Chapter 1

INTRODUCTION

The macropodine marsupials (Family Macropodidae; sub-Family Macropodinae) are an extremely successful group of herbivores that have radiated within Australia to occupy diverse ecological habitats in a manner parallel to the radiation of the eutherian herbivores on other continents. Physiological and behavioural adaptation among the macropodine species has resulted in a divergent array of habitat utilisation from arid and semi-arid zones to dense tropical rainforest.

Even today, under massive pressure from habitat destruction, changes in land use patterns, and competition from domestic livestock, there still exist more than thirty species of these herbivorous macropodines on the Australian continent (Frith, 1973).

The successful radiation by the macropodines may be partly attributed to their mode of digestion which has been described as "ruminant-like" by Moir, Somers and Waring (1956). Extensive microbial fermentation of ingested food in the voluminous cranial region of the stomach results in the digestion of considerable quantities of plant structural carbohydrates and the production of microbial protein, providing energy and amino acids for the host animal. Macropodines, like most ruminants, are thus able to utilise natural diets which may be both high in fibre content and low in protein.

Earlier studies have determined that macropodines utilise high roughage diets less efficiently than ruminants such as the sheep (Foot and Romberg, 1965; McIntosh, 1966; Forbes and Tribe, 1970). This was attributed to a faster rate of passage through the digestive tract, the assumption being that there is less time available for microbial attack on thethe less digestible structural carbohydrates within the stomach. Thus the macropodines are generally considered to be intermediate between

ruminants and the hindgut fermenters in efficiency of utilisation of roughage diets (Moir, 1965).

Recent evidence indicates an order of gradation among some species of macropodines in both the efficiency of utilisation of dietary fibre (Hume, 1974) and the contribution of microbial fermentation to the overall digestive process (Hume, 1977a). Hume (1977b) suggested that species adapted physiologically and behaviourally to the drier and less predictable climatic zones may be more efficient in digestion of low quality forage, and in metabolism, than those species restricted to closed forest habitats.

1.1 The research topic

Although differences in the efficiency of apparent digestion of diets have been reported between macropodines and sheep, and between two species of macropodines (Hume, 1974), detailed information on the factors affecting and controlling fermentation within the macropodine stomach is sparse. Previous investigations on digestion in macropodines were based on the assumption that factors governing microbial digestion within the stomach are essentially the same as those operating within the ruminant stomach.

The aim of this thesis is to investigate and compare in detail some of the fundamental aspects of the physiology of microbial digestion in three species of macropodines that represent a wide range of habitat preference. Some direct comparisons were also made between these species and sheep. Where applicable, reference is made in the thesis to observations on other species of macropodines.

The three macropodine species of primary interest in this study were *Thylogale thetis* (red-necked pademelon), *Macropus eugenii* (tammar wallaby) and *Macropus giganteus* (eastern grey kangaroo).

T. thetis is a small wallaby (3-8 kg) restricted to

rainforest and wet sclerophyll forest habitat along the eastern escarpment of the Great Dividing Range in south-east Queensland and eastern New South Wales. M. eugenii is a wallaby of similar size that is adapted to a more severe, drier climate and its range is now restricted mainly to some of the offshore islands in the south-west region of Western Australia and South Australia. M. giganteus, one of the largest species, has a preferred habitat of open woodland and is widely distributed throughout most of eastern Australia (Ride, 1970).

The first stage of this work consisted of an examination of the digestive tract of the above three species, and in addition, M. rufogriseus (red-necked wallaby) and M. robustus robustus (eastern wallaroo). Species-specific differences in stomach structure were apparent in all five species, but were more marked among T. thetis, M. eugenii and M. giganteus. Since these three species are also found in markedly different habitats, and were more readily obtainable, they were selected for the major part of this comparative study.

The use of radiographic and radioisotopic marker techniques revealed that the pattern of flow of digesta in each species could be related to stomach structure. In addition, the pattern of flow of digesta through the digestive tract of all three species was very different from that observed in the sheep. Further, these differences could be attributed to the mode of flow of digesta through the stomach.

Subsequent experiments were carried out to determine the extent of digestion in the whole digestive tract and within the stomach, and to investigate the effects of the pattern of flow of digesta through the stomach on microbial activity and digestion.

Chapter 2

REVIEW OF THE LITERATURE

Moir (1965, 1968) and Tyndale-Biscoe (1973) reviewed aspects of the anatomy of the digestive tract, the mode of digestion, and metabolism in macropodines and suggested that these herbivores are comparable to the ruminants. More recently, microbial activity in the macropodine stomach was discussed by Bauchop (1977), in a review on foregut fermentation.

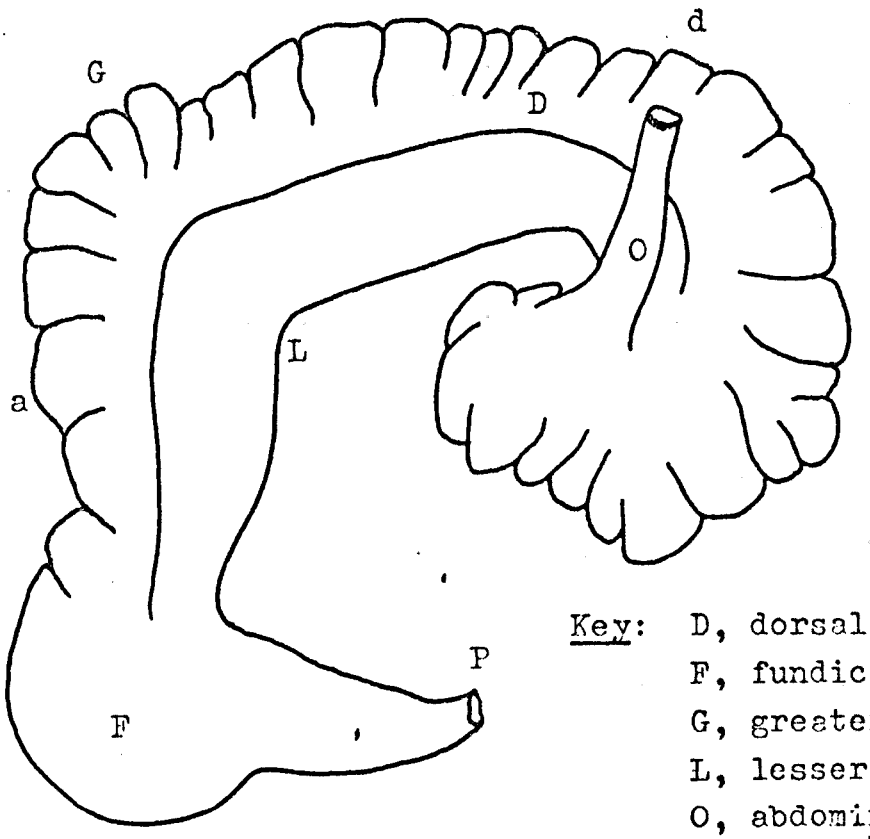
This review discusses the present state of knowledge of the physiology of microbial digestion in macropodine marsupials, and the direct comparisons that have been made between macropodines and sheep.

2.1 Anatomy of the digestive tract

Recorded descriptions of the anatomy of the macropodine digestive tract highlight the presence of a capacious stomach of complex structure, and a relatively small caecum and proximal colon. The dimensions of these structures and the presence of a gastric sulcus (oesophageal groove) within the stomach were suggested by Owen (1834, quoted by Moir, 1965), Owen (1868) and Moir, Somers and Waring (1956) as structural features analogous to those of the ruminant digestive tract.

2.1.1 Anatomy of the stomach

The macropodine stomach (Fig. 2.1), in contrast to the ruminant stomach (Nickel, Schummer and Seiferle, 1973), is a "long tubular colonlike organ - the contents of which may account for some 15% of the body weight" (Moir, 1968). In situ, the stomach is coiled spirally and occupies the entire ventral floor of the abdomen (MacKenzie, 1918). At the cardiac end of the stomach, a blind sac, positioned ventro-medially to the entrance of the oesophagus, is continuous with the main tubular body of the stomach which in turn consists of a left descending limb and a right



Key:
- D, dorsal taenia
- F, fundic region
- G, greater curvature
- L, lesser curvature
- O, abdominal oesophagus
- P, pylorus
- d, descending limb
- a, ascending limb

Fig. 2.1

Stomach of Macropus giganteus, the eastern grey kangaroo (Redrawn from MacKenzie, 1918).

ascending limb terminating at the gastric region and pylorus. The wall of the greater curvature of the left descending limb and part of the ascending limb of the stomach wall is deeply folded, whereas the wall of the lesser curvature and the gastric region is smooth. These haustrations are bordered by three bands of longitudinal muscle fibres, taeniae, which extend along most of the length of the stomach (Owen, 1868). It is the presence of these taenia, and the associated haustrations that give the macropodine stomach the colon-like appearance.

On the inner wall of the stomach, the gastric sulcus is continuous with the cardia (Owen, 1868), and extends caudally along the lesser curvature of the left descending limb. The gastric sulcus is considered to be present in all macropodines (Brown, 1964) but its length and definition apparently vary among species (Moir, 1968).

The lining of the stomach is composed mainly of glandular cardiac epithelium (Schäfer and Williams, 1876; Griffiths and Barton, 1966; Gemmel and Engelhardt, 1977). The structure of the tubular glands, and the finding that the surface cells elaborate a material stained by periodic acid-Schiff reagents, suggest that this mucosa secretes mucin (Griffiths and Barton, 1966; Gemmel and Engelhardt, 1977).

Non-secretory squamous epithelium is present on the floor and walls of the sulcus, the area adjacent to the cardia, and may also extend into the blind sac (Schäfer and Williams, 1876; MacKenzie, 1918). From literature descriptions of macropodine stomach histology (Schäfer and Williams, 1876; MacKenzie, 1918; Griffiths and Barton, 1966; Gemmel and Engelhardt, 1977), it is apparent that the extent and distribution of this squamous epithelium vary among species.

Parietal and chief cells are confined to an isolated area in the fundus of the stomach of adult macropodines (Griffiths and Barton, 1966). Thus, similar to ruminants,

only a small proportion of the total stomach contents in the caudal region of the organ are exposed to peptic digestion prior to passage into the duodenum.

Although minor differences in both the gross structure of the stomach and the distribution of the epithelial cell types among some species were noted by Owen (1868), Schäfer and Williams (1876) and MacKenzie (1918), macropodine stomach structure and digestive physiology have been considered as generally similar in all species (Moir, Somers and Waring, 1956; Brown, 1964).

2.1.2 The large intestine

The caecum is a relatively small, tubular structure, similar to that of ruminants, with dimensions somewhat variable among species (MacKenzie, 1918). Taeniae are present on the caecal wall and extend along the wall of the proximal colon. However, they are not well developed and associated haustrations are poorly defined. The distal colon has a small diameter (MacKenzie, 1918), similar to the distal colon of the sheep, and is relatively short.

2.2 Passage of digesta through the digestive tract

Moir, Somers and Waring (1956) demonstrated extensive microbial activity in the cranial regions of the stomach of Setonix brachyurus (quokka). Since microbial digestion of plant structural carbohydrates is relatively slow (Hungate, 1966) and thus, in part, a function of prolonged residence time, it became of interest to have some estimate of the flow of digesta in macropodines. The rate of passage of food residues was measured in S. brachyurus (Calaby, 1958), Megaleia rufa (red kangaroo) (Foot and Romberg, 1965; McIntosh, 1966; Forbes and Tribe, 1970) and Macropus giganteus (eastern grey kangaroo) (Forbes and Tribe, 1970) fed on roughage diets. In the latter three studies direct comparisons were made with sheep fed the same diets. Stained hay particles were used as a marker (similar to the methods of Castle (1956a)), and recovery of these

particles in the faeces was used to calculate percentage times of excretion.

Data from animals fed chopped lucerne hay (Table 2.1) indicate that, even on a lower voluntary food intake, excretion times in both Me. rufa and M. giganteus were shorter than in the sheep. Similar differences were found between the two macropodine species and the sheep when the animals were fed a chopped oaten hay diet. In all of these investigations it was generally observed that marker excretion times were increased when food intake was restricted or voluntary food intake decreased.

These results were estimates of the retention of the stained hay particles in the entire digestive tract. Since the stomach is much larger than the caecum-proximal colon it is likely that the retention time of digesta in the stomach of macropodines is, like ruminants, much longer than in any other region of the digestive tract.

2.2.1 Pattern of excretion of the marker

Not only did the excretion times differ, but there were also differences in the pattern of excretion of the marker between the macropodines and the sheep. For instance, the time difference between 50% excretion time and 90% excretion time was considerably shorter in the macropodines (10 to 12 h) than in the sheep (26 to 37 h) for both chopped lucerne hay and chopped oaten hay diets. This is evident in the steeper cumulative percentage excretion curves for both Me. rufa and M. giganteus.

Calaby (1958) also noted relatively short 95% excretion times in S. brachyurus. Both he and McIntosh (1966) concluded that this was possibly because of less complete mixing of ingested food in the macropodine stomach than would be expected in the ruminoreticulum of sheep. Further investigations on the pattern of flow of digesta through the digestive tract of macropodines to elucidate this have not been undertaken.

Table 2.1 Dry matter intake, 50% excretion times (50%ET) and 90% excretion times (90% ET) in three species of macropodines and in sheep fed chopped lucerne hay ad libitum. Stained hay particles were used as the marker.

Species	Dry matter intake ($g/kgW^{0.75}/d$)	50% ET (h)	90% ET (h)	Reference(1)
S. brachyurus	47	--	38[a]	1
Me. rufa	58	35	45	2
" "	38	41	58[a]	3
" "	63	28	39	4
M. giganteus	49	39	50	4
Macropodine mean:	52	36	48	
Sheep	72	41	67	2
"	64	38	69[a]	3
"	67	52	89	4
Sheep mean:	67	44	75	

a, measured as 95% ET

(1), Data from 1, Calaby (1958),
2, Foot and Romberg (1965),
3, McIntosh (1966),
4, Forbes and Tribe (1970).

2.2.2 Validity of the stained hay particle technique

In ruminants, larger particles of food have a longer retention time in the ruminoreticulum than do small particles (Blaxter, Graham and Wainman, 1956). The majority of large particles must be reduced to a minimum size by rumination and by microbial digestion before passage through the reticulo-omasal orifice to the omasum and the abomasum (Reid, Ulyatt and Monro, 1977). Thus the size of the stained hay particles could influence the rate of passage measurements. Such an effect was demonstrated by Castle (1956b) in her experiments with goats. Whether flow of food particles through the macropodine stomach is governed by a similar form of control is not known; macropodines occasionally display a form of regurgitation but they do not ruminate (Barker, Brown and Calaby, 1963).

Stained particles as a marker for digesta flow can only be indicative of the flow behaviour of those digesta particles with similar physical characteristics. In recent years, chemical and radioisotopic markers have been shown to be a more valid and discriminating measure of the flow of the fluid and the particulate fractions of the digesta, and have been extensively reviewed (see Hydén, 1961; Kotb and Luckey, 1972; Engelhardt, 1974; Faichney, 1975a).

The dual markers ^{51}Cr-EDTA (the ^{51}chromium complex of ethylenediaminetetra-acetic acid; Downes and McDonald, 1964), and ^{103}Ru-P (^{103}ruthenium labelled tris-(1,10-phenanthroline)-ruthenium-II chloride; Tan, Weston and Hogan, 1971), have been established as one of the most suitable available systems for the concurrent measurement of the flow of both the fluid and the particulate fractions of digesta, respectively, in sheep (Faichney, 1975a).

Warner (1977) used these same two markers to examine rate of passage in the digestive tract of Macropus eugenii (tammar wallaby) fed pelleted lucerne hay. He found significant differences in overall mean retention times of the two markers; 9 h and 16 h for ^{51}Cr-EDTA, and 16 h and 29 h for ^{103}Ru-P in animals fed continuously and once daily,

respectively. Measurement of marker concentrations in digesta samples from slaughtered animals indicated that separation of the two markers occurred in the stomach. The degree of separation of the two markers was considerably greater, and the mean retention time of the ^{51}Cr-EDTA marker was shorter, than would be expected to occur in the ruminoreticulum of sheep on similar dietary regimes.

2.3 Food intake and digestion

Food intake parameters (e.g. dry matter, organic matter, nitrogen, energy) and apparent digestibility coefficients have been previously measured in S. brachyurus (Calaby, 1958); M. robustus cervinus (western euro) (Brown, 1964, 1968; Hume, 1974); Me. rufa (Foot and Romberg, 1965; McIntosh, 1966; Forbes and Tribe, 1970; Hume, 1974); M. giganteus (Forbes and Tribe, 1970); M. eugenii (Lintern-Moore, 1973; Hume 1977b); and T. thetis (red-necked pademelon) (Hume, 1977b). These experiments have helped to define basal maintenance requirements of nitrogen and energy of the macropodines, and to compare the ability of macropodines and sheep to digest high roughage diets and to retain nitrogen at low dietary nitrogen intakes.

2.3.1 Intake requirements

Brown (1968), and others, have shown that macropodines generally consume less dry matter than sheep (on a metabolic body weight basis) when offered high roughage diets ad libitum, and concomitantly have a lower intake of digestible energy and nitrogen, but still maintain body weight. As pointed out by Hume (1977b), this is in agreement with the findings of Dawson and Hulbert (1970) that the basal metabolic rate of marsupials is only 70% of that of eutherian mammals.

Maintenance nitrogen requirements have been estimated for M. robustus cervinus (Brown and Main, 1967); M. eugenii (Barker, 1968; Hume, 1977b); and T. thetis (Hume, 1977b). The estimates for M. robustus cervinus and

M. eugenii are both lower than maintenance nitrogen requirements for the sheep, and Brown (1968) suggested that this is directly related to the lower metabolic rate of macropodines. However, the maintenance nitrogen requirement estimated for T. thetis (Hume, 1977b) is higher, and similar to that of the sheep. Hume suggested that the available evidence indicates that T. thetis has a more limited microbial digestion (Hume, 1977a), compared with macropodine species from more arid environments, rather than a higher metabolic rate.

2.3.2 Digestibility of dry matter and fibre

From balance experiments, Calaby (1958) determined that S. brachyurus is capable of digesting 29 to 40% of the crude fibre fraction of high roughage diets, and suggested that this species was intermediate between the hindgut fermenters and ruminants in its ability to digest fibre. Other balance experiments have shown that, compared with sheep, two of the larger species of macropodines, Me. rufa and M. giganteus generally digest less dry matter, and a smaller percentage of fibre of high roughage diets (Foot and Romberg, 1965; McIntosh, 1966; Forbes and Tribe, 1970; Hume, 1974; Kempton, Murray and Leng, 1976) (see Table 2.2). M. robustus cervinus, on the other hand, has been shown to be superior to Me. rufa and equal to the sheep in digestion of acid-detergent fibre (cellulose plus lignin) (Hume, 1974) of similar diets, although all three species digested similar quantities of hemicellulose.

The lower efficiency of digestion of dry matter, and particularly of fibre, in the macropodines is thought to be associated with the faster rate of passage of digesta in these species compared with the sheep (Foot and Romberg, 1965; McIntosh, 1966; Forbes and Tribe, 1970). The underlying assumption has been that in the digestive tract of macropodines, particularly in the stomach, there is less time available for digestion of the less tractable dietary structural carbohydrates. This premise also assumes that the mode of microbial digestion is similar in macropodines and sheep. Although Moir, Somers and

Table 2.2 Dry matter intake and apparent digestibility of dry matter and fibre in macropodines and in sheep fed chopped lucerne hay.

Species	Dry matter intake ($g/kgW^{0.75}/d$)	Digestibility of		Reference (1)
		Apparent dry matter (%)	Fibre (%)	
S. brachyurus	47	59	30	1
Me. rufa	58	54	33	2
" "	38	56	24	3
" "	63	51	32	4
" "	53	55	36	5
M. giganteus	48	54	38	4
" "	47	55	36	6
M. robustus cervinus	53	62	46	5
Sheep	72	63	46	2
"	64	57	39	3
"	67	58	43	4
"	92	61	44	5
"	48	62	48	6

(1), Data from: 1, Calaby (1958) - crude fibre,
2, Foot and Romberg (1965) - crude fibre,
3, McIntosh (1966) - crude fibre,
4, Forbes and Tribe (1970) - crude fibre,
5, Hume (1974) - acid detergent fibre,
6, Kempton, Murray and Leng (1976) - crude fibre.

Waring (1956) reported cellulolytic activity by microbes taken from the stomach of S. brachyurus, no quantitative estimates of the amounts of structural carbohydrates digested in the stomach of macropodines, or in the caecum-proximal colon, have been reported.

2.4 Microbial digestion in the stomach

As a result of microbial digestion in the proximal regions of the stomach it is probable that, similar to ruminants, only a small, variable proportion of the dietary soluble protein and little of the soluble carbohydrate fraction are directly available to the host animal. Thus, macropodines presumably rely on absorption of volatile fatty acids as a major source of energy, and absorption of microbial amino acids as a source of energy and for protein synthesis. Factors influencing the degree and efficiency of fermentative digestion and protein production by the stomach microbiota have been thoroughly investigated in domestic ruminants but have received little attention in macropodines.

2.4.1 Stomach microorganisms

Bacteria exist in the pregastric regions of the stomach, and in the large intestine, but no detailed information on the types or populations have been published. Moir, Somers and Waring (1956) examined stomach contents from S. brachyurus and observed a dense bacterial population of some fifteen types, mainly Gram negative rods and cocci, with a few spiral forms. Gram positive rods were also present, and dominated the population when the pH was below 5.5. Although fewer bacterial types were found than would be expected in ruminants, the total density of the population (10^{10}/ml) and the proportion of cellulolytic bacteria was similar (R.E. Hungate, pers. comm. to Moir, 1968).

Protozoa may occur less consistently in the macropodine

stomach. Ciliates were not found in S. brachyurus maintained in captivity, but three unidentified ciliates, ranging in total number 0.5 to 1.3 x 10^6/g, were noted in grazing animals (Moir, Somers and Waring, 1956). Ciliates have also been found in adult Me. rufa (Harrop and Barker, 1972) and in M. eugenii (Lintern-Moore, 1973).

2.4.2 The stomach environment

Conditions within the macropodine stomach favour the establishment and maintenance of a microbial population. Ingested food is retained for a considerable time, and is only exposed to acid conditions in the distal region of the organ (Griffiths and Barton, 1966). The pH of the digesta in the proximal regions of the stomach is approximately neutral, indicating that the stomach fluid is well buffered, although the pH may vary with time of last feeding (Moir, Somers and Waring, 1956).

The parotid salivary glands are large and produce a buffered serous secretion similar to that of ruminants (Forbes and Tribe, 1969). The secretion is alkaline (Moir, Somers and Waring, 1956), and secretion rates are considered to be comparable to ruminants of similar size (Brown, 1964). The submaxillary glands also produce a buffered and predominantly serous secretion (Forbes and Tribe, 1969). It is possible that the tubular glands of the glandular epithelium in the proximal stomach region may also secrete a buffered solution, along with mucin, as in the forestomach of the llama (Rübsamen and Engelhardt, 1978).

2.4.3 Microbial activity in the stomach

a) Volatile fatty acid production

VFA produced during the microbial fermentation of ingested food are absorbed across the stomach wall (Moir, Somers and Waring, 1956), and should provide a major source of energy for the host animal. The rate of production of VFA in the macropodine stomach has so far

only been estimated using *in vitro* incubation techniques. Moir (1968) reported a production rate of 20 μM/ml/h in stomach contents taken from *S. brachyurus*. Hume (1977a) incubated stomach contents *in vitro* from *T. thetis*, *Macropus rufogriseus* (red-necked wallaby) and sheep fed chopped lucerne hay and estimated production rates of 39, 52 and 23 μM/ml/h of total VFA for these three species, respectively. It was calculated that the VFA would provide 21% of the digestible energy intake of *T. thetis*, 42% in *M. rufogriseus* and 29% in the sheep. Molar proportions of the individual acids in these species were similar, except for acetic acid which was lower in *M. rufogriseus* than in the other two species.

Measurement of production rates of VFA in digesta samples incubated *in vitro* may correlate well with estimations *in vivo* (El-Shazly and Hungate, 1965), but the technique may significantly underestimate the true VFA production rate (Whitelaw, Hyldgaard-Jensen, Reid and Kay, 1970). Soluble components of food ingested by ruminants are rapidly digested by the microorganisms (Hungate, 1966), and concomitant production of VFA can be appreciable and very rapid for a short period of time (Sutherland, 1963). Such a short term, fast production of VFA is unlikely to be accounted for in an *in vitro* incubation since there is always an appreciable time lag from when the animal was last fed until the *in vitro* zero-time sample is collected. However, the use of such a technique for comparisons among species, fed the same diet, should be valid (Hume, 1977a).

b) Ammonia

Ammonia is produced by many gastrointestinal bacteria (Prins, 1977), mainly from the deamination of dietary and microbial amino acids, and from urea. This ammonia can be a major source of nitrogen for bacterial protein synthesis in macropodines (Kennedy and Hume, 1978) as it is in the sheep (Nolan and Leng, 1972).

Some of the ammonia is absorbed across the stomach wall and a proportion of this will be recycled to the

stomach as urea in the saliva (Brown, 1964), and possibly also across the stomach wall.
The importance of urea recycling to the stomach increases in animals that are fed, or only have access to, diets of low nitrogen content (Kinnear and Main, 1975).

c) Gas production

The microbial fermentation also results in the production of considerable quantities of gas, which in S. brachyurus was found to contain 65 to 75% CO_2, and hydrogen and methane (Moir, 1968); the concentrations of hydrogen and methane in the stomach gas were not reported. Kempton, Murray and Leng (1976) could not detect any methane in respired gas collected from M. giganteus fed chopped lucerne hay, nor in the gas produced from incubation in vitro of stomach contents from other M. giganteus fed the same, diet.

Microbial fermentation in ruminant animals results in the production of considerable quantities of hydrogen, most of which is normally converted to methane gas by methanobacteria (Hungate, 1966). The rate of production of methane is closely related to the rate of fermentation (Murray, Bryant and Leng, 1976). Kempton, Murray and Leng (1976) postulated that the absence of methane production in M. giganteus may be due to oxygen entering the stomach along with the food, or across the stomach wall, resulting in a low redox potential, thus inhibiting fermentation and methane production.

If the fermentation is anaerobic with the pattern of VFA produced in the absence of appreciable methane there is a problem of accounting for an excess of reducing equivalents (excess hydrogen). Some of the excess hydrogen may be present in the form of formic acid, but it is more likely that most of the hydrogen would be removed as hydrogen gas either by absorption across the stomach wall, or by physical transfer of the gas through eructation or by passage to the intestine and absorption; any gas absorbed would be eliminated in respired air.

2.4.4 Microbial protein production

It has been estimated that some 64 to 85% of ingested plant crude protein (N x 6.25) is converted to microbial protein in the stomach of M. eugenii (Lintern-Moore, 1973), an estimate similar to that obtained for sheep using the same technique (Weller, Pilgrim and Gray, 1962). In the same species, a considerable proportion of the microbial protein nitrogen is derived from utilisation of ammonia nitrogen (Kennedy and Hume, 1978). Thus, degradation of dietary protein, deamination of the resulting free amino acids, and synthesis of microbial amino acids and protein in the proximal regions of the stomach is likely to be extensive. In sheep fed various dried roughage diets, 11 to 25 g of microbial protein may be produced for every 100 g of organic matter fermented in the ruminoreticulum (see Hume, 1976); no such estimates for microbial protein production in the macropodine stomach have been reported.

2.5 Summary

Although the physiology of digestion has only been investigated in any detail in a few of the extant species of macropodines, these herbivores are generally classified as somewhat less efficient than ruminants in their digestion of roughage diets. Apart from one species, M. robustus cervinus, the macropodines generally digest less dry matter and fibre than sheep. In addition, macropodines have higher plasma glucose concentrations (Moir, Somers and Waring, 1956) and a lower degree of saturation of body fats (Hartman, Shorland and McDonald, 1955). The inference is that microbial fermentation in the macropodine stomach is more limited than in the ruminants and this is, in part, attributed to a faster rate of passage of food through the digestive tract.

Investigations on digestion in forestomach fermenters such as the macropodines appear to have assumed that microbial digestion in an enlarged proximal region must be basically similar to that in the ruminant stomach. However, two factors pertinent to the macropodines have not

been considered in any detail. These are:

(1) the gross structure of the macropodine stomach, which although large, is very different from the ruminant stomach - and this may be expected to have some effect on the pattern of flow of digesta.

(2) the faster rate of digesta flow, and any possible major effect of structure on the pattern of flow, could possibly result in microbial populations, and in patterns of microbial digestion, quite different from those in the ruminant stomach.

Chapter 3

MATERIALS AND METHODS

The materials and techniques used throughout this study are outlined in this chapter. Techniques applicable to individual experiments are described in the respective chapters.

3.1 Capture, care and maintenance of animals

All experiments were conducted with adult animals. Only adult non-lactating female *M. giganteus* (eastern grey kangaroo) were used whereas both sexes of *M. eugenii* (tammar wallaby), *T. thetis* (red-necked pademelon) and *M. robustus robustus* (eastern wallaroo) were included in experiments. The sheep used were Border Leicester x Merino wethers (24-30 months of age).

M. eugenii were obtained from a captive breeding colony maintained at CSIRO Division of Wildlife, Canberra. *M. giganteus* and *M. robustus robustus* were trapped near Jeogla, 80 km east of Armidale. *T. thetis* were trapped near Dorrigo, 150 km east of Armidale.

At the time of capture, macropodines were placed in open weave hessian bags and transported back to the laboratory as quickly as possible. The animals were housed in individual metabolism cages, in a quiet room maintained at 20 $\pm$ 2°C, with access to food and water. The cages were covered with open weave hessian bags for an initial period until the animals adjusted to their surroundings and the feeding and cleaning procedures. During the first 10 d, the bag coverings were gradually removed until only the rear portion of each cage was covered.

Now animals were daily offered chopped lucerne hay and mixed grain (crushed wheat, sorghum and soybean meal) plus a mineral/vitamin mix. During the first two weeks, fresh grass was also offered. Most animals accepted food

within 48 h. Adjustment to and acceptance of feeding and cleaning procedures was usually apparent within two weeks, but animals were always maintained for at least eight weeks before use in experiments. Of the four macropodine species, M. giganteus and M. robustus robustus were always more apprehensive of undue and unexpected noise, and to unfamiliar people. M. eugenii were generally more excitable than T. thetis. The latter species settled extremely quickly to maintenance procedures after capture.

Between experiments, reserve animals were kept in external, mesh enclosed yards with access to shelter, food and water. In the enclosures, the animals were fed lucerne in the long hay and chopped form, and mixed grain.

3.2 The experimental diets

Two diets were used in the experiments. These were chopped lucerne hay and a fresh grass diet, Phalaris aquatica. The chemical composition of the two diets is presented in Table 3.1. A mineral/vitamin mix (Dynamin, Janos Chemicals Ltd., Newmarket, Victoria; see Table 3.1) was added daily (final concentration of 2% of wet weight offered) to the chopped lucerne hay for each animal.

The grass diet contained almost twice as much readily fermentable carbohydrate (soluble sugars plus pectin) as the chopped lucerne hay. The high silica content of the grass accounts for the lower organic matter content.

3.2.1 Chopped lucerne hay

A single commercial supply of 1.5 tonne of chopped lucerne hay was uniformly mixed, bagged and stored for use as the experimental diet. Other supplies of chopped lucerne hay were used for feeding new animals and those kept in reserve.

During experimental periods, the chopped lucerne hay weighed and offered daily to each animal was at least 30%

Table 3.1 Chemical compositions (% DM) of the two experimental diets, chopped lucerne hay and the fresh herbage Phalaris aquatica, and composition of the mineral/vitamin mix.

	Lucerne	Phalaris
Readily fermentable carbohydrates	10.0	19.1
Hemicellulose	13.7	20.1
Cellulose	26.6	18.1
Acid-detergent fibre	35.1	29.5
Lignin	8.4	3.5
Silica	0.3	7.8
Organic matter	90.4	82.6
Total nitrogen	3.0	2.4
Crude protein	19.0	15.1
Total lipid	3.8	4.3
Energy (kJ/g DM)	18.4	17.5
Dry matter (% wet weight)	93.8	20 to 24

Mineral/vitamin mix (as units/kg chopped lucerne hay).
Minerals (mg/kg): Ca, 4900; Fe, 3; Cu, 3; Co, 1; Mg, 211; Mn, 3; Zn, 2; I, 1.
Vitamins (iu/kg): A, 35210; D, 211; E, 21.
Vitamins (mg/kg): B complex, 6; C, 14; Pantothenic acid, 6; Niacin, 11; Choline, 633; K, 0.1; Biotin, 0.1.

in excess of the previous day's intake. This was because of the ability of most animals to select against some of the larger stem material of the diet. Hume (1977a) similarly determined that T. thetis actively selects from chopped lucerne hay offered. In the present study it was apparent that this was an individual behavioural adaptation of the majority of animals of all four macropodine species. For this reason, chemical analysis of both lucerne offered and refusals from each animal during collection periods was necessary to allow determination of the actual intake of dietary constituents.

3.2.2 Phalaris aquatica

The fresh grass diet was harvested daily, from self-sown plants adjacent to the animal laboratory, during the period September-October, 1977. Dried growth from the previous summer was burnt off in the winter months and the subsequent spring growth allowed harvesting of the herbage in a young vegetative state uncontaminated with other pasture species. To avoid lush growth the area was deliberately not fertilised, although some irrigation was required.

The herbage was harvested by hand at 0800 to 0900 h each morning and the leaf material cut to a maximum length of 15 cm. Daily samples were used to estimate dry matter content during the experimental periods. Excess feed, determined by the previous day's intake, for each animal, was weighed and stored in a plastic bag below 10°C. Because of the bulky nature of the herbage, the animals were offered the weighed food at least twice daily. Since the grass offered contained no stem material, selection within the diet was not possible. Thus, after determination of total dry matter of the daily food refusals from each animal, the refusals were discarded.

3.3 Experimental feeding and collection procedures

Prior to and during experiments the sheep were housed in standard metabolism crates and the macropodines were

housed in individual metabolism cages enclosed with wire mesh. Feed and water were available ad libitum through openings at the front of the cages. The food and water containers were placed outside the cages for easy access and to minimise spillage and contamination of excreta. Faeces and urine were collected separately from inclined trays placed under the cages. The urine drained into a polythene bottle, under each tray, that contained glacial acetic acid to maintain pH below 4 to prevent loss of nitrogen as ammonia, and to inhibit bacterial growth.

The experimental diet was always offered to animals for a minimum period of three weeks before experiments commenced. Usually seven days before, and during experiments, food was replaced at least twice daily, or delivered from moving belt feeders, in an attempt to attain steady state conditions of fermentation and digesta flow in the digestive tract. Under ad libitum feeding conditions, it was noted that all four macropodine species consumed food more slowly than is normally seen in sheep. Thus, even when food was replaced only twice daily the intake of diet throughout the day was relatively constant.

Animals were weighed at the beginning and at the end of each balance period.

During experimental collection periods, daily samples (100 g) of chopped lucerne hay were kept and bulked for later analysis. A daily sample of the Phalaris diet (200 g) was stored at -10°C and a second sample (200 g) was dried to determine daily dry matter content.

Daily refusals of chopped lucerne hay from each animal were weighed and bulked over the collection period. Fresh grass refusals from each animal were dried each day to determine total dry daily dry matter intake and then discarded.

Faeces were collected each day, weighed, and stored at -10°C. The volume of urine collected each day was recorded and the urine stored at -10°C. Bulked samples of feed offered, feed refusals (lucerne) and faeces from each

collection period were subsampled and dried. These subsamples and those from the bulked urines from each animal were used for analyses.

3.4 Sedation, anaesthesia and surgery

For the majority of the radiographic observations (see Chapter 4), and prior to anaesthesia, macropodines were sedated with ketamine hydrochloride (Ketalar, Parke Davis, Sydney, NSW). In the slaughter experiments, animals were killed, after sedation, with an overdose of pentobarbitone sodium (Lethobarb, V.S. Supplies, Melbourne, Victoria) administered by cardiac puncture. For surgical procedures anaesthesia was maintained with halothane (Fluothane, I.C.I., Melbourne, Victoria).

3.4.1 Sedation and euthanasia

As found by Denny (1973) ketamine hydrochloride, administered intramuscularly, proved to be an effective and safe immobilisation agent for all macropodine species over a wide range of dose rates (10 to 30 mg/kg body weight). Respiratory inhibition was evident only at dose rates in excess of 25 mg/kg. At the higher dose rates, a plane of anaesthesia was attained that allowed cutaneous and subcutaneous surgical procedures to be performed with ease and safety.

Animals were always placed in an open weave hessian bag prior to injection and during induction. Induction from intramuscular injection required 2 to 3 min and if the animals were allowed to move freely during the induction phase, or were in a state of excitement before injection, higher dose rates were required to attain sedation, often with less predictable results. During the recovery period (15 to 30 min) from the effects of ketamine hydrochloride, and after surgery, animals were always kept in a hessian bag until they had regained consciousness. This allowed the animals to recover quietly, and prevented them from moving freely, before they were capable of maintaining

balance in a standing position. This minimised possible physical injury.

At low dose rates ketamine hydrochloride did not appear to inhibit the swallowing reflex nor, as far as could be judged from radiographic observations, was gut motility adversely affected or inhibited. This agent was thus always used prior to euthanasia with pentobarbitone sodium for the slaughter experiments. Euthanasia with pentobarbitone sodium alone often resulted in abnormally strong contractions of the gut wall.

3.4.2 Surgical anaesthesia and techniques

For surgical implantation of infusion catheters in the stomach of T. thetis and M. eugenii, individual animals were initially sedated with ketamine hydrochloride (10 mg/kg) and anaesthesia was then attained and maintained with a mixture of halothane and oxygen from a vapouriser (Fluotec, Cyprane Ltd., Keighley, England). After sedation, halothane (4 to 5% in oxygen) was administered through a face mask untill full anaesthesia was induced. The larynx and the rear of the mouth were then sprayed with lignocaine hydrochloride (Xylocaine, Astra Chemicals, Ryde, NSW) before intubation; without this, a laryngospasm was often induced during attempted intubation. Anaesthesia was then maintained with halothane (1 to 2% in oxygen).

Induction of anaesthesia with halothane alone usually resulted in undue excitement and spasmodic respiratory inhibition. This was not evident if the animal was previously sedated with ketamine hydrochloride.

For infusion experiments, narrow bore catheter tubing (1.0 mm internal diameter Silastic Tubing, Dow Corning Corporation, Michigan, USA) was implanted in the proximal region of the forestomach (see Chapter 8) or in the peptic region of the stomach (see Chapter 4) of T. thetis and M. eugenii. The catheter was implanted through a ventral midline incision in the abdominal wall and routed

subcutaneously to exit dorsally in the upper thoracic region. A short sleeve (10 mm) of larger bore tubing, 15 mm from the end of the catheter, was sewn to the stomach wall to prevent displacement of the catheter. A second short sleeve near the external end of the catheter was sewn subcutaneously to prevent movement of the tubing at the skin surface. Alternatively, a short length of stainless steel hypodermic needle tubing was embedded in a small, circular, epoxy resin plate (20 mm diameter, 2.5 mm depth). The proximal end of the catheter, at the skin surface, was tightly fitted over the steel tubing and the plate implanted subcutaneously. A short length of infusion tubing was then fitted over the external end of the steel tubing and stopped until required.

3.5 Analytical methods

For chemical analyses, samples of the chopped lucerne hay offered, refusals and faeces were dried at 70°C for 48 h; samples of Phalaris grass and the faeces from Experiment 4b (Chapter 6) were freeze-dried. Dried feed, refusals and faeces were milled through a 1 mm screen. Analyses of whole digesta samples were undertaken with wet digesta or with small samples dried at 70°C for 24 h. All analyses were done in at least duplicate.

a) Dry matter, organic matter and gross energy

Dry matter of lucerne offered, refusals and faeces was determined by drying at 100°C for 24 h.

Dry matter content of fresh Phalaris grass offered and refusals were determined by drying in a microwave oven. Samples were weighed into tared 1 litre glass beakers and placed in the oven along with a smaller beaker containing 150 ml of water. The samples were exposed to microwave radiation for periods of no longer than 2 min. At the end of each period the heated water was replaced with cold water; this method prevented overheating of the samples. A total weight of up to 300 g of fresh grass was usually

dried to a constant weight in 5 or 6 heating periods.

Organic matter was determined on 2 g samples heated to 550°C for 3 h.

The heat of combustion of feed, feed residues and faeces was determined using an adiabatic bomb calorimeter (Gallenkamp, England).

b) Total nitrogen

Total nitrogen was determined by Kjeldahl oxidation using H_2SO_4 and Se catalyst followed by either alkaline steam distillation and titration (as for ammonia N) or by the method of Clare and Stevenson (1964) using an auto-analyser (Technicon Instruments, New York). Approximately 0.2 g samples of dried feed and faeces, or an equivalent weight of wet digesta, were subsampled and oxidised.

c) Ammonia N

Ammonia N was obtained by steam distillation of 0.2 to 3 mg N from samples made alkaline with NaOH (30% w/v) or sodium tetraborate (5% w/v). The ammonia was collected into boric acid (2% w/v) and the ammonia-boric acid mixture titrated to pH 5 (Autoburette ABU 12, Radiometer, Copenhagen) with 0.0175 M H_2SO_4. The titration value obtained for a reagent blank was always subtracted from the sample value. Recovery of ammonia from a standard $(NH_4)_2SO_4$ solution was checked several times daily.

d) Urea N

Plasma, urine and the supernatant fraction of digesta were analysed for urea N using the autoanalyser diacetyl monoxime method of Marsh, Fingerhut and Kirsch (1957).

e) Chromium sesquioxide (Cr_2O_3)

Samples of feed, feed residues, faeces and digesta were ashed and digested (Stevenson and DeLangen, 1960) and the

Cr^{+++} content determined on an atomic absorption spectrophotometer (Perkin-Elmer, Model 360, Connecticut, USA) using an acetylene-nitrous oxide flame.

f) Volatile fatty acids

Total VFA concentration in digesta supernatant samples was estimated by steam distillation (Annison, 1954), and individual VFA proportions were determined by gas-liquid chromatography (Erwin, Marco and Emery, 1961) using a dual column gas chromatograph (Model 803, Packard Instrument Co., Illinois, USA).

g) Soluble and structural carbohydrates

The readily fermentable carbohydrates and hemicellulose, cellulose and lignin of diets and digesta samples were analysed by the methods of Bailey (1967). Acid-detergent fibre (ADF) content of feed, feed residues, digesta and faeces was determined by the method of Van Soest (1963).

h) Lipid and silicon content of the diets

Total lipid content of the two diets was determined by the method of Folch, Lees and Sloane Stanley (1957).

To determine silicon content of the diets, 0.05 g of ground, freeze-dried samples were fused with 1 g NaOH in a nickel crucible, with the addition of KNO_3 to oxidise carbon particles. Silicon was then measured as silicate by the molybdenum blue method of Mullin and Riley (1955).

i) Gas analyses

Samples of stomach gas were measured (similar to the methods of Tadesse and Eastwood, 1978) by gas chromatography (Pye Series 104, Pye Unicam, England) using molecular sieve packed glass columns and a katharometer. Carrier gas was argon (oxygen free) at a flow rate of 60 ml/min; oven temperature 30°C; detector temperature 100°C; bridge current 105 mA. This method allowed detection and accurate

measurement of CH_4, H_2, O_2, CO_2 and N_2 in low concentrations (below 20%), but with CO_2 at a higher concentration the estimates were less accurate.

j) ^{51}Cr-EDTA and ^{103}Ru-P

The radioactivity of ^{51}Cr-EDTA and ^{103}Ru-P in digesta, faeces and diluted infusion solutions was determined using a dual-channel autogamma spectrometer (Model 3002, Packard Instrument Co., Illinois, USA). The ^{51}Cr-EDTA was counted using Channel A set with an energy spectrum of 0.25 to 0.33 MeV and the ^{103}Ru-P counted in Channel B in an energy spectrum of 0.41 to 0.50 MeV. Individual standards of ^{51}Cr-EDTA and ^{103}Ru-P were used to check the channel spectrums and to calculate the proportion of ^{51}Cr-EDTA cpm appearing in Channel B (negligible) and ^{103}Ru-P cpm appearing in Channel A (approximately 16%). The correction method of Tan, Weston and Hogan, (1971) was used.

Differences in efficiency of counting occurred with variation in height of sample in the gamma tubes. Samples were thus packed to a constant height, or a constant volume.

No correction for isotope decay was necessary as all samples and standards were counted within 10 h. Alternatively, with greater numbers of samples, duplicate samples, pertaining to each animal, were counted in reciprocal order.

The background was estimated from empty tubes and subtracted from sample counts. Samples were usually counted for 20 min, or if the sample count was low, counting time was increased to ensure a machine error of less than $\pm$ 3% (Kobayashi and Maudsley, 1974). Samples with less than 50 cpm above background were discarded.

All measurements were made on samples of wet faeces and digesta. The plastic gamma tubes were then heated at 70°C for 6 d to obtain the dry weight of samples.

k) ^{14}C urea and tritiated water

The specific radioactivity of urinary urea was determined on diluted samples (0.2 ml urine + 1.8 ml water) using the $Ba(OH)_2$-$ZnSO_4$ deproteinisation method and the Toluene-triton X scintillation mixture as described by Nolan and Leng (1970) with a Tricarb Scintillation Spectrophotometer (Model 3320, Packard Instrument Co., Illinois, USA).

Water was isolated from urine samples by vacuum sublimation (Vaughan and Boling, 1961) and 0.5 ml was counted in 10 ml of a scintillation mixture (Bray, 1960) using the same scintillation spectrophotometer. The channels ratio method described by Hendler (1964) was used to correct for quenching.

l) ^{14}C labelled volatile fatty acids

Individual VFA in digesta supernatant samples were separated by the liquid/liquid chromatography method of Leng and Leonard (1965). Of each bulked fraction collected, 5 ml was titrated for acid concentration and 5 ml was assayed for ^{14}C radioactivity.

m) ^{15}N analysis

The ^{15}N abundance of samples was determined by mass spectrometry using the methods of Nolan (1972) as described by Nolan and Leng (1972).

The ammonia N distillation method described above was used to isolate the N as ammonium sulphate. Ammonia N in digesta supernatant was isolated by distillation of the supernatant. Kjeldahl oxidation was used for bacterial N and total N prior to distillation. To obtain N from urea in urine, samples were made alkaline with NaOH, distilled to remove any ammonia N and then adjusted to pH 6.8 to 7.0 with 0.33 M H_2SO_4. Phosphate buffer (0.2 M K_2HPO_4 + 0.2 M KH_2PO_4, pH 6.8) was added before the addition of

urease (Water Melon Seed, British Drug Houses, Ltd., England) to hydrolyse the urea. A second distillation isolated the ammonia derived from the urea. Before isolation of urea N from plasma, 5 ml samples were deproteinised with successive additions of water (10 ml), 10% w/v $NaWO_4$ (5 ml), and 0.33 M H_2SO_4 (5 ml).