in section 2.2.2.7(1 to 5).
Amino acid measurement details are in section 2.2.2.8.

Enzyme specific activity values are the mean ± the standard error of the mean from triplicate assays; amino acid values are the means of duplicate measurements which were within 10 % of each other.

* indicates undetected activity.

(a) GS γ-GT (•) and forward (◦) specific activities for diet D.

(b) AsS specific activities for diet D.

(c) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for diet D.

(d) GDH NADH- (■) and NADPH-dependent (▫) specific activities for diet D.

(e) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for diet D.

(f) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for diet D.

(g) GS γ-GT (•) and forward (◦) specific activities for diet E.

(h) AsS (■) specific activities for diet E.

(i) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for diet E.

(j) GDH NADH- (■) and NADPH-dependent (▫) specific activities for diet E.

(k) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for diet E.

(l) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for diet E.

DIET D
DIET E
GS γ-GT Specific Activity
(nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
AsS Specific Activity
(nmoles ß-aspartyl hydroxamate produced min.-1 (mg protein)-1)
GOGAT Specific Activity
GDH Specific Activity
AlaDH Specific Activity
Glutamic Acid
(µmoles amino acid (litre of final sample)-1)
(µmoles NADH oxidised min.-1 (mg protein)-1)
(a)
(b)
(c)
(d)
(e)
(f)
NOT DETECTED
GS Forward Specific Activity
(nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
(g)
(h)
(i)
(j)
(k)
(l)
GOGAT Specific Activity
GDH Specific Activity
AlaDH Specific Activity
Aspartic Acid & Alanine
(µmoles amino acid (litre of final sample)-1)
(µmoles NADPH oxidised min.-1 (mg protein)1)
DAY

Figure A1.6 : Rumen fluid pH and free protein levels, specific activities of AsS, GOGAT, GDH and AlaDH, and intracellular levels of glutamic acid, aspartic acid and alanine, before, and at 30, 60, 120 and 240 mins after, bolus additions of "synthetic saliva" only (control "pulse" - a) and "synthetic saliva" plus 0.005 moles NH_4Cl (low ammonia "pulse" - b) to the rumen of diet D (experiment 2).

Rumen fluid samples (150 ml) from each animal were taken before the addition of the bolus (0), and at 30, 60, 120 and 240 mins afterwards. pH was determined immediately, and the sample divided into three 50 ml aliquots. Two aliquots were processed into cell-free bacterial extracts for GS, AsS, GOGAT, GDH and AlaDH assays, and measurement of the levels of glutamic acid, glutamine, aspartic acid and alanine, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.

Full details of rumen fluid sampling, and pH and free protein measurements are in sections 2.2.2.5 and 2.2.2.6 respectively. Enzyme assay details are in section 2.2.2.7(1 to 5). Amino acid measurement details are in section 2.2.2.8.

pH values are from single measurements; free protein and enzyme activity values are the mean ± the standard error of the mean from triplicate assays; amino acid values are the mean of duplicates which were within 10 % of each other.

(a-i) pH values (•) and free protein levels (◦) for the control bolus added to diet D.

(a-ii) AsS specific activities for the control bolus added to diet D.

(a-iii) GOGAT NADH- (■) and NADPH-dependent (□) specific activities for the control bolus added to diet D.

(a-iv) GDH NADH- (■) and NADPH-dependent (□) specific activities for the control bolus added to diet D.

(a-v) AlaDH NADH- (■) and NADPH-dependent (□) specific activities for the control bolus added to diet D.

(a-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the control bolus added to diet D.

(b-i) pH values (•) and free protein levels (◦) for the low ammonia bolus added to diet D.

(b-ii) AsS specific activities for the low ammonia bolus added to diet D.

(b-iii) GOGAT NADH- (■) and NADPH-dependant (□) specific activities for the low ammonia bolus added to diet D.

(b-iv) GDH NADH- (■) and NADPH-dependant (□) specific activities for the low ammonia bolus added to diet D.

(b-v) AlaDH NADH- (■) and NADPH-dependant (□) specific activities for the low ammonia bolus added to diet D.

(b-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the low ammonia bolus added to diet D.

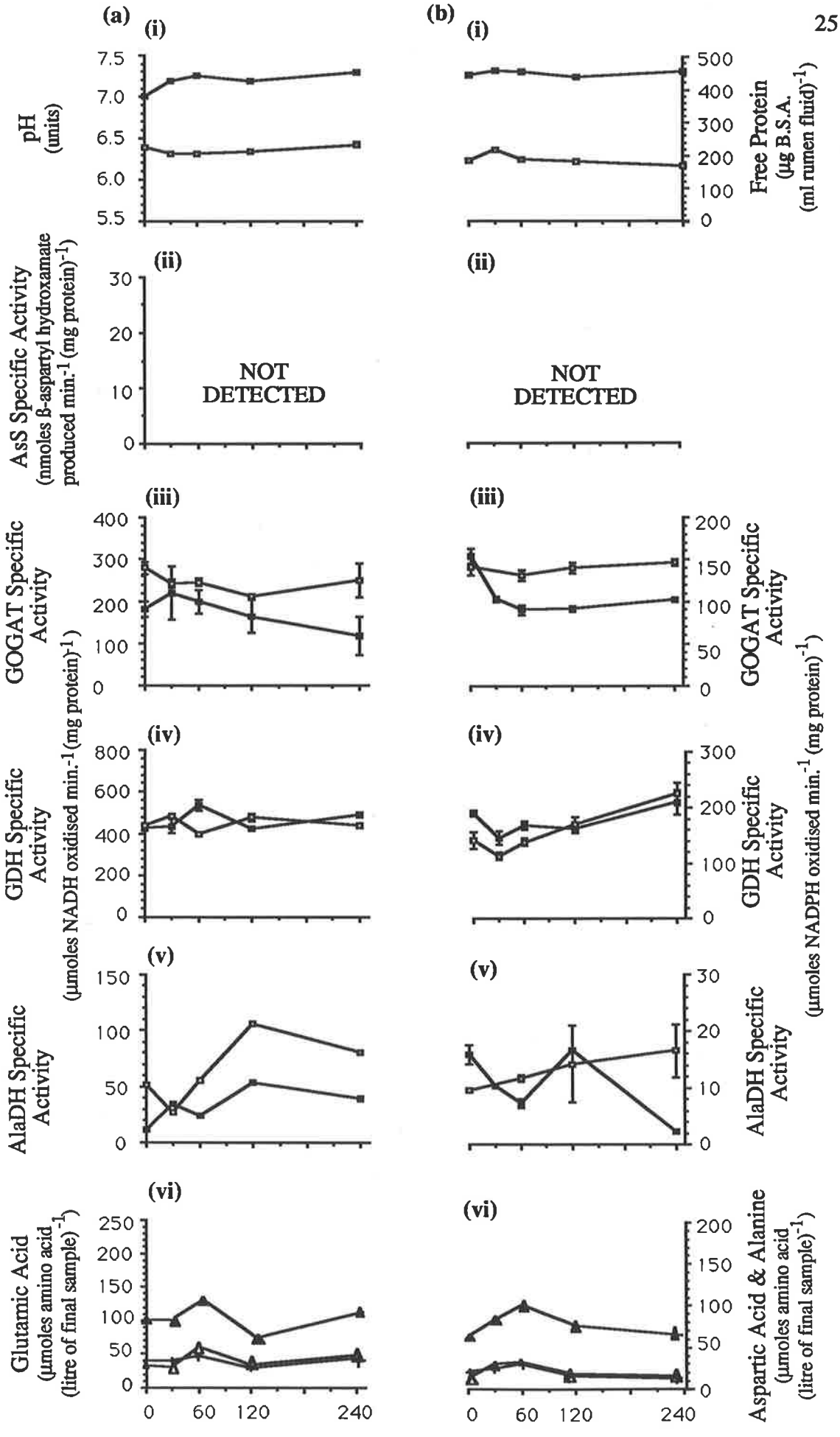

(a)
(b)
(i)
pH (units)
Free Protein (µg B.S.A. (ml rumen fluid)$^{-1}$)
(ii)
AsS Specific Activity (nmoles β-asparty1 hydroxamate produced min.$^{-1}$ (mg protein)$^{-1}$)
NOT DETECTED
(iii)
GOGAT Specific Activity
(µmoles NADH oxidised min.$^{-1}$ (mg protein)$^{-1}$)
(µmoles NADPH oxidised min.$^{-1}$ (mg protein)$^{-1}$)
(iv)
GDH Specific Activity
(v)
AlaDH Specific Activity
(vi)
Glutamic Acid (µmoles amino acid (litre of final sample)$^{-1}$)
Aspartic Acid & Alanine (µmoles amino acid (litre of final sample)$^{-1}$)
0 60 120 240
Time after addition of bolus (min.)

Figure A1.7 : Rumen fluid pH and free protein levels, specific activities of AsS, GOGAT, GDH and AlaDH, and intracellular levels of glutamic acid, aspartic acid and alanine, before, and at 30, 60, 120 and 240 mins after, bolus additions of "synthetic saliva" plus 0.5 moles NH_4Cl (high ammonia "pulse" - a) and "synthetic saliva" plus 0.5 moles glucose (glucose "pulse" - b) to the rumen of diet D (experiment 2).

Rumen fluid samples (150 ml) from each animal were taken before the addition of the bolus (0), and at 30, 60, 120 and 240 mins afterwards. pH was determined immediately, and the sample divided into three 50 ml aliquots. Two aliquots were processed into cell-free bacterial extracts for GS, AsS, GOGAT, GDH and AlaDH assays, and measurement of the levels of glutamic acid, glutamine, aspartic acid and alanine, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.

Full details of rumen fluid sampling, and pH and free protein measurements are in sections 2.2.2.5 and 2.2.2.6 respectively. Enzyme assay details are in section 2.2.2.7(1 to 5). Amino acid measurement details are in section 2.2.2.8.

pH values are from single measurements; free protein and enzyme activity values are the mean ± the standard error of the mean from triplicate assays; amino acid values are the mean of duplicates which were within 10 % of each other.

** in (a-v) indicates a missing value for the NADPH-dependent activity at 120 mins.

(a-i) pH values (•) and free protein levels (○) for the high ammonia bolus added to diet D.

(a-ii) AsS specific activities for the high ammonia bolus added to diet D.

(a-iii) GOGAT NADH- (■) and NADPH-dependent (□) specific activities for the high ammonia bolus added to diet D.

(a-iv) GDH NADH- (■) and NADPH-dependent (□) specific activities for the high ammonia bolus added to diet D.

(a-v) AlaDH NADH- (■) and NADPH-dependent (□) specific activities for the high ammonia bolus added to diet D.

(a-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the high ammonia bolus added to diet D.

(b-i) pH values (•) and free protein levels (○) for the glucose bolus added to diet D.

(b-ii) AsS specific activities for the glucose bolus added to diet D.

(b-iii) GOGAT NADH- (■) and NADPH-dependent (□) specific activities for the glucose bolus added to diet D.

(b-iv) GDH NADH- (■) and NADPH-dependent (□) specific activities for the glucose bolus added to diet D.

(b-v) AlaDH NADH- (■) and NADPH-dependent (□) specific activities for the glucose bolus added to diet D.

(b-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the glucose bolus added to diet D.

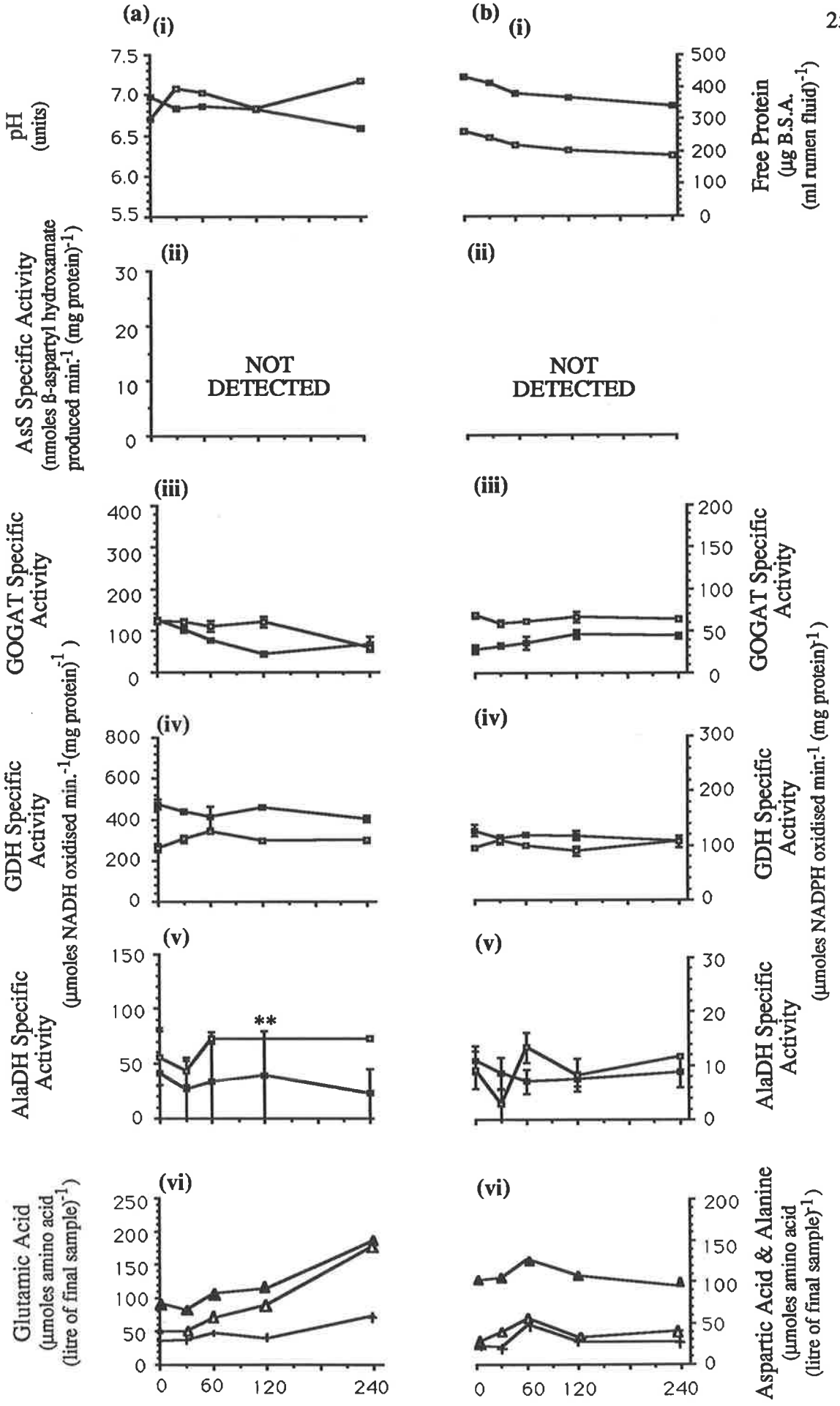

(a)
(b)
(i)
(ii)
(iii)
(iv)
(v)
(vi)
pH (units)
Free Protein (µg B.S.A. (ml rumen fluid)-1)
AsS Specific Activity (nmoles β-aspartyl hydroxamate produced min.-1 (mg protein)-1)
NOT DETECTED
GOGAT Specific Activity
GDH Specific Activity (µmoles NADH oxidised min.-1(mg protein)-1)
GDH Specific Activity (µmoles NADPH oxidised min.-1 (mg protein)-1)
AlaDH Specific Activity
**
Glutamic Acid (µmoles amino acid (litre of final sample)-1)
Aspartic Acid & Alanine (µmoles amino acid (litre of final sample)-1)
Time after addition of bolus (min.)

Figure A1.8 : Rumen fluid pH and free protein levels, specific activities of AsS, GOGAT, GDH and AlaDH, and intracellular levels of glutamic acid, aspartic acid and alanine, before, and at 30, 60, 120, 240 and 480 mins after, a bolus addition of "synthetic saliva" plus 0.5 moles NH_4Cl and 0.5 moles glucose (ammonia plus glucose "pulse") to the rumen of diet D (experiment 2).

Rumen fluid samples (150 ml) from each animal were taken before the addition of the bolus (0), and at 30, 60, 120 and 240 mins afterwards. pH was determined immediately, and the sample divided into three 50 ml aliquots. Two aliquots were processed into cell-free bacterial extracts for GS, AsS, GOGAT, GDH and AlaDH assays, and measurement of the levels of glutamic acid, glutamine, aspartic acid and alanine, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.

Full details of rumen fluid sampling, and pH and free protein measurements are in sections 2.2.2.5 and 2.2.2.6 respectively. Enzyme assay details are in section 2.2.2.7(1 to 5). Amino acid measurement details are in section 2.2.2.8.

pH values are from single measurements; free protein and enzyme activity values are the mean ± the standard error of the mean from triplicate assays; amino acid values are the mean of duplicates which were within 10 % of each other.

(i) pH values (•) and free protein levels (◦) for the ammonia plus glucose bolus added to diet D.

(ii) AsS specific activities for the ammonia plus glucose bolus added to diet D.

(iii) GOGAT NADH- (■) and NADPH-dependent (□) specific activities for the ammonia plus glucose bolus added to diet D.

(iv) GDH NADH- (■) and NADPH-dependent (□) specific activities for the ammonia plus glucose bolus added to diet D.

(v) AlaDH NADH- (■) and NADPH-dependent (□) specific activities for the ammonia plus glucose bolus added to diet D.

(vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the ammonia plus glucose bolus added to diet D.

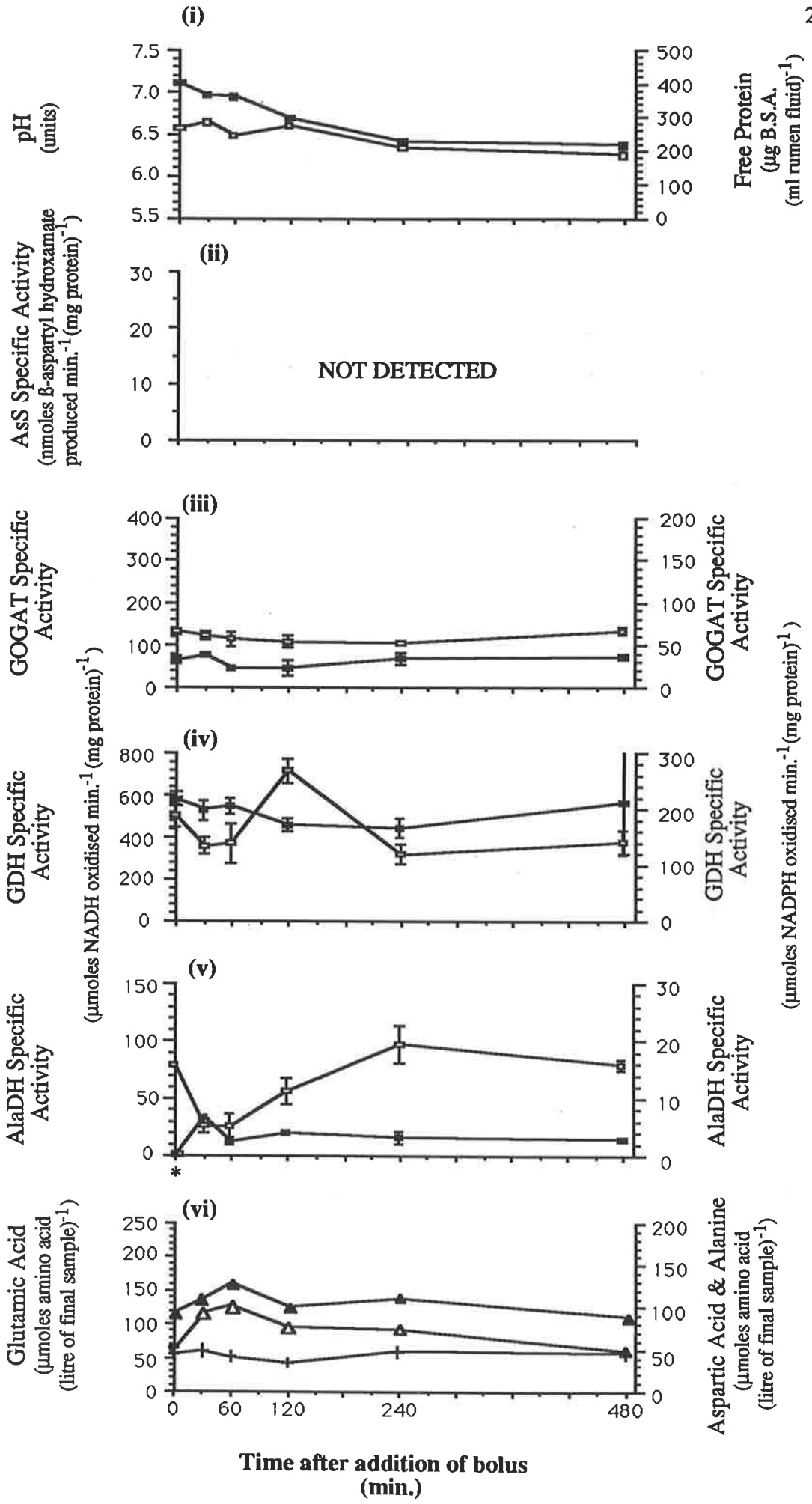

(i)
pH (units)
Free Protein (µg B.S.A. (ml rumen fluid)$^{-1}$)
(ii)
AsS Specific Activity (nmoles β-aspartyl hydroxamate produced min.$^{-1}$(mg protein)$^{-1}$)
NOT DETECTED
(iii)
GOGAT Specific Activity
(µmoles NADH oxidised min.$^{-1}$(mg protein)$^{-1}$)
(µmoles NADPH oxidised min.$^{-1}$(mg protein)$^{-1}$)
(iv)
GDH Specific Activity
(v)
AlaDH Specific Activity
*
(vi)
Glutamic Acid (µmoles amino acid (litre of final sample)$^{-1}$)
Aspartic Acid & Alanine (µmoles amino acid (litre of final sample)$^{-1}$)
0 60 120 240 480
Time after addition of bolus (min.)

Figure A1.9 : Rumen fluid pH and free protein levels before, and at 30, 60, 120 and 240 mins after, bolus additions of "synthetic saliva" only (control "pulse" - a), "synthetic saliva" plus 0.5 moles NH_4Cl (ammonia "pulse" - b), "synthetic saliva" plus 0.5 moles glucose (glucose "pulse" -c) and "synthetic saliva" plus 0.5 moles NH_4Cl and 0.5 moles glucose (ammonia plus glucose "pulse" - d) to the rumen of diet E (experiment 2).

Rumen fluid samples (150 ml) from each animal were taken before the addition of the bolus (0), and at 30, 60, 120 and 240 mins afterwards. pH was determined immediately, and the sample divided into three 50 ml aliquots. Two aliquots were processed into cell-free bacterial extracts for assays of GS, AsS, GOGAT, GDH and AlaDH activity, and measurement of the levels of glutamic acid, glutamine, aspartic acid and alanine, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.

Full details of rumen fluid sampling, and pH and free protein measurements are in sections 2.2.2.5 and 2.2.2.6 respectively.

pH values are from single measurements; free protein values are the mean ± the standard error of the mean from triplicate assays.

(a) pH values (■) and free protein levels (▫) for the control bolus.

(b) pH values (■) and free protein concentrations (▫) for the ammonia bolus.

(c) pH values (■) and free protein levels (▫) for the glucose bolus.

(d) pH values (■) and free protein concentrations (▫) for the ammonia plus glucose bolus.

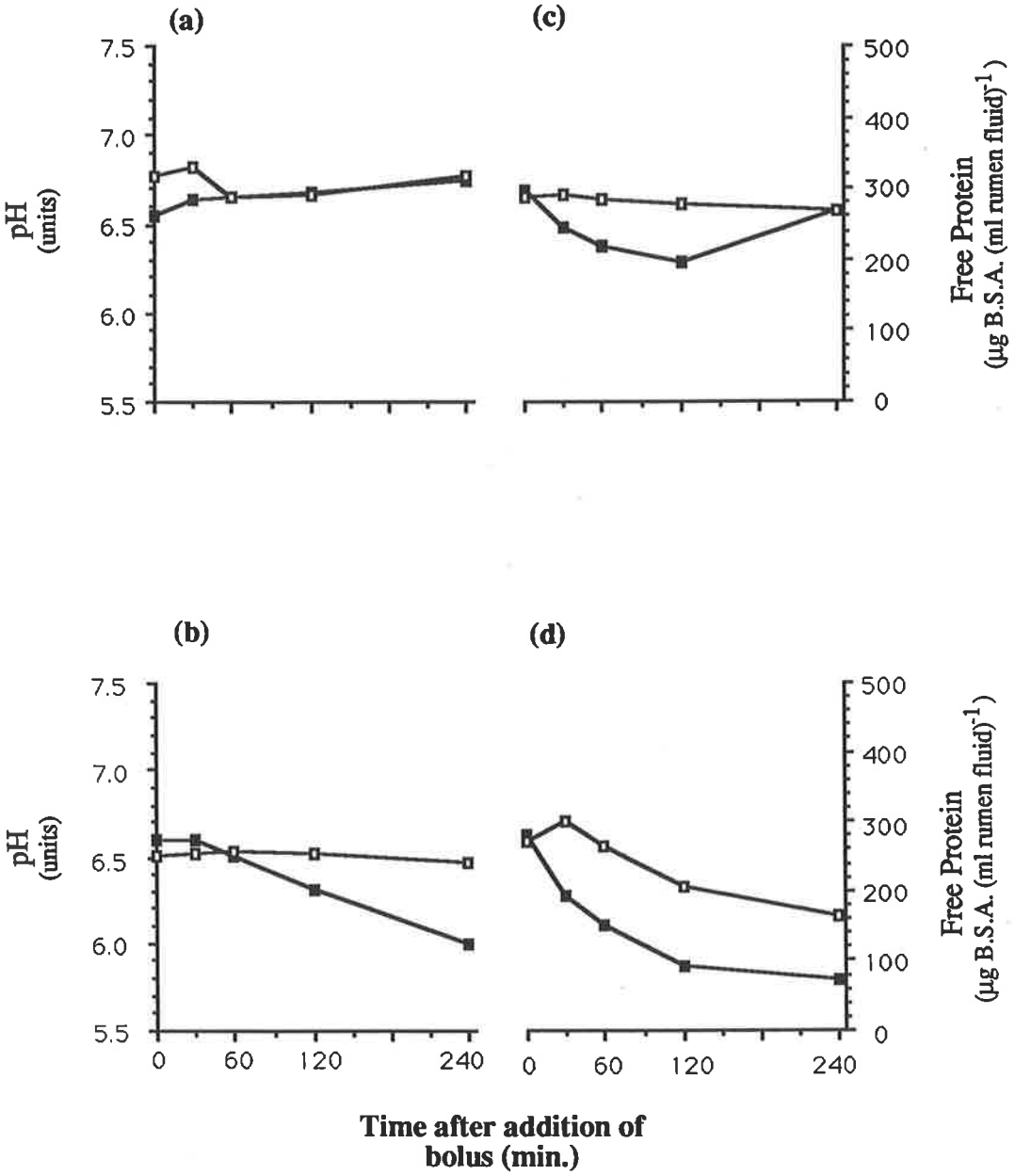

(a)
(c)
(b)
(d)
pH (units)
Free Protein (µg B.S.A. (ml rumen fluid)$^{-1}$)
7.5
7.0
6.5
6.0
5.5
500
400
300
200
100
0
0
60
120
240
Time after addition of bolus (min.)

Figure A1.10 : Specific activities of GS, AsS, GOGAT, GDH and AlaDH, and intracellular levels of glutamic acid, aspartic acid and alanine, before, and at 30, 60, 120 and 240 mins after, bolus additions of "synthetic saliva" only (control "pulse" - a) and "synthetic saliva" plus 0.5 moles NH_4Cl (ammonia "pulse" - b) to the rumen of diet E (experiment 2).

Rumen fluid samples (150 ml) from each animal were taken before the addition of the bolus (0), and at 30, 60, 120 and 240 mins afterwards. pH was determined immediately, and the sample divided into three 50 ml aliquots. Two aliquots were processed into cell-free bacterial extracts for assays of GS, AsS, GOGAT, GDH and AlaDH activity, and measurement of the levels of glutamic acid, glutamine, aspartic acid and alanine, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.

Full details of rumen fluid sampling, and pH and free protein measurements are in sections 2.2.2.5 and 2.2.2.6 respectively. Enzyme assay details are in section 2.2.2.7(1 to 5). Amino acid measurement details are in section 2.2.2.8.

pH values are from single measurements; free protein and enzyme activity values are the mean ± the standard error of the mean from triplicate assays; amino acid values are the mean of duplicates which were within 10 % of each other.

(a-i) GS γ-GT (♦) and forward (◇) specific activities for the control bolus added to diet E.

(a-ii) Asparagine synthetase (x) specific activities for the control bolus added to diet E.

(a-iii)GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for the control bolus added to diet E.

(a-iv) GDH NADH- (■) and NADPH-dependent (▫) specific activities for the control bolus added to diet E.

(a-v) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for the control bolus added to diet E.

(a-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the control bolus added to diet E.

(b-i) GS γ-GT (♦) and forward (◇) specific activities for the ammonia bolus added to diet E.

(b-ii) AsS (x) specific activities for the ammonia bolus added to diet E.

(b-iii) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for the ammonia bolus added to diet E.

(b-iv) GDH NADH- (■) and NADPH-dependent (▫) specific activities for the ammonia bolus added to diet E.

(b-v) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for the ammonia bolus added to diet E.

(b-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the ammonia bolus added to diet E.

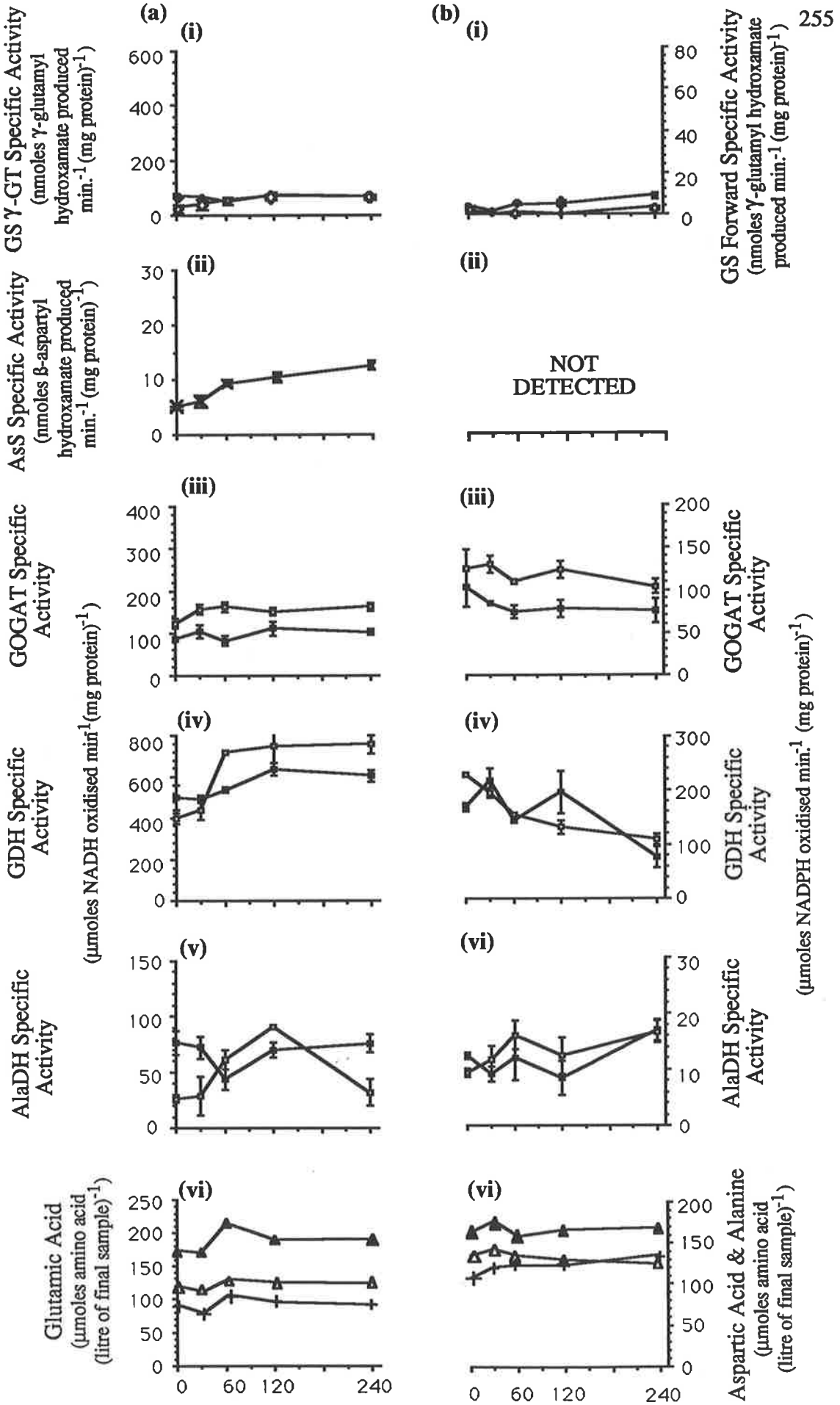

(a)
(b)
(i)
(ii)
(iii)
(iv)
(v)
(vi)
GS γ-GT Specific Activity (nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
GS Forward Specific Activity (nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
AsS Specific Activity (nmoles β-aspartyl hydroxamate produced min.-1 (mg protein)-1)
NOT DETECTED
GOGAT Specific Activity
GDH Specific Activity
AlaDH Specific Activity
(μmoles NADH oxidised min-1 (mg protein)-1)
(μmoles NADPH oxidised min.-1 (mg protein)-1)
Glutamic Acid (μmoles amino acid (litre of final sample)-1)
Aspartic Acid & Alanine (μmoles amino acid (litre of final sample)-1)
0 60 120 240
Time after addition of bolus (min.)

Figure A1.11 : Specific activities of GS, AsS, GOGAT, GDH and AlaDH, and intracellular levels of glutamic acid, aspartic acid and alanine, before, and at 30, 60, 120 and 240 mins after, bolus additions of "synthetic saliva" plus 0.5 moles glucose (glucose "pulse" - a) and "synthetic saliva" plus 0.5 moles NH_4Cl and 0.5 moles glucose (ammonia plus glucose "pulse" - b) to the rumen of diet E (experiment 2).

Rumen fluid samples (150 ml) from each animal were taken before the addition of the bolus (0), and at 30, 60, 120 and 240 mins afterwards. pH was determined immediately, and the sample divided into three 50 ml aliquots. Two aliquots were processed into cell-free bacterial extracts for assays of GS, AsS, GOGAT, GDH and AlaDH activity, and measurement of the levels of glutamic acid, glutamine, aspartic acid and alanine, respectively (section 2.2.2.7 (1 to 5) and section 2.2.2.8 for full details). The third aliquot was processed for storage and subsequent measurement of ammonia, free protein and carbohydrates levels.

Full details of rumen fluid sampling, and pH and free protein measurements are in sections 2.2.2.5 and 2.2.2.6 respectively. Enzyme assay details are in section 2.2.2.7(1 to 5). Amino acid measurement details are in section 2.2.2.8.

pH values are from single measurements; free protein and enzyme activity values are the mean ± the standard error of the mean from triplicate assays; amino acid values are the mean of duplicates which were within 10 % of each other.

* indicates undetected activity

(a-i) GS γ-GT (♦) and forward (◇) specific activities for the glucose bolus added to diet E.

(a-ii) AsS (x) specific activities for the glucose bolus added to diet E.

(a-iii) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for the glucose bolus added to diet E.

(a-iv) GDH NADH- (■) and NADPH-dependent (▫) specific activities for the glucose bolus added to diet E.

(a-v) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for the glucose bolus added to diet E.

(a-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the glucose bolus added to diet E.

(b-i) GS γ-GT (♦) and forward (◇) specific activities for the ammonia plus glucose bolus added to diet E.

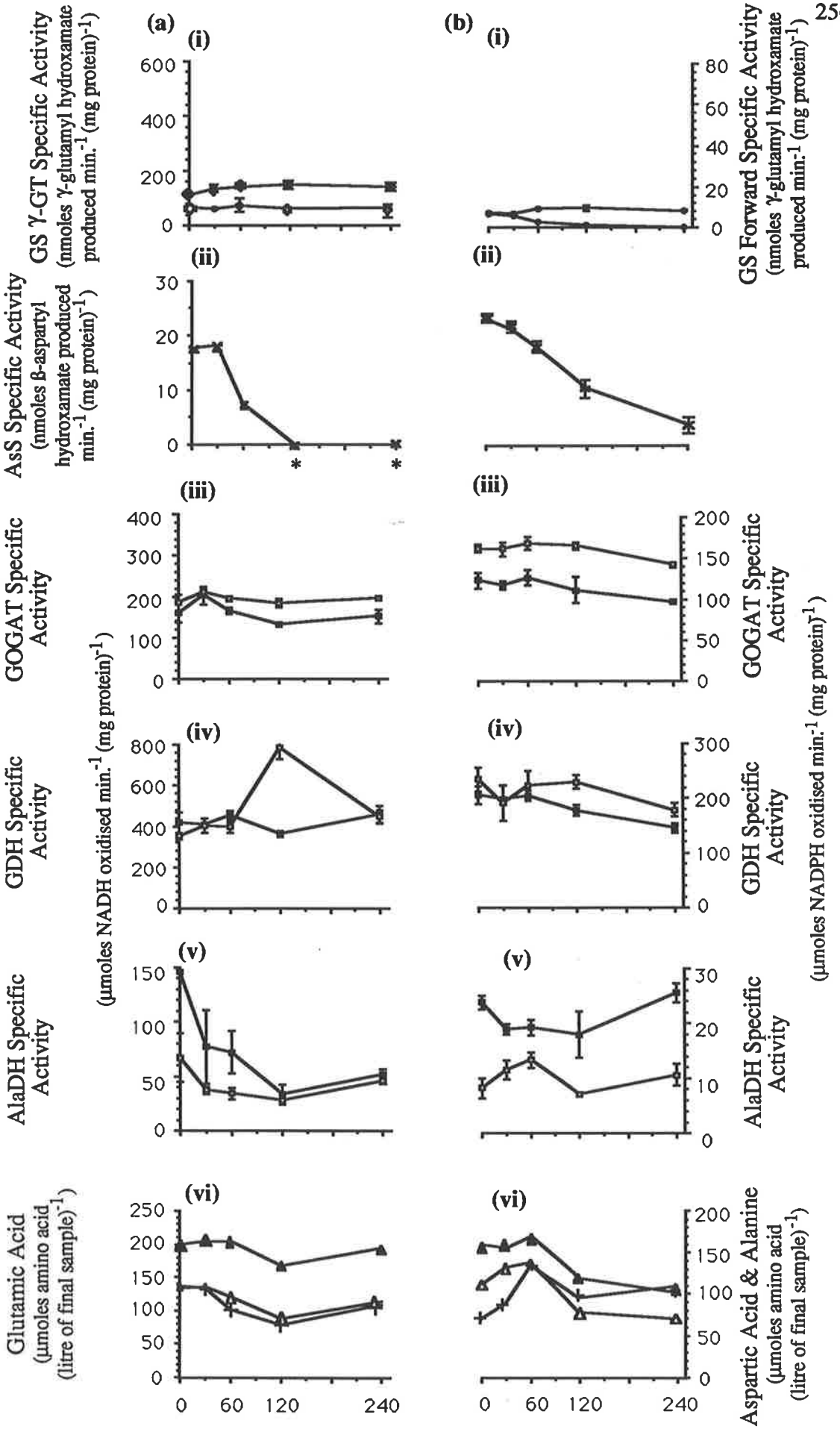
(b-ii) AsS (x) specific activities for the ammonia plus glucose bolus added to diet E.

(b-iii) GOGAT NADH- (■) and NADPH-dependent (▫) specific activities for the ammonia plus glucose bolus added to diet E.

(b-iv) GDH NADH- (■) and NADPH-dependent (▫) specific activities for the ammonia plus glucose bolus added to diet E.

(b-v) AlaDH NADH- (■) and NADPH-dependent (▫) specific activities for the ammonia plus glucose bolus added to diet E.

(b-vi) Levels of glutamic acid (+), aspartic acid (▲) and alanine (△) for the ammonia plus glucose bolus added to diet E.


(a)
(b)
(i)
(ii)
(iii)
(iv)
(v)
(vi)
GS γ-GT Specific Activity (nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
GS Forward Specific Activity (nmoles γ-glutamyl hydroxamate produced min.-1 (mg protein)-1)
AsS Specific Activity (nmoles β-aspartyl hydroxamate produced min.-1 (mg protein)-1)
GOGAT Specific Activity
GDH Specific Activity
AlaDH Specific Activity
(μmoles NADH oxidised min.-1 (mg protein)-1)
(μmoles NADPH oxidised min.-1 (mg protein)-1)
Glutamic Acid (μmoles amino acid (litre of final sample)-1)
Aspartic Acid & Alanine (μmoles amino acid (litre of final sample)-1)
Time after addition of bolus (min.)

APPENDIX 2

(Chapter 3)

Figure A2.1 : Effects of MSX (■) and azaserine (□) on growth of cultures of *B. ruminicola* BA25, *S. ruminantium* MP72, *S. ruminantium* ME13, *B. ruminicola* subsp. *ruminicola* and *Butyrivibrio fibrisolvens* 49 in NFMS media supplemented with 8 mM NH_4Cl (control cultures (○) with no inhibitor added were also grown).

Nine "Hungate" tube cultures (10 ml) of the appropriate NFMS media supplemented with 8 mM NH_4Cl were inoculated at 1 % of final culture volume from an overnight culture of the species being studied, grown in NFMS media supplemented with 8 mM NH_4Cl. Optical density (600 nm) was measured following inoculation, and at 30 or 60 min. intervals thereafter, directly in the "Hungate" tubes using a special holding cage fitted into the spectrophotometer. When obvious culture growth was evident from these optical density measurements (indicated by the arrow (↓) on each graph; see section 3.2.2.2 for definitions of "obvious culture growth"), 100 µl of 0.1 M MSX was added to three of the tubes and 100 µl of 0.01 M azaserine was added to three other tubes. The remaining three cultures were continued as controls. Growth was continued until stationary phase in the control cultures, when the used media was harvested. Clarified media was stored for ammonia level measurements as outlined in section 3.2.2.2.

Results are the mean ± the standard error of the mean from duplicate measurements (n = 2). Optical density values were adjusted by subtraction of initial optical density values for each culture (i.e. zero time values) from subsequent measurements, hence all curves start with initial optical density values of zero units.

E = early log phase of culture growth; M = mid log phase; L = late log phase; S = stationary phase. The actual time at which different cultures reached each of these phases varied somewhat with growth conditions.

a) Growth of *B. ruminicola* BA25 in NFMS media supplemented with 8 mM NH_4Cl.

b) Growth of *S. ruminantium* MP72 in NFMS media supplemented with 8 mM NH_4Cl.

c) Growth of *S. ruminantium* ME13 in NFMS media supplemented with 8 mM NH_4Cl.

d) Growth of *B. ruminicola* strain *ruminicola* in NFMS media supplemented with 8 mM NH_4Cl and 0.1 % yeast extract.

e) Growth of *Butyrivibrio fibrisolvens* 49 in NFMS media supplemented with 0.15 % Trypticase and 8 mM NH_4Cl.

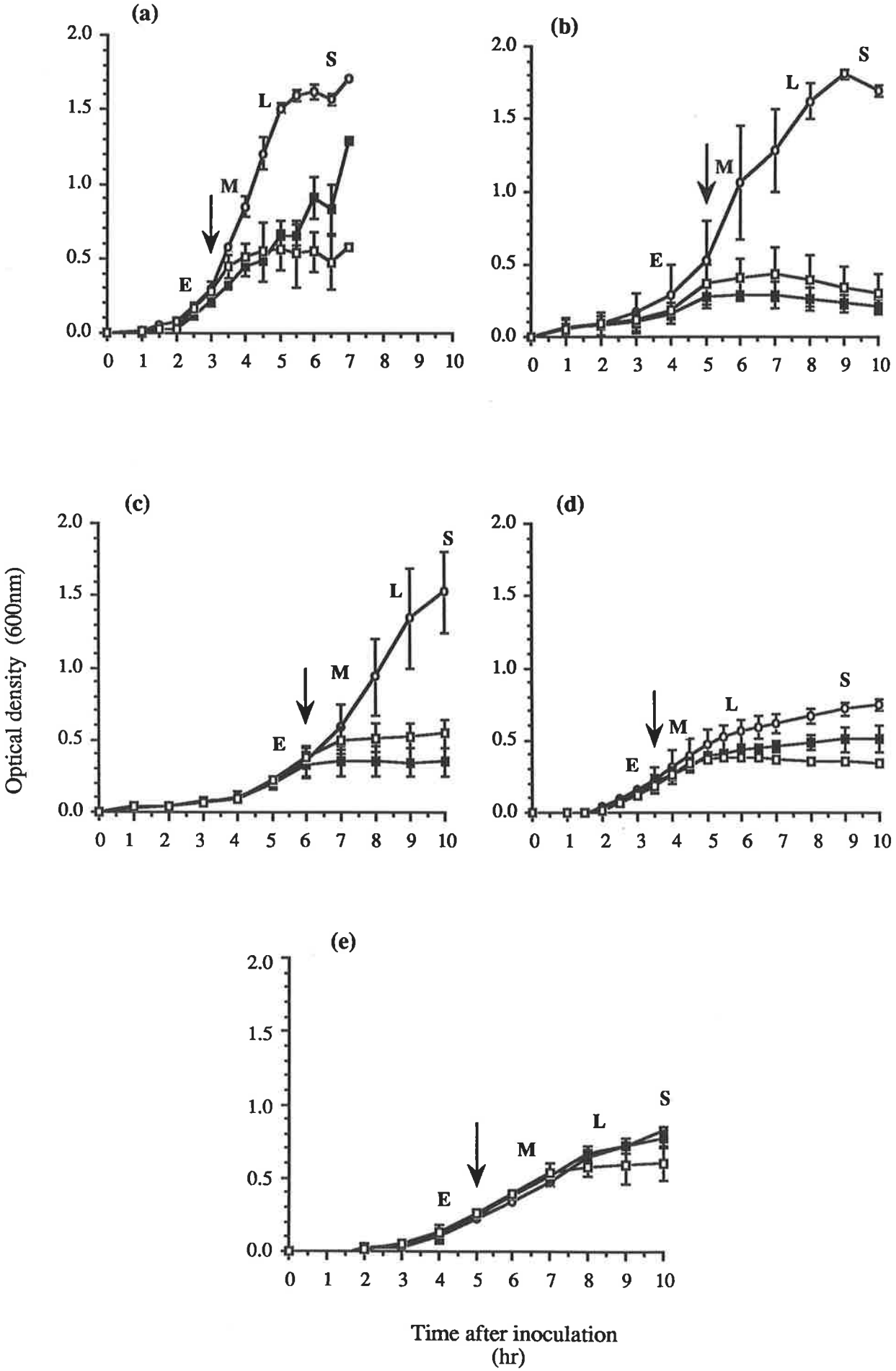

(a)
(b)
(c)
(d)
(e)
S
L
M
E
Optical density (600nm)
Time after inoculation
(hr)

Table A2.1 : Casamino Acids, Tryptone and casein levels for *B. ruminicola* BA25 cultures in NFMS media supplemented with 0.05 % [w/v] or 0.5 % [w/v] glucose and various nitrogen sources, individually and in combinations.

Serum bottle cultures (130 ml) of *B. ruminicola* BA25, inoculated from an overnight culture in NFMS media supplemented with 8 mM NH_4Cl, were grown in the appropriate NFMS medium (see Table 3.2) supplemented with one of the nitrogen source treatments listed in Table 3.10, and either 0.05 % [w/v] or 0.5 % [w/v] glucose. Growth was monitored at 30 or 60 min. intervals by optical density measurements of 1 ml sub-samples from the cultures. Casamino Acids, Tryptone and casein levels in the media were measured in samples which were not inoculated, as well as in sub-samples of the media harvested at late log of culture growth (Figure A2.1 (a), Appendix 2) in the serum bottle cultures.

Casamino Acids, Tryptone and casein concentrations are the mean ± the standard error of the mean from triplicate measurements. Different superscripts within each glucose level indicate significant differences at the 5% level of significance (Student's T-tests were not performed for between glucose level or between treatment comparisons).

† The NFMS medium (Table 3.2) was modified with the nitrogen source & glucose level listed.

TREATMENT †		Sample	Casamino Acids Concⁿ (mg (ml media)$^{-1}$)	Tryptone Concⁿ (mg (ml media)$^{-1}$)	Casein Concⁿ (mg (ml media)$^{-1}$)
Nitrogen Source	Glucose Concⁿ				
NH_4Cl	0.05 %	pre	1.63 ± 0.205 [a]	1.08 ± 0.028 [a]	0.03 ± 0.028 [a]
		late log	6.24 ± 0.901 [b]	0.00 ± 0.000 [b]	0.00 ± 0.000 [a]
	0.5 %	pre	0.47 ± 0.090 [a]	0.99 ± 0.057 [a]	0.04 ± 0.025 [a]
		late log	1.53 ± 0.557 [a]	0.06 ± 0.035 [b]	0.00 ± 0.000 [a]
Casamino Acids	0.05 %	pre	12.5 ± 1.71 [a]	4.97 ± 0.074 [a]	0.21 ± 0.012 [a]
		late log	12.4 ± 0.47 [a]	4.10 ± 0.834 [b]	0.00 ± 0.000 [b]
	0.5 %	pre	12.9 ± 1.71 [a]	5.47 ± 0.243 [a]	0.31 ± 0.041 [a]
		late log	11.2 ± 1.09 [a]	4.13 ± 0.072 [b]	0.15 ± 0.005 [b]
Tryptone	0.05 %	pre	6.71 ± 0.875 [a]	12.3 ± 0.18 [a]	0.31 ± 0.028 [a]
		late log	4.27 ± 0.277 [a]	10.4 ± 0.21 [b]	0.00 ± 0.000 [b]
	0.5 %	pre	4.68 ± 0.254 [a]	12.9 ± 0.20 [a]	0.28 ± 0.037 [a]
		late log	4.42 ± 1.005 [a]	10.8 ± 0.09 [b]	0.32 ± 0.014 [a]
Casein	0.05 %	pre	0.68 ± 0.375 [a]	1.90 ± 0.083 [a]	19.0 ± 0.50 [a]
		late log	0.31 ± 0.159 [a]	0.50 ± 0.041 [b]	17.2 ± 0.15 [b]
	0.5 %	pre	0.00 ± 0.000 [a]	1.99 ± 0.111 [a]	18.7 ± 0.91 [a]
		late log	0.57 ± 0.528 [a]	1.92 ± 0.052 [a]	15.9 ± 0.46 [a]
NH_4Cl + Casamino Acids	0.05 %	pre	12.4 ± 2.30 [a]	5.12 ± 0.037 [a]	0.24 ± 0.005 [a]
		late log	13.5 ± 1.56 [a]	3.25 ± 0.010 [b]	0.00 ± 0.000 [b]
	0.5 %	pre	9.99 ± 0.774 [a]	5.50 ± 0.103 [a]	0.28 ± 0.031 [a]
		late log	9.99 ± 0.349 [a]	4.06 ± 0.127 [b]	0.00 ± 0.000 [b]
NH_4Cl + Tryptone	0.05 %	pre	4.73 ± 0.710 [a]	12.8 ± 0.32 [a]	0.38 ± 0.016 [a]
		late log	5.98 ± 1.374 [a]	10.5 ± 0.14 [b]	0.42 ± 0.046 [a]
	0.5 %	pre	4.63 ± 0.280 [a]	12.2 ± 0.19 [a]	0.55 ± 0.045 [a]
		late log	4.53 ± 0.964 [a]	11.12 ± 0.13 [b]	0.21 ± 0.063 [b]
NH_4Cl + Casein	0.05 %	pre	1.09 ± 0.292 [a]	2.05 ± 0.564 [a]	17.8 ± 0.37 [a]
		late log	1.56 ± 1.039 [a]	0.53 ± 0.061 [a]	16.7 ± 0.69 [a]
	0.5 %	pre	1.04 ± 0.710 [a]	2.04 ± 0.164 [a]	19.0 ± 0.37 [a]
		late log	1.20 ± 0.785 [a]	2.08 ± 0.071 [a]	14.5 ± 0.37 [b]
Casamino Acids + Tryptone	0.05 %	pre	12.0 ± 0.60 [a]	15.9 ± 0.30 [a]	0.53 ± 0.012 [a]
		late log	10.9 ± 0.13 [a]	14.2 ± 0.05 [b]	0.49 ± 0.097 [a]
	0.5 %	pre	12.0 ± 1.59 [a]	15.9 ± 0.73 [a]	0.56 ± 0.005 [a]
		late log	14.4 ± 0.91 [a]	14.3 ± 0.35 [a]	0.59 ± 0.104 [a]
NH_4Cl + Casamino Acids + Tryptone	0.05 %	pre	18.1 ± 0.87 [a]	16.4 ± 0.10 [a]	0.52 ± 0.019 [a]
		late log	16.5 ± 1.98 [a]	14.4 ± 0.39 [b]	0.32 ± 0.115 [a]
	0.5 %	pre	9.88 ± 0.681 [a]	16.4 ± 0.36 [a]	0.66 ± 0.028 [a]
		late log	10.0 ± 0.52 [a]	14.8 ± 0.25 [b]	0.32 ± 0.063 [b]
NH_4Cl + Cas Acids + Tryptone + Casein	0.05 %	pre	24.4 ± 1.09 [a]	16.2 ± 0.53 [a]	18.7 ± 0.50 [a]
		late log	19.3 ± 0.43 [b]	14.3 ± 0.28 [a]	19.6 ± 0.76 [a]
	0.5 %	pre	24.5 ± 2.10 [a]	17.3 ± 0.12 [a]	19.2 ± 0.39 [a]
		late log	16.1 ± 1.57 [a]	14.6 ± 0.04 [b]	19.1 ± 0.30 [a]
RGM	0.2 %	pre	3.28 ± 0.710 [a]	4.07 ± 0.027 [a]	0.15 ± 0.041 [a]
		late log	3.69 ± 0.286 [a]	2.81 ± 0.066 [b]	0.00 ± 0.000 [b]

CHAPTER 6

Bibliography

"We stand upon the intellectual shoulders of the giants of bygone days "

(Claude Bernard, 1855)

Czerkawski (1986)

Chapter 6

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