METABOLISM IN RUMEN EPITHELIUM OF THE SHEEP

by

Brendan Leighton, B.Sc.

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DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree of qualification of this or any other University or other institution of learning.

The studies reported in this thesis were carried out in the Department of Biochemistry, University of Manchester from October 1980 to September 1983.

B. Leighton.

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CONVENTIONS AND ABBREVIATIONS

The conventions of the Biochemical Journal with regard to units and abbreviations have been used throughout. Additional abbreviations are explained in the text and are listed below:

AOA	Aminooxyacetate
BSA	Bovine serum albumin
BM	Butylmalonate
CHC	2-Cyano-4-hydroxy cinnamate
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanosulphonic acid
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
MPA	3-Mercaptopicolinate
NCS	Nuclear Chicago solubiliser
PAGE	Polyacrylamide gel electrophoresis
PCA	Perchloric acid
PEP	Phosphoenolpyru vate
PEPCK	Phosphoenolpyruvate carboxykinase
SDS	Sodium dodecylsulphate
TCA	Trichloroacetic acid
TOFA	5-(Tetradecyloxy)-2-furoic acid
TEA	Triethanolamine
VFA	Volatile fatty acid (i.e. short-chain, steam volatile fatty acids)

To Mary Louise

ABSTRACT

This investigation confirms that the epithelium lining the rumen is an extremely metabolically active tissue. Although rates of fatty acid and sterol synthesis *in vitro* are low in rumen papillae, ketogenesis, glycolysis, protein synthesis, proteolysis and propionate conversion to L-lactate all occurred at significantly high rates.

A study of subcellular enzyme distribution shows that the location of the pathway of ketogenesis is mitochondrial. In particular, 3-hydroxy-3-methylglutaryl-CoA lyase and D(-)-3-hydroxybutyrate dehydrogenase are exclusively mitochondrial; the latter enzyme is particulate and not easily solubilised. Rates of ketogenesis are insensitive to (-)-hydroxycitrate - an inhibitor of an enzyme necessary for the operation of the cytoplasmic route, i.e. ATP:citrate lyase - confirming conclusions regarding the subcellular location of the pathway.

Butyrate is the only quantitatively significant substrate for ketogenesis. Propionate, acetate and long-chain fatty acids are much less effective. Butyrate-dependent ketogenesis in rumen papillae is insensitive to insulin, glucagon, and both α- and β-agonists. Vasopressin increased flux by about 14%. Pyruvate, lactate and fructose, known antiketogenic substrates in liver cells, have no effect on ruminal ketogenesis. Also, glucose is not antiketogenic but this carbohydrate increases CO_2 production from butyrate. Ammonia is a ketogenic effector, albeit a weak stimulator. However, high concentrations of propionate produced a significant decrease of butyrate-dependent ketogenesis but an increase in butyrate conversion to CO_2.

Propionate oxidation to CO_2, and conversion to lactate is stimulated by butyrate. The rate of lactate production is unaffected by n-butylmalonate, 3-mercaptopicolinate and 2-cyano-4-hydroxycinnamate. Results suggest that the pathway involves 'malic'-enzyme and not phosphoenolpyruvate carboxykinase; this latter enzyme is exclusively mitochondrial but there was, however, no formation of glucose in rumen papillae from radiolabelled propionate.

Lactate and CO_2 production from glucose is decreased by short- and long-chain fatty acids. Evidence for the possible involvement of pyruvate dehydrogenase as a control site between the oxidative fatty acid pathway and glucose oxidation is presented.

Rumen epithelium converts glutamine mainly to glutamate with some synthesis of other amino acids. Propionate enhances the formation of these other amino acids. Release of essential amino acids indicated substantial proteolysis; this observation is confirmed with studies of [^{3}H]leucine released from prelabelled epithelial protein. The rates of incorporation of [^{3}H]leucine into protein are high, and stimulated by both butyrate and propionate and, especially glucose. Acetate is an inhibitor, an effect possibly correlated with decreases in ATP content. The pattern of labelling newly-synthesised proteins does not support the view that prekeratin is a major component.

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INTRODUCTION

1.1 *Introductory Remarks*

As well as providing draught power in many areas of the world, domesticated ruminants supply most of the meat and milk consumed by mankind. These animals thrive on high-fibre low protein diets because of a large population of symbiotic microorganisms in the rumen; which is the largest chamber of the fore-stomach of the ruminant mammal. Rumen microbial activity digests most of the forage ingested by the animal and it is the end-products of this metabolism that supply most of the metabolisable energy to the host animal.

Lining the rumen is a stratified squamous epithelium which plays a key role in transporting nutrients from the rumen to the portal blood. Furthermore, unlike the protective epithelium of the oesophagus and stomachs of other animals, the rumen epithelium is an extremely metabolically active tissue. The purpose of this introductory chapter is to outline the major digestive pathways (and main end-products formed) in the rumen, the morphology of the rumen epithelium and, lastly, the absorptive and metabolic processes operating in the epithelium.

1.2. *Fermentation in the Rumen*

Three separate chambers make up the ruminant fore-stomach, namely, the abomasum, omasum, reticulum and the rumen. The position of the rumen anterior to the abomasum (or true-stomach), the presence of the omasum and the reticulo-rumen orifice, which prevents large particles from entering the omasum and the lower gut, are all important components of the fore-stomach (see Fig. 1.1 and Fig. 1.2). A comprehensive description of the functional anatomy of the stomach appears in Hungate's (1966) written exposition and a review by Ruckebusch (1980).

Ruminants obtain their food by browsing and grazing; they therefore subsist largely on plant material. Ingested forage is subjected to little chewing, just enough to mix it with saliva to form a bolus; this passes rapidly down the esophagus and is ejected into the rumen with considerable force (Ash and Kay, 1959). Regular contractions of the muscular tissue surrounding the stomach ensures that mixing of solid digesta and microbial fluid occurs, although stratification has been observed in the bovine rumen (Smith *et al.*, 1956).

An analogy between the ruminant fore-stomach and a fermentation vessel is often made. One main reason for this parallelism is the large numbers of bacteria (10^9 - 10^{11} per g rumen contents) and ciliate protozoa (10^6 - 10^7 per g rumen contents) it supports (Galyean *et al.*, 1981). Carbohyrates and proteins are catabolised by microorganisms to short-chain volatile fatty acids (VFAs) and amino acids

Fig. 1.1. Diagram of the Ruminant Fore-Stomach (Hungate, 1966)

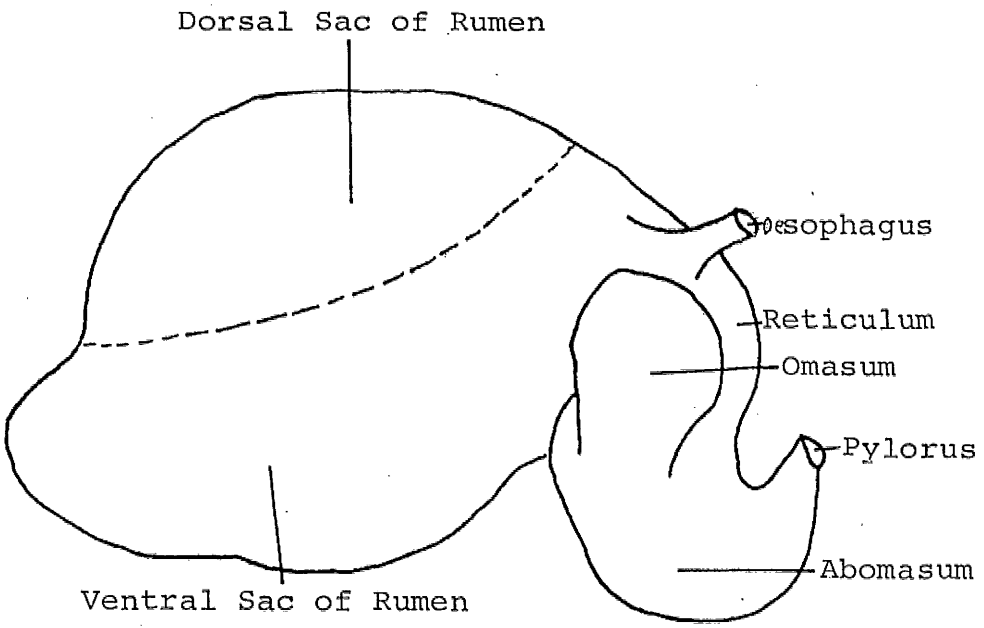
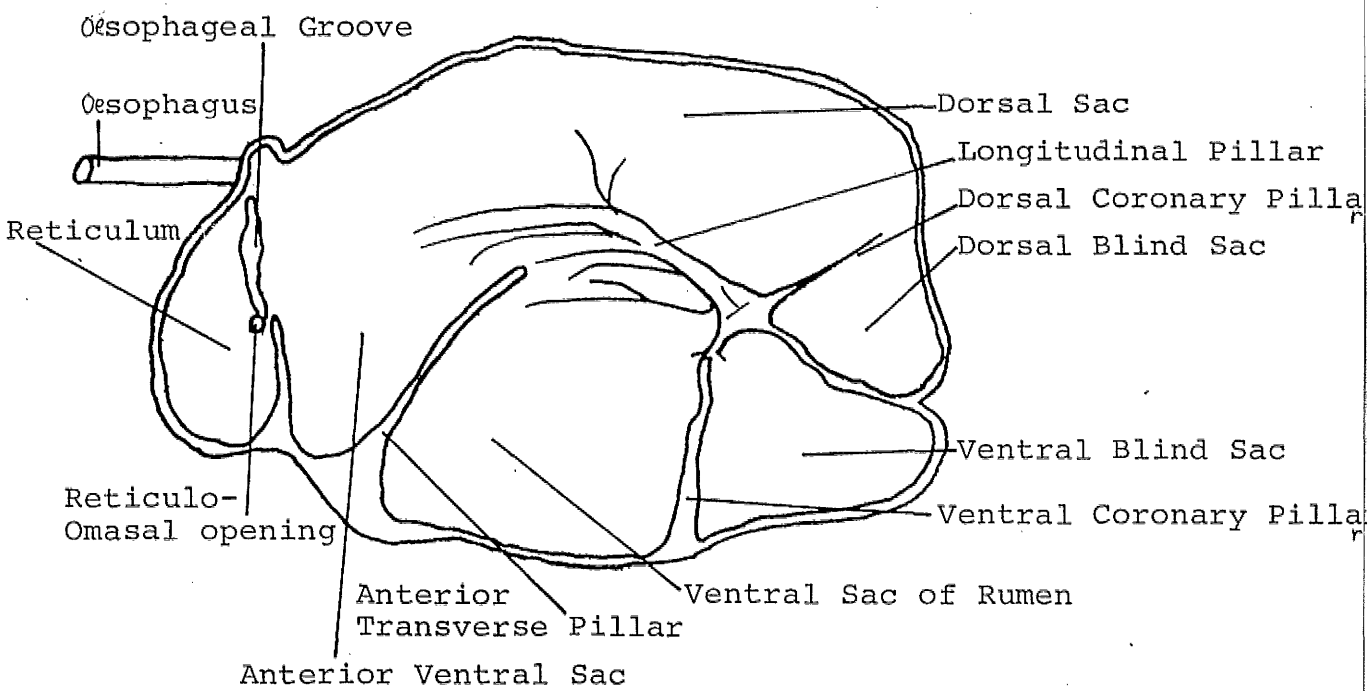


Fig. 1.2. The Right Half of the Rumen as seen from the Inside (Hungate, 1966)



and ammonia respectively. Degradation of long-chain fatty acids in the rumen is limited (Garton, 1965) but unsaturated lipids undergo biohydration, a process which is responsible for the largely saturated nature of ruminant adipose tissue and milk fat.

Carbohydrates are important constituents of the diet because not only do they generate energy for microbial anabolic processes (Leng, 1973) but their metabolic end-products, VFAs, are extensively absorbed by the rumen epithelium. VFAs normally account for 70-80% of the total digestible energy intake of ruminants (McAnally and Phillipson, 1942).

Amino acids and ammonia, the end-products of dietary protein proteolysis, become substrates for microbial protein synthesis. Consumed bacteria are the normal source of nitrogen for protozoa (Wolin, 1981). Bacteria, but not protozoa (Weller and Pilgrim, 1974), are diluted out of the rumen to the lower gut, where microbial protein is degraded and amino acids are absorbed in an analagous manner to the processes in monogastric small intestine (Bondi, 1981). Therefore, it is bacterial protein that is the major nitrogen source for ruminants.

The culminating effects of this exhaustive fermentation are that it creates a highly anaerobic and reducing milieu. This means that the rumen epithelium is served on one side by oxygenated blood and on the other by the acidic rumen liquor, whose physical properties (e.g. temperature, pH, redox potential, osmolarity etc.) can fluctuate considerably. For instance, the pH of rumen liquor is largely governed by the entry of saliva (7.4-9.2 L/day; Stevens *et al.*, 1979), which contains large amounts of bicarbonate and phosphate, and the rate of production of VFAs - principally acetate, propionate and butyrate. The net production of VFAs increases in sheep rumen as the concentration of concentrates in the diet increases (see Table 1.1). From Table 1.1, it can be seen that the percentage molar proportions of individual VFAs can change too, depending on the concentrate/roughage ratio (Weiss *et al.*, 1967). Acetic, propionic and butyric acids account for 90-95% of all the VFAs produced in the rumen; n-valeric, isovaleric, isobutyric and 2-methylbutyric acids make up the remainder.

1.3.1. Morphology of Rumen Epithelium

The rumen is divided internally, by the anterior transverse fold, into distinct portions (see Fig. 1.2) and subdivided by muscular pillars which aid mixing of digesta. A sizeable part of the whole gut is formed by the rumen wall (Sander *et al.*, 1959; Warner *et al.*, 1956). The wet weight of the epithelium, from non-pregnant sheep, ranges from 305g

	Total Volatile Fatty Acids	Acetic Acid (%)	Propionic Acid (%)	Butyric Acid (%)
		100% HAY		
Net Production	3.56	73	20	7
Concentration	59.6	74	19	7
		40% HAY : 60% CONCENTRATES		
Net Production	5.13	60	22	18
Concentration	75.3	66	19	15
		10% HAY : 90% CONCENTRATES		
Net Production	6.15	61	30	10
Concentration	97.4	57	31	12

TABLE 1.1. The Rate of Volatile Fatty Acid Production in Sheep Rumen

Net production (mol/day) and concentration (mmol/L) to total volatile fatty acids and the percentage molar proportions of individual volatile fatty acids produced in the rumen of sheep receiving three different diets. Results are taken from Sutton and Morant (1978).

(Graham *et al.*, 1982) to 381 g (Weekes, 1971), for animals of about 50 kg live weight. Although, the mucosal weight increases significantly (426g) in lactating ewes (Weekes, 1971).

Covering the lumen side of the rumen wall are projections (papillae) which vary in size, from 1-10mm in diameter and 3-10mm in length, and shape, from small, short semiconicals to larger nipple-like structures. The larger papillae, normally found in contact with rumen liquor, effectively increase the surface area of the epithelium. A richly vascularised core of connective tissues is contained innermost in the papillae (Cheetham and Stevens, 1966; see Fig. 1.3). Free nerve endings are reported to originate from the core and enter the surrounding epithelium (Hill, 1959; Message, 1966). Many of the fibres appear intercellular in position and terminate below the outermost keratinised layer of epithelial cells. The submucosal layer contains only a single substantial capillary bed, which is applied to the base of the epithelium to serve an absorptive function.

An indigenous population of bacteria is associated with the epithelial surface (Bauchop *et al.*, 1975). Polysaccharide glycocalyx fibres (Bennett, 1963) are found surrounding adherent bacteria and are attached to epithelial cells. Although Henrikson (1970) thought the role of the glycocalyx was to influence the movement of substrates through the intracellular spaces, McCowan

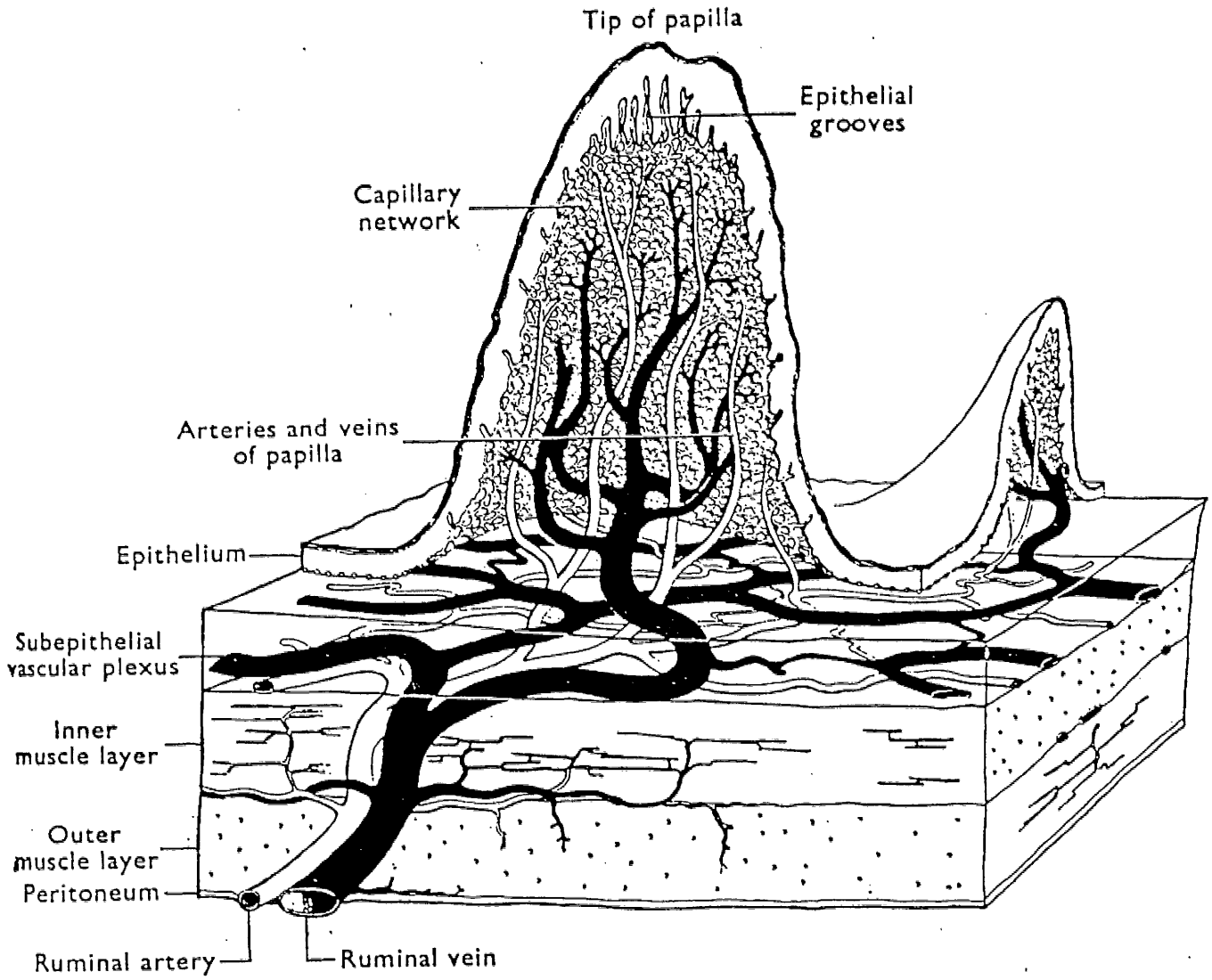


Fig. 1.3. Schematic Diagram of the Blood Supply to a Section of the Rumen Wall (Cheetham & Stevens, 1966)

et al. (1978) propose that the acid polysaccharides mediate attachment of bacteria to the epithelium, to food particles and to each other so that microcolonies are formed.

Even though the rumen wall represents an aerobic-anaerobic interface, 165 adherent bacterial strains have been isolated under anaerobic conditions (Mead and Jones, 1981). Of the bacteria isolated from the population adhering to the rumen wall some 25-50% are facultative anaerobes (Wallace *et al.*, 1979).

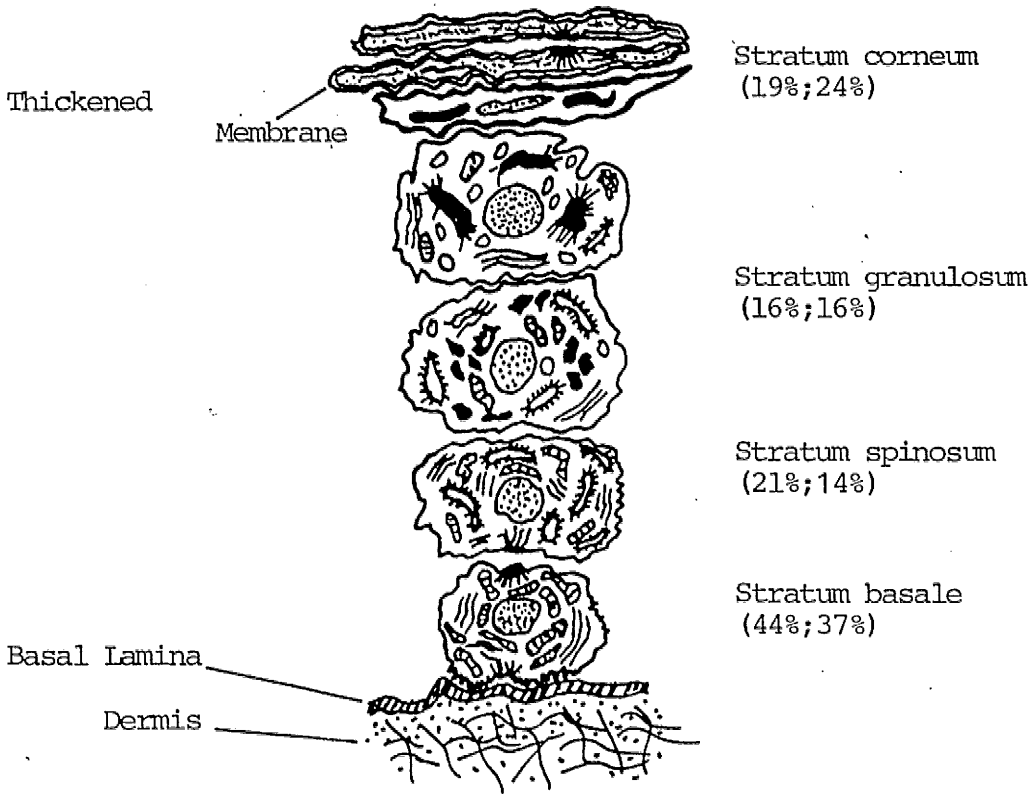
Rumen mucosal bacteria contribute to ruminant digestive processes in a variety of ways (Cheng and Costerton, 1979). First of all, bacteria attached to abraded keratinised cells degrade epithelial protein to a usable form for the bacterial biomass. Cheng and Costerson (1979) estimate that adherent bacteria contribute more than 10% to total rumen protease activity. In addition to this the epithelial bacteria hydrolyse urea absorbed through the rumen wall, from the blood(Cheng and Wallace, 1979), and may even regulate urea transport through the epithelium (Wallace *et al.*, 1979). Attached microorganisms utilise oxygen diffusing through rumen epithelium, thereby obtaining a more favourable energy yield from rumen reducing equivalents and, more importantly, protecting sensitive anaerobic bacteria from damage.

1.3.2. *Histology of Rumen Muscosa*

Ovine rumen epithelial fine structure has been described by Lavker and Matoltsy (1970) and Henrikson (1970). Similar studies on other species have been reported by Lindhé and Sperber (1959), Hyden and Sperber (1965), Lavker *et al.* (1969) and Escobar (1972,1981). Rumen epithelial cell layers are labelled with the same nomenclature used for identifying epidermal cell layers. There are, however, cytological differences between rumen mucosa and epidermal tissue (Fell and Weekes, 1975).

The epithelium consists of five or six layers of nucleated cells and a thin layer of keratinised cells, the whole being about 50nm thick (see Fig. 1.4). The surfaces of the majority of cells in the basale layer are covered by an irregular, microvillus-like array of cell processes which touch the electron-translucent basement membrane (Henrikson, 1970). A dense population of mitochondria are found in the cytoplasm of the basale cells, although the Golgi apparatus appears to be poorly developed. These cells have ovoid nuclei. Lanthanum and horseradish peroxidase (EC 1.11.1.7) readily penetrate the non-keratinised layers, indicating that there is no barrier to free diffusion at these levels (Henrikson and Stacy, 1971). The basale cells are the proliferative fraction of the epithelium as the relative number of cells with nuclei in the S and G_2 phases was by far the largest in the layer (Ohwada and Tamate, 1979).

Fig. 1.4. Diagramatic Cross-Section of Rumen Epithelium



The nomenclature for the different layers of cells are given on the right hand side of the diagram. Below each name (in parentheses) are values for the percentage of cells in the various rumen epithelial layers. The values on the left and right are for sheep fed on roughage-based and concentrates-based diets, respectively (Goolad, 1981).

Key: , mitochondria; , rough endoplasmic reticulum; , lysosome-like bodies; , keratohyalin granules; , nucleus; , Golgi apparatus.

Another occasionally observed stratum basale cell type is identifiable by its dendritic shape (Gemmell, 1973). These dendritic cells (called Langerhans cells) contain mitochondria and are capable of phagocytosis of cell debris when the epithelium is damaged; e.g. by raising the osmolarity of rumen liquor (Gemmell, 1973). Branching cells in the stratum basale layer of sheep rumen mucosa (Marshall and Stevens, 1969; Sagebeil, 1972) may be the Langerhans cells found by Gemmell (1973).

As cells from the stratum basale layer move upwards, away from the basement membrane (see Fig. 1.4), they begin to differentiate. Cells in the stratum spinosum layer are mainly cells with nuclei in the G_1-phase, so these cells are non-proliferative (Ohwada and Tamate, 1979). Spinous cells also contain finger-like processes, although these are a lot shorter and less numerous than those covering cells in the basale layer. Maculae occludentes and desmosomes are observed where the processes from adjacent cells are close together. The intracellular space is still prominent at this layer (Henrikson, 1970). Ribosomes are found in the spinosum cells and it is proposed that prekeratin synthesis proceeds at a fast rate in these cells (Fraser *et al.*, 1972).

The next two layers of cells, the stratum granulosum, are more compactly arranged, with drastically diminished intracellular spaces. These cells contain keratohyalin granules (electron-dense bodies which have an avidity for OsO_4 and heavy metal ions of uranium and lead), membrane-

bound mucous granules and a prominent endoplasmic reticulum, the cisternae of which are dilated and filled with a material referred to as ER-protein.

Granular cells that undergo transformation (T-cells) have filament bundles accumulated at the cell periphery and lysosome-like vesicles appear. An increase in the number of lysosome-like bodies is associated with a degradation of mitochondria, mucous granules and ER-protein. Later stages of T-cell transformation include further degradation of cell organelles, including the nucleus , thickening of the plasma membrane and, finally, filling of the cytoplasm which keratin to form horny cells (Lavker and Matoltsy, 1970). Desmosomal attachments prevent separation of T-cells and, as a result, deep interdigitations are formed between cells of the stratum corneum.

Keratins are fibrous proteins formed from a precursor molecule, namely prekeratin, which is synthesised in more metabolically active cells (Bowden *et al.*, 1983). Lavker and Matoltsy (1970) propose that in rumen epithelium, and maybe other keratinised epithelia, the endoplasmic reticulum produces a protein which becomes admixed with keratohyalin granules to produce a keratin complex.

Cells from the stratum basale and spinosum layers of bovine rumen epithelium have been isolated, after fractionated trypsinisation, and cultured as monolayers (Galfi *et al.*, 1981a). After culturing for 16 days monolayer

cells undergo keratinisation patterns similar to rumen epithelium *in situ* (Galfi *et al.*, 1981b). This procedure may prove to be a valuable technique for future metabolic studies on rumen epithelial tissue.

1.3.3. *Abnormal Rumen Mucosal Development*

Undoubtedly the 'soft' keratins, present in cells of the stratum corneum, act as a defensive mechanism against the abrasive rumen contents. Sheep sustained entirely on liquid nutrients had abnormally underdeveloped gut tissue (Ørskov *et al.*, 1979) and during similar infusion studies solid inert objects are placed in the rumen to stimulate epithelial development (Wallace *et al.*, 1979).

Ruminants fed on high energy diets, low in fibre content, have rumen epithelium that develop parakeratosis (Jensen *et al.*, 1958), i.e. incomplete keratinisation of stratum corneum cells and an absence of a granular layer (Graham, 1972). This condition is thought to be the result of an increase in the rate of upward migrating epithelial cells (Lever, 1967). A decrease in the rate causes excessive thickening of the stratum corneum and granulosum or hyperkeratosis (Fell and Weekes, 1975).

Lesions of rumen epithelium, namely rumenitis, are frequently found in animals fed on concentrate rations lacking fibre (Szemedy and Raul, 1976). Excessive consumption of forage leading to acute acidosis also causes rumenitis, which often takes the form of clubbed papillae and rugae of the mucosa (Fell & Weekes, 1975).

The rate of cell division and transit time determine the thickness of rumen epithelium. When sheep that are fed on a roughage only diet are gradually switched to a concentrate diet there is an increase in the mitotic index of epithelial cells, albeit transitory, when the diets are interchanged (Goodland, 1981). Also the rate of incorporation of $[6\text{-}^{3}H]$ thymidine into epithelial DNA is highest in sheep fed on concentrate diets, compared to roughage fed animals (Rowe and Janes, 1981).

A self-regulatory mechanism for controlling rumen epithelial mass is suggested (Goodlad, 1981) as a reason for the tendency of the mitotic index to decline after it has been stimulated (Sakata and Tamate, 1978a, 1979; Fell and Weekes, 1975; Goodlad, 1981). A circadian rhythm also exists for mitotic indicies of ovine rumen epithelium (Sakata and Tamate, 1978b). Cell deletion and shrinkage necrosis may act as a counter measure to mitosis in regulating epithelial cell populations (Fell and Weekes, 1975; Tamate and Fell, 1978).

1.4. *Absorption and Transport of Nutrients Across Rumen Epithelium*

The subject of nutrient absorption and transport across rumen mucosa, both *in vivo* and *in vitro*, would require another chapter if it were to be covered adequately. Therefore, in this section I will summarise the information about the uptake of quantitatively important ruminal and blood substrates used in experiments reported in this thesis. A review by Dobson and Phillipson (1968) discusses the advantages of various techniques for studying absorption from the rumen *in vivo*.

Barcroft *et al.* (1944) demonstrated that large amounts of VFAs are absorbed from the rumen into the portal blood, the rate of absorption being elevated with increasing VFA chain length. However, the VFA concentrations in blood draining the rumen is in the reverse order, i.e. acetate > propionate > butyrate (Mason and Phillipson, 1951). This finding implied that VFAs are metabolised on passage through the rumen wall.

Increased VFA absorption from ovine rumen at lower rumen pH indicated greater epithelial permeability to the undissociated form of the acid (Danielli *et al.*, 1945). An increase in pH from the range normally associated with rumen contents (i.e. pH 6-7) leads to decreased VFA

absorption rates. With an average pKa of 4.8, VFAs will be about 95% ionised at the pH range found in the rumen (Cummings, 1981). Thus, the ionised form of the fatty acid is absorbed in quantity even from rumen liquor at pH 7 or above. The extent that the ionized form penetrates the epithelium is difficult to assess (Dobson and Philipson, 1968). Although Annison (1965) states that at neutrality about 50% of VFAs are absorbed in the undissociated form, Stevens and Stettler (1966b) suggest that the epithelium facing the lumen side is relatively impermeable to the ionised VFAs. The differences in the rates of absorption of each VFA has been related to the rate at which each acid is metabolised in the rumen wall. This proposition was based on observations of VFA transport across isolated sheets of anoxic epithelium (Hird and Weidmann, 1964a; Stevens and Stettler, 1966a).

As portal vein concentrations of acetate, propionate and butyrate are low (2.35, 0.28 and 0.06mM, respectively; Ryan, 1980), compared to rumen liquor concentrations (see Table 1.1), Annison (1965) believes that the concentration gradients between rumen and blood is evidence enough to consider that absorption of dissociated and undissociated VFAs occurs only by simple diffusion. The greater the metabolism of each VFA in the epithelium, the higher the concentration gradient between rumen and blood.

However, Hegner and Anika (1975) propose that HCO_3^--stimulated ATPase, (EC 3.6.1.8) located in the plasma membrane of stratum basale cells (Hegner and Anika, 1975), is involved in the active transport of acetate (Stevens and Stettler, 1967) and Cl^- (Sperber and Hyden, 1952; Stevens, 1964; Scott, 1970) across rumen epithelium. The source of HCO_3^- is either from the plasma, or hydration of CO_2 by carbonate dehydratase (EC 4.2.1.1):

$$H_2O + CO_2 \xrightleftharpoons{\text{carbonate dehydratase}} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Ash and Dobson (1963) concluded that hydration of CO_2 in sheep rumen provided a source of H^+ ions for the production of undissociated VFAs. This would favour absorption of these fatty acids and accumulation of HCO_3^- within the rumen. Yet, rumen epithelial carbonate dehydratase activity has been measured in extracts obtained from various ruminant species (Aafjes, 1967; Stevens and Stettler, 1967; Carter, 1971) and the activity is located in all cell layers except the stratum corneum (Galfi *et al.*, 1982). One suggestion is that hydration occurs in the tissue, which could create an acid microclimate at the rumen epithelial surface, thus favouring absorption of the unionised form of the acid (Cummings, 1981). Perhaps the acid polysaccharide (glycocalyx) also plays a role in mucosal absorption. These suggestions illustrate that the exact mechanism(s) of VFA absorption across the epithelium still requires further investigation.

Microbial fermentation of carbohydrates in the rumen means that ruminants, on an ordinary diet, absorb little or no glucose (Bergman *et al.*, 1970). However, dietary starch escaping rumen fermentation is hydrolysed and absorbed in the small intestine of sheep (Thivend, 1974). The quantity of starch passing through the rumen is greater in ruminants fed on concentrate diets (barley or maize based), compared to animals fed on roughage diets (Piperova and Pearce, 1982). Therefore, glucose is mainly supplied to the animal by gluconeogenesis from non-carbohydrate sources. Considerable amounts of glucose are taken up by sheep portal drained viscera *in vivo*; the amount as a percentage of total glucose turnover, is between 20% (Bergman *et al.*, 1970) and 35% (Weekes and Webster, 1975). Some of the glucose taken up may be metabolised in rumen epithelium as rates of glucose uptake *in vitro* have been recorded for sheep (27μmol/h per g wet wt.; Pennington and Sutherland, 1956a) and ox mucosa (110μmol/h per g dry wt.; Weekes, 1974).

Lactate, which arises from carbohydrate digestion in the rumen, is a potential precursor for gluconeogenesis in ruminants. Both D- and L-isomers of lactate are synthesised in ovine rumen, resulting in concentrations of 2 and 6 mmol/L, respectively (Giesecke and Stangassinger, 1979). Both isomers are absorbed more rapidly from the small intestine than the rumen (Dunlop, 1970). However, disappearance of D-lactate from the rumen is slightly faster than that of the L-isomer (Williams and

MacKenzie, 1965); although rumen epithelium oxidises little or no D-lactate (Hinkson *et al.*, 1967; Preston and Noller, 1973).

Like lactate, it is thought that absorption of amino acids from the rumen is relatively unimportant (Dobson and Phillipson, 1968). The evidence for this suggestion is the lack of an increased level of amino acids in sheep portal blood after feeding (Annison, 1956; Shimbayashi *et al.*, 1975). Wright and Hungate (1967b) also found that there were only small transient increases in the normally low ruminal amino acid concentrations after feeding. The quantitative significance of absorption of amino acids from the rumen must be considered in relation to the high turnover and degradation rates of amino acids in the rumen (Portugal and Sutherland, 1966; Wright and Hungate, 1967a).

Rumen epithelium has availability to a supply of amino acids, from blood serving the tissue. Barej *et al.* (1973) showed that transfer of ^{14}C-labelled lysine, injected intravenously into fed goats, from plasma to rumen is minimal. Using goats *in vivo*, Teinkäö, (1972) showed that plasma amino acids accumulate in an electrolyte solution in the washed out rumen; glycine, alanine and glutamate are present at the highest concentrations. This is considered to be a leakage problem but with reservations concerning the greater inflow of glycine, alanine and glutamate.

In contrast to *in vivo* data, the transport of histidine from the buffer bathing the muscle side of isolated sheets of rumen epithelium to the luminal buffer is proportional to the initial mucosal concentration and is an energy dependent process (Liebholz, 1971).

It is also proposed that ruminal Na^+ and Cl^- absorption is accomplished by active transport (Stevens *et al.*, 1979), and that this is partially due to Na^+-H^+ and Cl^--HCO_3^- exchanges. Net Na^+ and Cl^- fluxes *in vitro* are decreased by treatment of the sheep epithelium with acetazolamide, a carbonate dehydratase inhibitor (Emanovic *et al.*, 1976); implying that Na^+ and Cl^- transport is mediated through 'low-activity' carbonate dehydratase. Stevens *et al.* (1969) have suggested a model to explain electrolyte transport across rumen mucosa. The absorption of Na^+ and Cl^- by rumen epithelium is important because it recycles NaCl secreted into the rumen in saliva.

The rate of K^+ absorption from the rumen is directly related to its concentration in the rumen fluid (Scott, 1967). Recently, it has been reported that the rumen wall is the main site for entry of Mg^{2+} both *in vivo* (Tomas and Potter, 1978) and *in vitro* (Martens, 1983). Absorption is postulated to be by an active Na^+-linked process, although the gassing of rumen contents with CO_2 aids Mg^{2+} efflux (Martens, 1983). There is no significant movement of Ca^{2+} (Phillipson and Storry, 1965) or phosphate (Dobson and Phillipson, 1968) across rumen epithelium.

1.5. *Intermediary Metabolism in Rumen Epithelium*

Research carried out by Pennington and colleagues pioneered the study of intermediary metabolism in rumen mucosa (Annison and Pennington, 1954; Pennington, 1952, 1954; Pennington and Sutherland, 1956a, 1956b; Pennington and Pfander, 1957). Since those investigations the metabolic pathways responsible for ketone body synthesis and L-lactate production have received most attention. These metabolic routes are, quantitatively, of great significance to both the epithelial tissue and the animal itself, and are discussed below. Also, other reported anabolic or catabolic processes present in rumen epithelium are described.

1.5.1. *Ketone Body Production*

Ketone bodies (acetoacetate and D(-)-3-hydroxybutyrate) are important metabolic fuels; the ruminant heart and skeletal muscles being the major sites which utilise them (Lindsay and Setchell, 1976; Pethwick and Lindsay, 1982). Leng and Annison (1964) demonstrated that there is substantial production of D(-)-3-hydroxybutyrate in fed sheep *in vivo*; the rumen epithelium is the main site for this production (Roe *et al.*, 1966). Ketogenesis in rumen mucosa *in vitro* is greatest with butyrate as the only exogenous substrate (Pennington, 1952; Pennington and Sutherland, 1956a). Bergman and Wolff (1971) found that 90% of butyrate absorbed from the rumen was not transported to the portal blood and it is estimated that of the total amount of D(-)-3-hydroxybutyrate produced in rumen

epithelium, between 74% and 94% arises from butyrate (Leng and West, 1969). However, ketogenesis also occurs in omasal mucosa and might contribute to ketone body production in fed sheep *in vivo* (Hird and Symons, 1959; Jayner *et al.*, 1963; Jackson *et al.*, 1968).

In fasted sheep, there is progressive mobilisation of lipid reserves and an increase in fatty acid conversion to ketone bodies in the liver (Bouchat *et al.*, 1981). In the starved state, the increase in plasma concentrations of non-esterified fatty acids, associated with decreased rates of VFA absorption from the rumen (Katz and Bergman, 1969), results in the liver becoming the major ketogenic organ. Interestingly, lipolysis in bovine adipose slices is inhibited by butyrate (Metz and Van den Bergh, 1972, 1974).

A proposed pathway for butyrate metabolism in rumen mucosa is illustrated in Fig. 1.5. Hird and Symons (1961) studied the labelling patterns of acetoacetate synthesised from ^{14}C-labelled butyrate (specifically labelled at each one of the carbon atoms) in rumen epithelium. Their results are construed as evidence that the main route for ruminal ketogenesis involves the hydroxymethylglutaryl-CoA (HMG-CoA) pathway (Lynen *et al.*, 1958; see Fig. 1.5). Acetoacetyl-CoA thiolase (EC 2.3.1.9), HMG-CoA synthase (EC 4.1.3.5) and HMG-CoA lyase (EC 4.1.3.4) activities have been measured in bovine rumen epithelial extracts (Baird and Hibbit, 1969; Baird *et al.*, 1970).

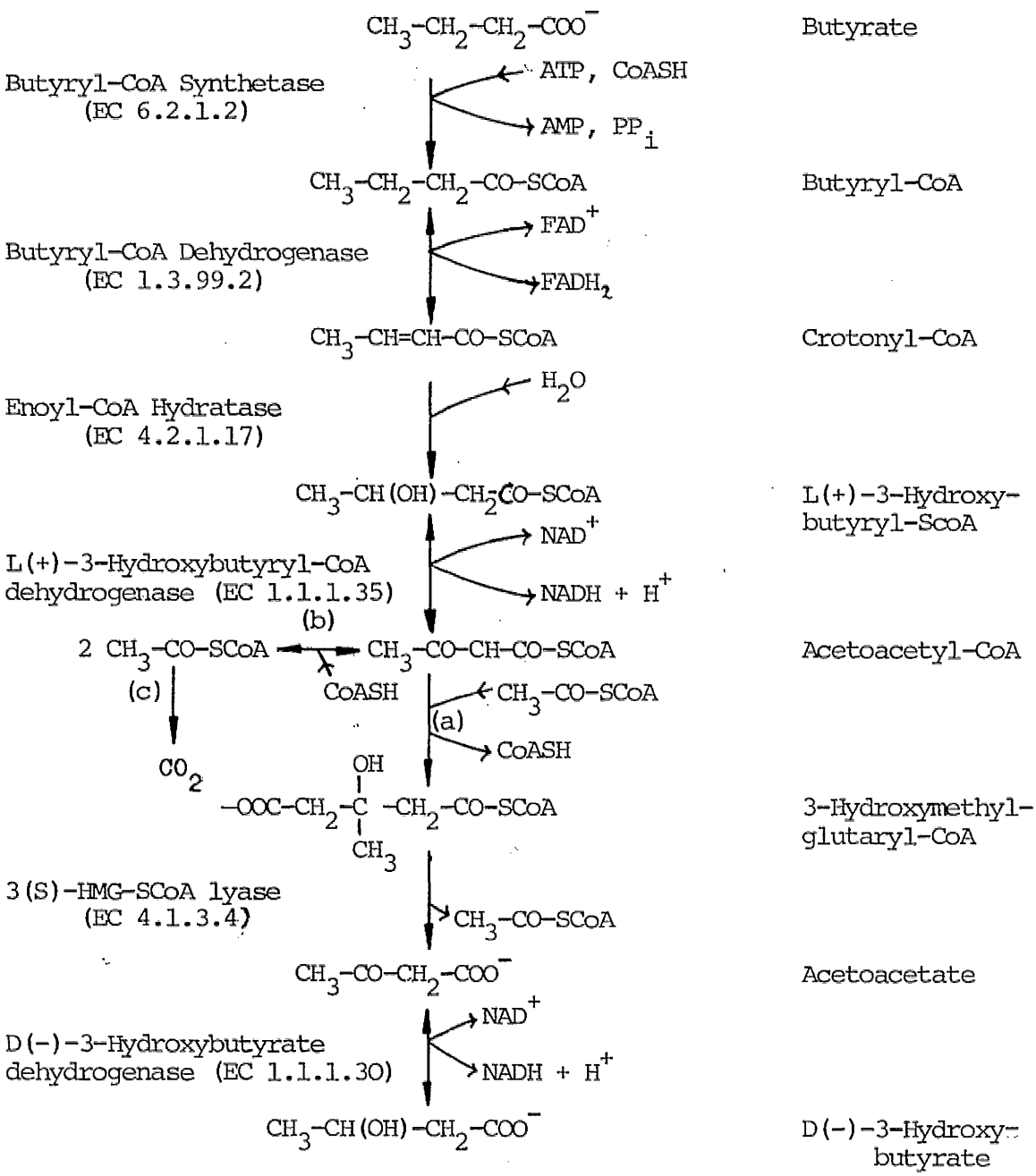


Fig. 1.5. Possible Pathway of Butyrate Metabolism in Rumen Epithelium

(a), 3(S)-Hydroxymethylglutaryl-CoA (HMG-CoA) Synthase (EC 4.1.3.5); (b), Acetoacetyl-CoA thiolase (EC 2.3.1.9); (c), Tricarboxylic acid cycle.

Entry of butyrate into metabolically active rumen epithelial cells is likely to be by diffusion across the plasma membrane. Once inside the cell it is not known if butyrate becomes bound to a specific binding protein, like the butyrate-binding protein that exists in mouse liver (Morioka and Ono, 1978). Nonetheless, activation of butyrate, catalysed by butyryl-CoA synthetase (EC 6.2.1.2), is confined to the mitochondrial space in sheep rumen mucosa (Cook *et al.*, 1969). Though, butyryl-CoA synthetase activity is reported to be evenly distributed between mitochondrial and cytoplasmic compartments in bovine rumen epithelium (Ash and Baird, 1973). Scaife and Tichivanga (1980) propose that there are two short-chain acyl-CoA synthetase enzymes present in ovine epithelium; one capable of activating acetate, propionate and butyrate and the other only accepting butyrate as a substrate. Acyl-CoA synthetase activity is most active when butyrate is the sole substrate, less active with propionate and least active when acetate is activated (Cook *et al.*, 1969; Ash and Baird, 1973; Scaife and Tichvangana, 1980).

Ovine rumen epithelial butyryl-CoA synthetase activity (0.93µmol/min per g wet wt.; Scaife and Tichvangana, 1980) is sufficient to account for the production rate of D(-)-3-hydroxybutyrate in fed sheep *in vivo* (0.43 - 1.23 µmol/min per g wet wt.; Leng and Annison, 1964; Roe *et al.*, 1966; Annison *et al.*, 1967; Leng and West, 1969; Katz and Bergman, 1969).

Activities for butyryl-CoA dehydrogenase (EC 1.3.99.2) and enoyl-CoA hydratase (EC 4.2.1.7) in rumen epithelium have, to date, not been reported. However, high L(+)-3-hydroxybutyryl-CoA dehydrogenase activity (EC 1.1.1.35) is found in the sheep tissue (7.73µmol/min per g wet wt.; Emmanuel *et al.*, 1982). Two distinct dehydrogenases exist, one inner mitochondrial membrane bound, specific for long-chain fatty acids, the other, a mitochondrial matrix enzyme which catalyses oxidation of short-chain fatty acids (El-Fakhri and Middleton, 1979). The final step in the proposed pathway is catalysed by D(-)-3-hydroxybutyrate dehydrogenase. Activities of this enzyme have been measured in ovine and bovine epithelial extracts by several research groups (Whanger and Church, 1970; Koundakjian and Snoswell, 1970; Watson and Lindsay, 1972; Weekes, 1974; Chandrasena *et al.*, 1979; Bush, 1982).

Acetate (Pennington, 1952; Seto *et al.*, 1955a), n-valeric, isovaleric (Annison and Pennington, 1954) and long-chain fatty acids (Jackson *et al.*, 1964; Hird *et al.*, 1966; Leng and West, 1969; Goosen, 1975) are also metabolised in rumen epithelium, with the formation of ketone bodies. These substrates are probably converted to acetyl-CoA, which can be converted to acetoacetyl-CoA by acetoacetyl-CoA thiolase and then subsequently metabolised to acetoacetate.

Alternative pathways for the formation of acetoacetate from acetoacetyl-CoA in rumen epithelium have been suggested (Bush and Milligan, 1971). These metabolic routes are either direct deacylation of acetoacetyl-CoA, by acetoacetyl-CoA hydrolase (EC 3.1.2.1; Stern and Miller, 1959), or by transfer of the CoASH moiety from acetoacetyl-CoA to succinate, a reaction catalysed by 3-ketoacid CoA-transferase (EC 6.2.1.5; Weidemann and Krebs, 1969).

In addition to this a completely different metabolic pathway (see Fig. 1.6) for D(-)-3-hydroxybutyrate production, in cow and sheep rumen mucosa, is suggested by Emmanuel *et al.* (1982). However, much more evidence is required before it can be determined if this is a quantitatively important route for D(-)-3-hydroxybutyrate synthesis in rumen epithelium *in vivo*.

1.5.2. L-Lactate Synthesis

Leng *et al.* (1967) concluded that 70% of propionate, utilised by sheep tissues for gluconeogenesis, is metabolised *via* L-lactate; the rumen epithelium was suggested as the most likely site for this conversion. There is other evidence for extensive metabolism of propionate in rumen mucosa (Stevens, 1970; Bergman, 1975).

Pathway A

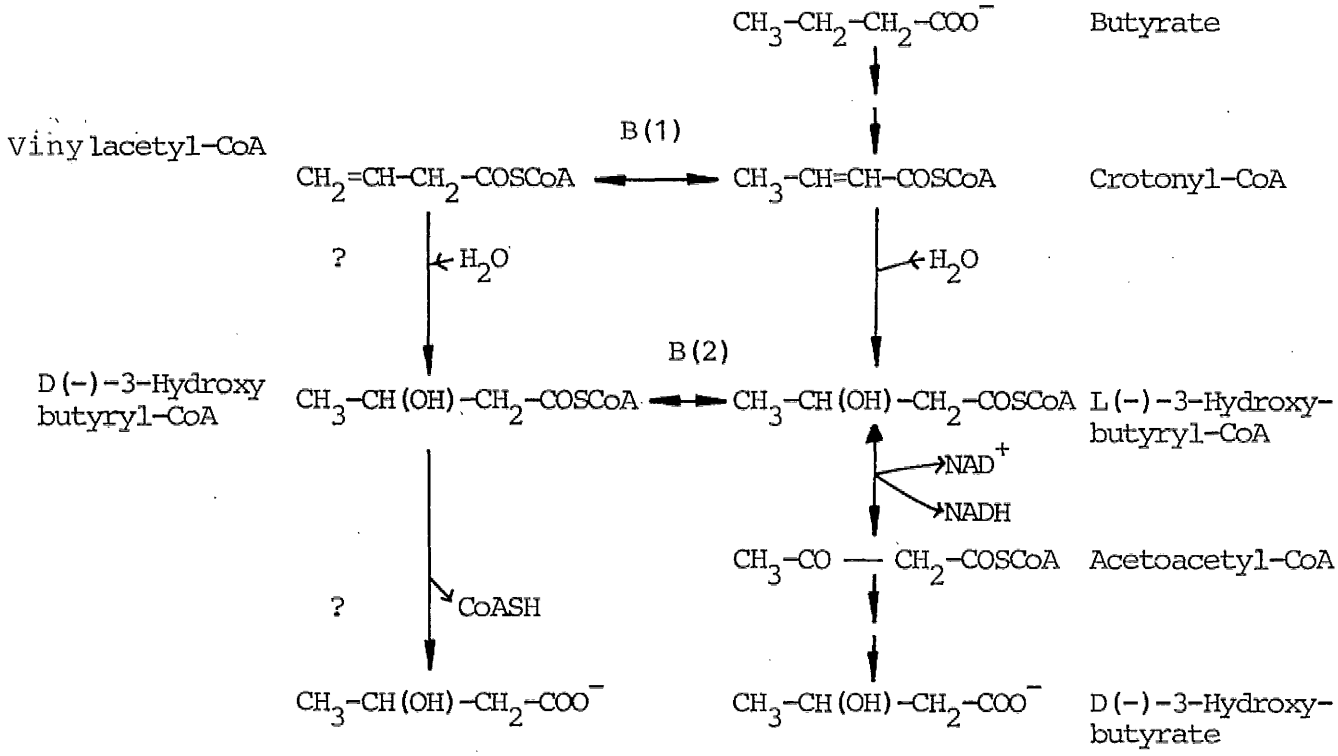


Fig. 1.6. An Alternative Pathway for D(-)-3-Hydroxybutyrate Synthesis in Rumen Epithelium (Emmanuel *et al.*, 1982)

Enzymes associated with pathway A are given in Fig. 1.5. Other enzymes concerned with D(-)-3-hydroxybutyrate synthesis are as follows: B(1), vinylacetyl-CoA isomerase (EC 5.3.3.3); B(2), 3-hydroxybutyryl-CoA racemase (EC 5.1.2.3); ?, no enzymes known which might catalyse these reactions.

However, this suggestion has been challenged by Weigland *et al.* (1972) who have calculated the true extent of propionate conversion to lactate, in rumen epithelium of calves, is about 2.3%. This calculation is corrected for the lactate production in the tissue, from glycolysis. A separate study found that the total lactate entering the portal blood accounts for about 15% of the propionate entry rate, if it is entirely derived from propionate (Baird *et al.*, 1975). Weekes and Webster (1975) concluded from their studies that lactate production by sheep rumen wall is of limited quantitative significance. Therefore, although 50% of propionate absorbed from the rumen is metabolised by tissues of the portal bed (Bergman and Wolff, 1971; Weekes and Webster, 1975) it remains unresolved how extensively propionate is oxidised or converted to other metabolites in rumen epithelium. Propionate metabolism in the epithelium is proposed as a source of extramitochondrial NADPH (Wolff and Bergman, 1972).

Pennington and Sutherland (1956b) showed that the methylmalonyl-CoA pathway is the route for propionyl-CoA conversion to succinate (see Fig. 3.4). Subsequent enzyme studies have supported this metabolic route as measurable activities of propionyl-CoA synthetase (EC 6.2.1.2) (Weekes, 1972; Cook *et al.*, 1969; Ash and Baird, 1973; Scaife and Tichvangana, 1980; Nocek *et al.*, 1980), propionyl carboxylase (EC 4.1.1.41) (Weekes, 1972) and succinate dehydrogenase (EC 1.3.99.1) (Ash and Baird, 1973; Hegner and Anika, 1975) are found in rumen epithelial extracts.

The exact metabolic pathway of succinate conversion to lactate is not known, although one possibility is that $NADP^+$-'malic' enzyme (E.C.1.1.1.40) catalyses a key reaction in the pathway (Young *et al.*, 1969). If this were so then malate would be converted to pyruvate in the cytoplasm but this suggestion is based on the finding that $NADP^+$-'malic' enzyme is about seven times more active than phosphoenolpyruvate carboxykinase (EC 4.1.1.32), another enzyme possibly involved in epithelial propionate metabolism.

1.5.3. Other Metabolic Pathways

Evidence for the operation of the complete tricarboxylic acid (TCA) cycle in rumen epithelium is presented by Pennington and Sutherland (1956a); these observations were later confirmed by Seto and Umezu (1959). The TCA cycle, or rather parts of it, are required for metabolism of propionate. However, oxidation of ^{14}C-labelled VFAs in rumen epithelium has been measured by several research groups (Hird and Symons, 1959; Seto *et al.*, 1973; Seto *et al.*, 1974a; 1974b).

Rates of $^{14}CO_2$ production from [^{14}C]propionate are higher than from [^{14}C]butyrate (Goosen, 1976); although both VFAs are oxidised at greater rates than [^{14}C]glucose (Hird and Symons, 1959). Increased $^{14}CO_2$ formation from [1-^{14}C]butyrate is produced by glucose, whilst butyrate depressed [U-^{14}C]glucose oxidation (Hird and Symons, 1959).

As well as oxidation of glucose in rumen epithelium *in vitro*, L-lactate, but little pyruvate, is also released by the tissue (Hird and Symons, 1959; Emmanuel, 1980c). There has been speculation that some glucose is metabolised through the hexose monophosphate shunt (Emmanuel, 1980c), as not all of the glucose taken up by the tissue can be accounted for as CO_2 and glycolytic end-products. High glucose-6-phosphate dehydrogenase activity (EC 1.1.1.49) has been measured in rumen epithelial extracts (Whanger and Church, 1970; Young *et al.*, 1969).

The transfer of ^{14}C from ^{14}C-labelled substrates into glucose, in bovine rumen epithelium *in vitro*, was reported by Seto *et al.* (1971). Of the gluconeogenic precursors tested propionate was the best substrate. Other studies have been unsuccessful in measuring gluconeogenesis in cow (Weekes, 1974) or sheep rumen mucosa *in vitro* (Emmanuel, 1980d).

Although it is questionable whether glucose is synthesised in rumen epithelium there is plenty of evidence to suggest that non-essential amino acids are synthesised by the tissue (McClaren *et al.*, 1961; Hoshino *et al.*, 1966; Boila and Milligan, 1980a, 1980b). As 14-15g NH_3-nitrogen/day (Nolan and Leng, 1972; Nolan *et al.*, 1976) are transported from the rumen to the portal blood incorporation of NH_3 into amino acids might prevent ammonia intoxication in the ruminant, which occurs if arterial concentrations exceed 0.8mmol per litre (Symonds *et al.*, 1981). Glutamate dehydrogenase (EC 1.4.1.3; Weekes, 1974),

alanine aminotransferase (EC 2.6.1.2; Weekes, 1972) and aspartate aminotransferase (EC 2.6.1.1., Nocek *et al.*, 1980) activities have been measured in rumen mucosal extracts.

Citrulline release, from bovine rumen papillae incubated with arginine, led Boila and Milligan (1981a) to suggest that this is evidence for a functional urea cycle operating in rumen epithelium. Even though appreciable activities of arginase (EC 3.5.3.1; Martincic and Krvavica, 1974; Kurelec *et al.*, 1968), ornithine carbamyl transferase (EC 2.1.3.3; Holtenius and Jacobson, 1966; Harmeyer *et al.*, 1968; Ide, 1969) and carbomoyl-phosphate synthetase (Salem *et al.*, 1973) have been recorded for rumen epithelial extracts the function of the complete urea cycle in rumen mucosa is insignificant compared to the ruminant liver (the cycle in this tissue is 850 times more active than the mucosal cycle, Emmanuel, 1980a). Ruminal carbamoyl-phosphate synthase activity has been implicated in nucleic acid synthesis (Emmanuel, 1980a).

Large amounts of different aminoacyl-tRNA synthetase (EC 6.1.1.–) and tRNA have been isolated from bovine rumen mucosa (Kalachnyuk *et al.*, 1973). The acceptor ability of many of the ruminal tRNAs exceed that of corresponding tRNAs in cow-liver (Kalachnyuk *et al.*, 1974a, 1974b). Kalachnyuk *et al.* (1975) propose that the genetic apparatus

of rumen epithelial cells might possess an ability for induction, resulting in synthesis of specific functional proteins which help cells to adapt to changes in the physical properties of liquids bathing the epithelium.

In this study many of the metabolic processes mentioned above were investigated. Also, where the operation of the metabolic pathways were quantitatively significant further experiments were carried out to determine the effect of physiological substrates on these pathways.

1.6. *Interactions of Volatile Fatty Acids with other Physiological and Metabolic Processes*

Because of the importance of VFAs to ruminants, the influence these acids have on other processes must be taken into account when considering or evaluating rumen epithelial metabolism in relation to whole body metabolism.

For instance, blood VFA levels control feed intake (Preston and Willis, 1974). Adams and Forbes (1982) have proposed that receptors which are sensitive to VFAs might form part of a feedback mechanism for control of feeding.

VFAs might aid absorption, as they stimulate blood flow both to the rumen (Dobson and Phillipson, 1956; Seller *et al.*, 1964; Trautman and Feiber, 1952) and colon (Krietys and Granger, 1979). Barnes *et al.* (1983) reported that blood flow to the rumen epithelium is 2-4 times greater after feeding. This observation is attributed

to the effects on the subepithelial capillary plexus of an increased absorption of butyrate and CO_2 (Sellers *et al.*, 1964).

Rapid administration of butyrate into the rumen results in significant increases, over control values, of the mitotic indices of rumen epithelial cells (Sakata and Tamate, 1978a). The stimulatory effects of acetate or propionate were less than that of butyrate (Sakata and Tamate, 1979). Intravenous infusion of insulin and glucose stimulated epithelial cell proliferation comparable with the effect observed with butyrate (Sakata *et al.*, 1980a). A proposal is that a humoral effector (e.g. insulin or gastrin) is released by VFAs directly or *via* an intermediary metabolite and that this stimulates cell proliferation (Sakata *et al.*, 1980b).

Intravenous infusion of VFAs produces an increase in insulin secretion in lambs, sheep and calves (Manns and Boda, 1967; Horino *et al.*, 1968; Johnson *et al.*, 1982). Manns *et al.* (1967) suggested that propionate and butyrate play a role in controlling insulin secretion. Furthermore, propionate, butyrate and n-valerate (but not acetate) were potent stimuli for secretion of insulin and glucagon in goats fed *ad libitum* (deJong, 1982). Whether low plasma concentrations of VFAs are hormonal secretagogues is still a controversial question (Bassett, 1975; Trenkle, 1978; Elliot, 1980).

Exclusion of butyrate from ruminant blood, by it being metabolised in the rumen wall, might prevent the disturbing effect butyrate has on glucose homeostasis (Potter, 1952; Jarrett *et al.*, 1952; Ash *et al.*, 1964; Phillips *et al.*, 1965; Anand and Black, 1970).

Exclusion would also prevent the many morphological and biochemical changes which butyrate might produce in ruminant cells. This assumes that the influences butyrate has on cultured eukaryotic cells (for reviews see, Kruh, 1982; Prasad, 1980), also happen to cells *in situ*. Constantly observed effects produced by butyrate are: regulatory control of gene expression and cell growth; an almost complete arrest of [^{3}H]thymidine incorporation into DNA; an inhibition of histone deacetylase, resulting in hyperacetylation of DNA; and an arrest of cell proliferation. Galfi *et al.* (1981c) measured incorporation of [^{3}H]thymidine into cultured rumen epithelial cells. This incorporation into epithelial DNA is inhibited by 65% and 92% by 2mM- and 10mM-butyrate, respectively.

C H A P T E R 2

MATERIALS AND METHODS

2.1. *Introduction*

This Chapter contains details of experimental procedures which were routinely used throughout the studies reported in this thesis. Additional methodology is described in Chapters 6 and 7. Sources of chemicals and enzymes are given in Appendix 1.

2.2. *Solutions*

All solutions were prepared in double-distilled water. The pH values were measured using a Corning PT1-6 Universal Digital pH Meter (Corning Ltd., Halstead, Essex) equipped with a glass electrode.

2.3. *Krebs-Henseleit Bicarbonate Buffer*

Krebs-Henseleit bicarbonate buffer (Krebs and Henseleit, 1932) was prepared as follows:-

Parts	%(w/v)	Chemicals
100	0.90	NaCl
4	1.15	KCl
1	2.10	KH_2PO_4
1	3.80	$MgSO_4.7H_2O$
21	1.30	$NaHCO_3$
3	1.22	$CaCl_2$

$CaCl_2$ was added after thorough gassing with O_2:CO_2 (95:5%); the final pH was 7.4. Penicillin G (0.5µg/ml) and streptomycin sulphate (1µg/ml) (Stevens and Stettler, 1966; Weekes, 1971) were added to the buffer (referred to in the text as 'modified Krebs-buffer').

2.4. *Transport of Sheep Forestomach to the Laboratory*

Rumens were obtained from sheep slaughtered at a local abattoir. The nutritional history of any of the animals prior to killing was not known. The contents of the pouches were removed before the fore-stomachs were placed in Krebs-buffer (20°C) (ice-cold buffer was used in enzyme studies) contained in a Dewar flask. Transport from the slaughterhouse to the laboratory took about 30 min.

2.5. *Preparation of Rumen Epithelium for Experiments*

In the laboratory a portion of the anterior ventral sac was resected from the rumen, washed with cold water and placed in modified Krebs-buffer. Isolated rumen papillae were obtained by shaving the tissue with a sharp razor blade. When strips of epithelium were required the mucosa was separated from underlying muscular layers before cutting into sections with a pair of scissors. Isolated tissue was always washed in at least three changes of modified Krebs-buffer.

2.6. *Homogenisation of Rumen Epithelium*

All solutions were kept at 4°C during operational proceedings. A weighed amount of isolated papillae was added to 2-3 volumes of extraction buffer (0.25M-sucrose/ 10mM-3-[N-morpholino]propane-sulphonic acid/3mM-Na_2 EDTA, pH 7.0 at 0°C); the tissue was homogenised with a Ystral homogeniser ['Polytron®-type'] (Ystral Gmbh, Ballrechten-Dottingen, German Federal Republic, through the Scientific Instrument Centre Ltd., Liverpool, U.K.) for approximately 30 sec. at low speed (setting 3-4) followed by 10 sec. at higher speed (setting 7-8). The homogenate was then filtered through nylon mesh (pore size 150μm); filtrate and washings were diluted with extraction buffer to give a final suspension of 10% (w/v).

2.7. *Subcellular Fractionation of Homogenates*

After removal of a sample (1ml) for enzyme assays the homogenate was centrifuged at 600g for 10 min, at 4°C (Beckman J2-21 centrifuge). The resulting supernatant was centrifuged at 32 000g for 15 min at 4°C; the pellets were designated the 'Nuclear' and 'Mitochondrial fractions', respectively. The new supernatant was deemed the 'Cytoplasmic fraction'. Both pellets were resuspended in 5ml of extraction buffer containing 0.2% (w/v)-Triton X-100; the sample of homogenate was diluted 1:1 (v/v) with the same solution. Occasionally the nuclear fraction was rehomogenised to facilitate maximum release of protein.

All fractions were passed through three cycles of freezing in liquid N_2 followed by thawing at 37°C, before being centrifuged at 12 000g for 5 min. The supernatant from this centrifugation step was used for all enzyme assays, unless otherwise stated.

2.8. *Isolation of Mitochondria*

Mitochondria were prepared from homogenates of rumen mucosa by centrifugation as described in sections 2.6 and 2.7. The following changes to the procedure were made: K_2-EDTA replaced the Na_2 salt; the second centrifugation step was at 9000g for 7 min at 4°C; the mitochondrial pellet was washed twice (first in extraction buffer and then in incubation medium). Mitochondrial pellets were resuspended, using a cold 'finger', in about three times their own volume of the following incubation buffer (pH 7.4) (Greksak *et al.*, 1982):

50mM	Sucrose
5mM	$MgCl_2$
2mM	K_2-EDTA
15mM	KCl
50mM	Tris HCl
30mM	KH_2PO_4
3mM	ATP

Mitochondrial suspensions (15 to 20mg protein/ml; Biuret assay) were kept ice-cold until used.

2.9. *Conditions for Incubations In vitro*

Incubations were carried out at 39°C (Pennington, 1952; Smith *et al.*, 1961) in Dubnoff-type shaking water baths (Mickle Engineering Ltd., Gomshall, Surrey) oscillating at between 100-120 cycles/min or in Orbital shaking water baths (F.T. Scientific Instruments Ltd., Tewkesbury, Glos.) rotating at between 150-170 rpm.

Modified Krebs-buffer was saturated with O_2:CO_2 (95:5%) before use, and, after sealing the incubation flasks, these too were gassed for 2 min with O_2:CO_2 (95:5%). All additions to sealed vials were made by injection using either microsyringes (Hamilton Syringes, London) or Gilford automatic dispensers (Anachem., Luton, Bedfordshire). Incubations were normally carried out in triplicate.

Tissue was either incubated in 2ml or 5ml of incubation medium in 10ml Kontes flasks (Kontes Glass Co., Vineland, NJ., U.S.A.) or 25ml Erlenmeyer flasks, respectively.

2.10. *Treatment of Incubation Material*

Incubations were normally terminated with 0.37M-$HClO_4$ (PCA) and, if metabolites released or utilised were measured, a sample of the medium was neutralised with 2M-KOH/0.5M-triethanolamine (TEA) (pH 7.4), containing Universal pH Indicator, to pH 7.0. Samples were normally kept on ice or stored at -80°C until assayed.

After any incubation period strips of epithelium were dried to a constant weight (dry weight). Normally papillae were homogenised, in 0.37M-PCA, using a ground glass homogeniser before DNA content was estimated as described in section 2.11.

2.11. DNA Assay

DNA was determined by the method of Burton (1956). The homogenate (see section 2.10) was heated at 80°C for 30 min. After brief centrifugation at 3000g a portion (5-40µg DNA) was removed and made up to 1ml with 5% (w/v) PCA; 2ml diphenylamine reagent was added, the contents were mixed on a rotamixer, and then left overnight at 30°C before the absorbance of each sample was measured at 595nm.

DNA content was estimated from a standard curve (0-50µg) prepared with calf thymus DNA (500µg/ml in 5% (v/v)-PCA) as standard. A standard curve was performed each time a batch of samples were assayed for DNA.

The diphenylamine reagent contained:1.5g diphenylamine in 100ml glacial acetic acid (AR), 1.5ml concentrated H_2SO_4 and 0.5ml acetaldehyde (diluted 1:50 with water).

2.12.1. Dye-Protein Assay (Bradford, 1976)

The protein reagent contained: 100mg Coomassie Brilliant Blue G 250 dissolved in 50ml 95% (v/v)-ethanol, this solution being made up to 100ml with 85%-(w/v) orthophosphoric acid before dilution to 1 litre. The

solution was filtered through Whatman No. 1 filter paper before use.

Solutions containing 0-40µg protein in a final volume of 0.1ml were pipetted into test tubes. To each tube was added 1.25ml of diluted Coomassie reagent, and after 5 min, the absorbance at 595nm was determined. Standard protein solutions (0-40µg bovine serum albumin (BSA)) were determined for each assay.

2.12.2. *Biuret Assay*

Biuret reagent (4ml) was mixed with 1ml of protein solution (1-10mg/ml), the mixture being allowed to stand for 30 min at 20°C before the absorbance at 540nm was measured. Protein standards (1-10mg BSA/ml) were run simultaneously.

The Biuret reagent contained: 0.15g $CuSO_4.5H_2O$ and 0.6g NaK tartrate dissolved in 50ml H_2O; 30ml 10% (w/v)-NaOH solution was added with thorough mixing. If a cuprous oxide precipitate is deposited in the solution, 1g KI may be added to prevent this happening.

2.13. *Determination of $^{14}CO_2$*

For determination of $^{14}CO_2$, after acidification of the incubation medium (i.e. 0.37M-PCA), 0.25ml 2-phenylethylamine/methanol (1:1, v/v) was added to plastic centre wells, containing a strip of filter paper (1 x 8cm), through the suba seal (Gallenkamp, Widnes, Cheshire). The flasks were

further incubated, at 20°C, in water baths oscillating at 75 cycles/min. After 60 min centre wells were removed and, before being added to 6ml Cocktail 'T' scintillation fluid in 20ml plastic vials, their outside walls were dried with tissue paper. Vials were counted for ^{14}C-radioactivity in either a Beckman LS 9800 (Beckman, High Wycombe, Bucks) or Intertechnique SP400 series with PG 400 (Plasir, France) scintillation counter. Appropriate controls (PCA added before ^{14}C-labelled substrates) were carried out for each experiment and these were taken into account in final calculations.

2.14. *Estimation of ^{14}C-labelled Ketone Bodies*

This method is a modification of the procedure described by Mayes and Felts (1967). Metabolism of ^{14}C-labelled substrates to ^{14}C-labelled acetoacetate, by ketogenic tissues, provides a means for estimating ^{14}C activity in this ketone body. Also this method allows the activity in the carboxyl carbon to be separated from the remainder of the molecule (see Fig. 2.1).

Incubation medium (5ml) containing ^{14}C-labelled substrates was incubated with rumen papillae, in 25ml Kontes flasks as described in section 2.7. After termination of the incubation (0.37M-PCA) flasks were shaken in water baths at 0°C for collection of respiratory $^{14}CO_2$ (section 2.13).

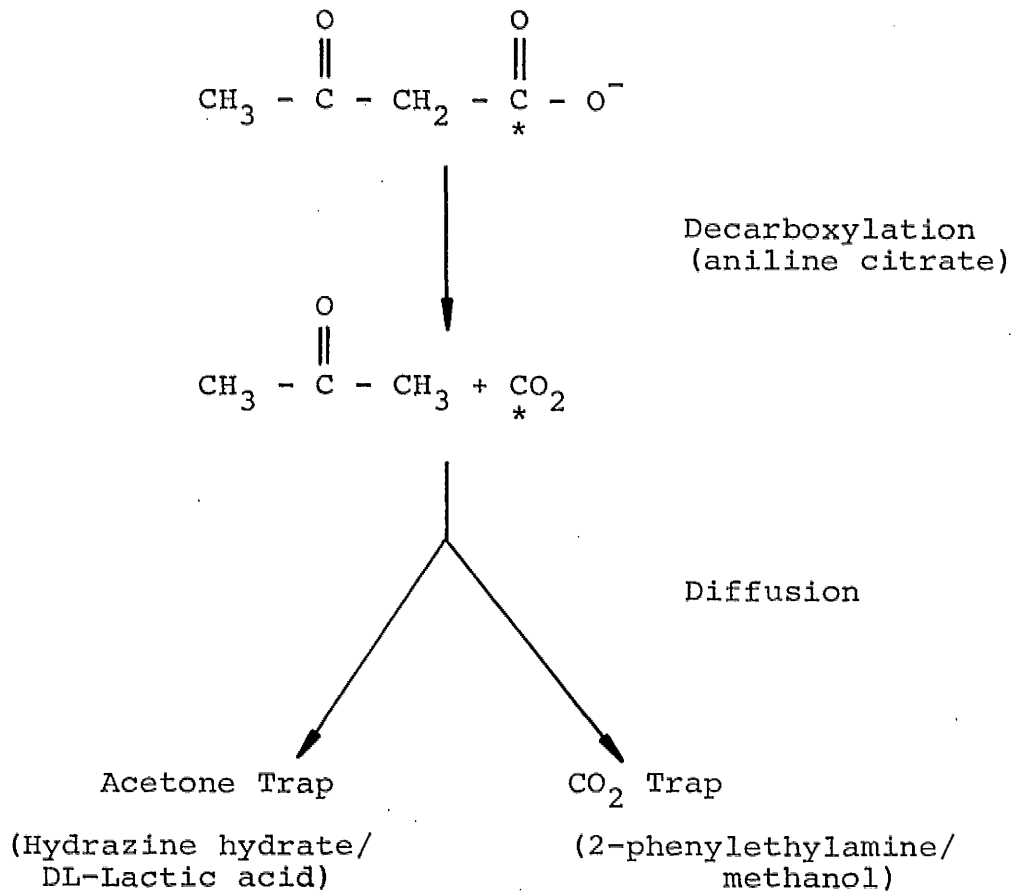


FIG. 2.1. Decarboxylation of Acetoacetate

The contents of each flask were tipped into plastic test tubes before brief centrifugation (3000g at 4°C). For each test tube the procedure was as follows: 1ml medium dispensed in three Kontes flasks (10ml); flasks were immediately stoppled with seals holding plastic centre wells (section 2.13); 0.25ml aniline citrate (4.5ml aniline plus 5.5ml 50% (w/v) - citric acid; Krebs and Eggleston, 1945; Ontko, 1964) was delivered to each using a needle and plastic syringe.

The triplicate centre wells for each sample contained one of the following solutions: a, 0.25ml 2-phenylethylamine/methanol (1:1, v/v); b, 0.125ml methanol; c, 0.25ml hydrazine hydrate/D,L-Lactic acid (2ml hydrazine hydrate titrated to pH 5 with about 8ml 90% (w/v)-D,L-Lactic acid solution; Mayes and Felts, 1967).

Flasks (a) and (b) were incubated for 1h at 20°C. [^{14}C]acetone was allowed to diffuse into the solution contained in centre wells of flask (c) for 24h. After the incubation periods centre wells were collected as described in section 2.13. All flasks were shaken during the diffusion step.

^{14}C activity measured in flask (b) served as control for radioactivity found in flask (a) (for the same sample) as appreciable diffusion of [^{14}C]acetone into the polar solvent occurred The significant quenching of hydrazine sulphate/lactic acid samples was taken into consideration in final calculations.

2.15. *Measurement of Fatty Acid and Cholesterol Synthesis*

To tared Erlenmeyer flasks, containing 2ml gassed modified-Krebs buffer plus substrates, were added washed and blotted rumen papillae. The flasks were reweighed before being placed in an orbital shaking water bath (at 39°C); preincubation was for 10 min before addition of $^{3}H_2O$ (0.1mCi/ml) or ^{14}C-labelled substrates (0.5µCi/ml). Incubations were ended by aspiration of medium from papillae after a sample of liquid was removed for counting for radioactivity.

Lipids were extracted from tissue, essentially by the method of Folch *et al.* (1957), as follows: tissue was homogenised (ground glass homogeniser) in 20% (w/v)-chloroform:methanol (2:1, v/v); homogenate was passed through glass wool, and washed with 0.2 times its volume of water; the interface was then washed three times with a small amount of pure solvents upper phase (i.e. chloroform-methanol-H_2O mixture; 8:4:3 (v/v/v) - collect upper phase). After the final wash the lower phase was made into one phase by addition of methanol.

The lipid containing extract was evaporated under nitrogen and the residue saponified with 2ml 5M-NaOH in 3% (v/v)-ethanol for 3h at 80°C (Brunengraber *et al.*, 1973).

Incorporation of radioactivity into cholesterol was measured as follows: 5ml distilled water was added to the alkaline solution; the mixture was extracted with 6ml

petroleum ether (40-60^{o}C fraction) three times; pooled extracts were evaporated under nitrogen to dryness; the solid was redissolved in 5ml acetone-ethanol (1:1, v/v), acidified with 1 drop of 10% (v/v)-acetic acid, and precipitated with 2ml 0.5% (w/v)-digitonin in 50% (v/v) ethanol.

The white flocculent precipitate was collected by centrifugation and the supernatant (non-saponifiable fatty acid fraction) was dried down in plastic scintillation vials, before addition of 5ml cocktail 'T' scintillation fluid. The pellet was washed with 6ml acetone:ether (1:2, v/v) followed by 6ml ether and finally 1ml methanol was added before radioactivity was measured in 5ml cocktail 'T' (cholesterol fraction).

Incorporation of radioactive precursors into fatty acids was estimated as follows: 1.25ml 2 M-H_2SO_4 was added to the aqueous residue (from the alkaline extraction step); this was extracted with 6ml petroleum ether three times; combined extracts were evaporated to dryness in scintillation vials (fatty acid fraction); 6ml cocktail 'T' was added before this fraction, and a sample of the aqueous extract, were counted for radioactivity.

Dilution of ^{14}C-labelled substrates, if used to measure fatty acid synthesis, by endogenous non-radioactive intermediates will not reflect the true rate of lipid synthesis. $^{3}H_2O$ is not diluted to the same extent (Jungas, 1968) and provides a better method for determining the rate

of fatty acid synthesis. The specific radioactivity of 'medium water' was estimated by assuming a water concentration of 52.8M (Brunengraber, 1973).

Rates of fatty acid synthesis are expressed as µmoles of ^{3}H incorporated per g wet wt. These values are converted to µmoles of acetyl groups incorporated into fatty acid of cholesterol by multiplying by 1.15 or 1.31, respectively (Brunengraber, 1973).

2.16. *Apparatus for Measuring Transport Across Rumen Epithelium*

A greater number of rumen epithelial strips (per rumen) may be used in this system unlike other reported methods (Hird & Weidemann, 1964; Hird *et al.*, 1966; Stevens, 1965; Jones and Kesler, 1969; Nocek *et al.*, 1980).

Sections of rumen epithelium, dissected free of muscle, were secured on to an 'open-ended' test tube by way of a plastic clip and tensioning gun (Fig. 2.2). Excess tissue, above the clip, was trimmed off with a sharp razor blade.

Gassing of buffer (3ml) placed in the test tube (Blood side) aided mixing of this solution and maintenance of pH. Modified-Krebs buffer (3ml) in the glass jar (Lumen side) had an equivalent amount of NaCl replaced by Na acetate (65mM), Na propionate (20mM), and Na butyrate (15mM). The tops of the flasks were screwed on to start the experiment and flasks were shaken at between 150-170 rpm in a water bath, at 39°C.

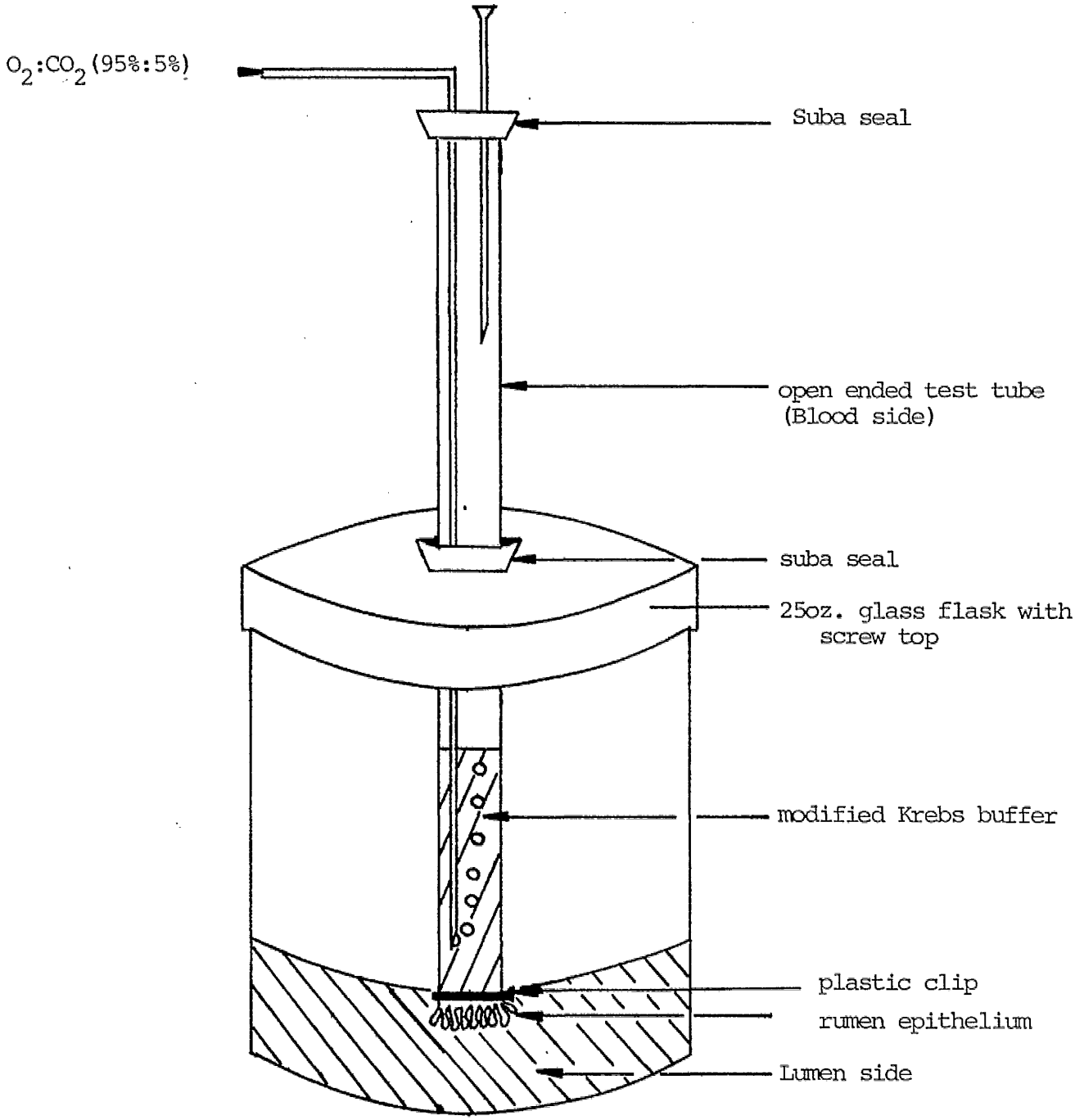


Fig. 2.2. Diagram of the Apparatus used for Transport Studies.

Samples (0.1-0.2ml) were taken from the blood side at predetermined times during the experiment. Portions of the lumen buffer were taken at the beginning and end of the experiment, as were measurements of the lumen buffer pH.

The lumen solution was buffered with Na 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) and not with $NaHCO_3$. Although lumen buffers were made up at 20°C the $\Delta pKa/^{\circ}C$ for Hepes (-0.014) was taken into account.

2.17. Enzyme Assays

2.17.1. Introduction

Continuous spectrophotometric assays were recorded on a Gilford 250 recording spectrophotometer (Corning Ltd., Halstead, Essex). All assays were carried out at 30°C (except phosphoenolpyruvate carboxykinase, 37°C) and on the same day as homogenisation of the tissue. Measurements were normally carried out in duplicate for each sample and appropriate blanks (as indicated in the text) were also performed .

As a matter of routine, enzyme extracts were first centrifuged (12 000g x 2 min) and then kept on ice before assaying. Samples were not centrifuged before assay of HMG-CoA synthase (Method B) and 3-hydroxybutyrate dehydrogenase (Method B). The molar absorption coefficient for NADH at 340nm was taken as 6.22×10^3 litre.mol^{-1}.cm^{-1}.

2.17.2. Acetoacetyl-CoA hydrolase (EC 3.1.2.11) and 3-keto acid CoA-transferase (EC 2.8.3.5)

3-Keto acid CoA-transferase was determined, as described by Tisdale and Brennan (1983), in the direction of succinyl-CoA formation. The reaction mixture contained in a final volume of 1ml:

50mM	Tris-HCl buffer, pH 8.5
10mM	Mg Acetate
5mM	Iodoacetamide
0.1mM	Li-Acetoacetyl-CoA

The change in absorbance at 303nm was recorded for 2 min (spontaneous hydrolysis of acetoacetyl-CoA); difference in absorbance over 3 min was measured after addition of extract (hydrolase activity); Na succinate (50mM) was added to complete the assay system (transferase activity).

Iodoacetamide is an inhibitor of HMG-CoA synthase and acetoacetyl-CoA thiolase activities (Stern and Miller, 1959). The molar absorbance coefficient of acetoacetyl-CoA was assumed to be 20.5 x 10^{-3} litre.mol^{-1}.cm^{-1}.

2.17.3. Acetoacetyl-CoA thiolase (Acetyl-CoA Acetyl-transferase; EC 2.3.1.9) [Assay A]

This enzyme was assayed by the method of Williamson *et al.* (1968), except the buffer pH was lowered from pH 8.5. In this system the molar absorbance coefficient of

acetoacetyl-CoA, at 303nm was taken as 13.8×10^3 litre. $mol^{-1}.cm^{-1}$ (Dashti and Ontko, 1979). Contained in a total volume of 0.5ml was:

150mM	Tris-HCl buffer, pH 8.2
5mM	$MgCl_2$
0.05mM	Li-Acetoacetyl-CoA
0.1mM	CoASH
	Tissue extract and H_2O

Extract and all reagents, except CoASH, were incubated for 4 min, the blank rate being recorded (spontaneous hydrolysis of acetoacetyl-CoA). After addition of CoASH the rate of decrease was measured (spontaneous hydrolysis plus thiolase activity).

2.17.4. Acetoacetyl-CoA thiolase [Method B] (Middleton, 1973)

In a final volume of 1ml was:

100mM	Tris-HCl buffer, pH 8.1
25mM	Mg Acetate
50mM	NaCl or KCl
0.01mM	Acetoacetyl-CoA
0.05mM	CoASH
	Enzyme and H_2O

All reagents, except CoASH, were incubated in the presence of extract for 5 min and the decrease in absorbance at 303nm was determined. Addition of CoASH caused the stimulation of thiolase activity. The molar absorbance coefficient, under assay conditions, was 16.9×10^3 litre.mol^{-1}.cm^{-1} (Middleton, 1973).

The mitochondrial acetoacetyl-CoA thiolase content (M) may be calculated from;

$$M = \rho\Delta/(\rho-1)$$ (Middleton, 1973).

Δ is the difference between thiolase activity measured in the presence of Na^+ and K^+; ρ is the observed K^+ effect. ρ is normally determined on semipurified thiolase. In this study ρ was taken as the mean of the observed effects of K^+ on acetoacetyl-CoA thiolase measured in the nuclear and mitochondrial fraction (see Table 2.1).

Cytoplasmic thiolase activity (C) was obtained by subtracting the total tissue thiolase activity (measured in K^+ containing medium) from the mitochondrial activity. This assumed that the tissue content of general oxoacyl-CoA thiolase was low.

Fraction	Activation by K^+
Homogenate	1.42 ± 0.01
Nuclear Fraction	1.88 ± 0.19
Mitochondrial Fraction	1.84 ± 0.25

Table 2.1. Activation of Acetoacetyl-CoA thiolase by K^+

Results are means ± S.D. for three independent tissue extractions.

2.17.5. *ATP-Citrate (pro-3S)-Lyase (EC 4.1.3.8)*

The assay procedure was based on the method of Srere (1959), as modified by Stanley (1980). The incubation medium, in 1ml total volume, consisted of:

200mM	Tris HCl buffer, pH 8.4
10mM	Dithiothreitol
5mM	Na_2 EDTA
0.15mM	NADH
10mM	$MgCl_2$
20mM	K Citrate
10mM	Phosphocreatine
0.2mM	CoASH
100µg	Creatine kinase (EC 2.7.3.2, from rabbit muscle)
2.5µg	Malate dehydrogenase (EC 1.1.1.37, from pig heart)
5µg	Rotenone (dissolved in absolute ethanol)

After preincubating the assay medium (minus CoASH), containing enzyme, for 3 min at 340nm, CoASH was added to start the reaction. A high background rate was observed if NH_4^+ ions were present or rotenone, an inhibitor of NADH dehydrogenase (EC 1.6.99.5) activity, was absent from the assay system. Malate dehydrogenase supplied as an ammonium sulphate suspension, was dialysed overnight against 0.2M-Tris HCl buffer (pH 8.4) before use.

A non-linear time course for the reaction was obtained if creatine kinase and phosphocreatine were omitted from the reaction medium. This was because of accumulation of ADP, a competitive inhibitor of ATP-citrate lyase (Hatefi *et al.*, 1962). The use of creatine kinase and phosphocreatine, as an ATP generating system, in this assay resulted in a linear time course.

2.17.6. Citrate Synthase (EC 4.1.3.7)

Citrate synthase activity was assayed as described by Srere *et al.* (1963). The reaction mixture (1ml) consisted of:

200mM	Tris-HCl buffer, pH 8.1
0.1mM	5,5'-dithiobis (2-nitrobenzoic acid)
47mM	Acetyl-CoA
0.23mM	Oxaloacetate (freshly prepared)

Extract was preincubated with all reagents, except oxaloacetate. The reaction was initiated by addition of this dicarboxylic acid and change of absorbance was recorded at 412nm. The molar absorbance coefficient of 5,5'-dithiobis (2-nitrobenzoic acid) was taken as 13.6 x 10^3 litre.mol^{-1}.cm^{-1}.

2.17.7. *Glutamate Dehydrogenase(EC 1.4.1.2)*

Glutamate dehydrogenase activity was assayed, as described by Longshaw *et al.* (1972). The reaction medium contained (in a final volume of 1ml):

100mM	Tris HCl buffer, pH 7.7
3mM	Na_2EDTA
1mM	Na_2ADP
10mM	Na_2 oxoglutarate (freshly prepared)
0.15mM	NADH
50mM	NH_4Cl

A preincubation period of 2 min for extract and reagents, except NH_4Cl, was observed, at 340nm, before addition of NH_4Cl to start the reaction. The endogenous rate was subtracted to give the activity of glutamate dehydrogenase in ruminal epithelial extracts.

2.17.8. *D(-)-3-Hydroxybutyrate Dehydrogenase (EC 1.1.1.30; Method A)*

D(-)-3-hydroxybutyrate dehydrogenase was assayed by two methods: a continuous optical assay system (A), as employed by Watson and Lindsay (1972); and a stopped assay (B) utilised by Chandrasena *et al.* (1978). In both assay systems enzyme activity was measured in the direction of acetoacetate reduction.

The conditions for the spectrophotometric assay were as follows:

100mM	Na Phosphate buffer, pH 6.8
50mM	Nicotinamide
0.15mM	NADH
5µg	Rotenone (dissolved in absolute ethanol)
2mM	Li-Acetoacetate (freshly prepared)

After preincubation, for 3 min, the reaction was allowed to proceed by addition of acetoacetate to give a final volume of 1ml. The rate of decrease in absorbance at 340nm, minus blank rate, was used to calculate D(-)-3-hydroxybutyrate hydrogenase activity.

2.17.9. D(-)-3-Hydroxybutyrate Dehydrogenase (Method B; Chandrasena et al., 1978)

The incubation mixture and volume described in Method A were used in this assay except that the final concentration of Tris HCl (pH 6.8) and NADH were 40 and 0.5mM, respectively.

The assay was started by addition of extract and incubated for 5 min (see Fig. 2.3) before addition of 0.2ml 2% (v/v)-PCA, to stop the reaction. After centrifugation at 12000g for 2 min a portion of the supernatant was

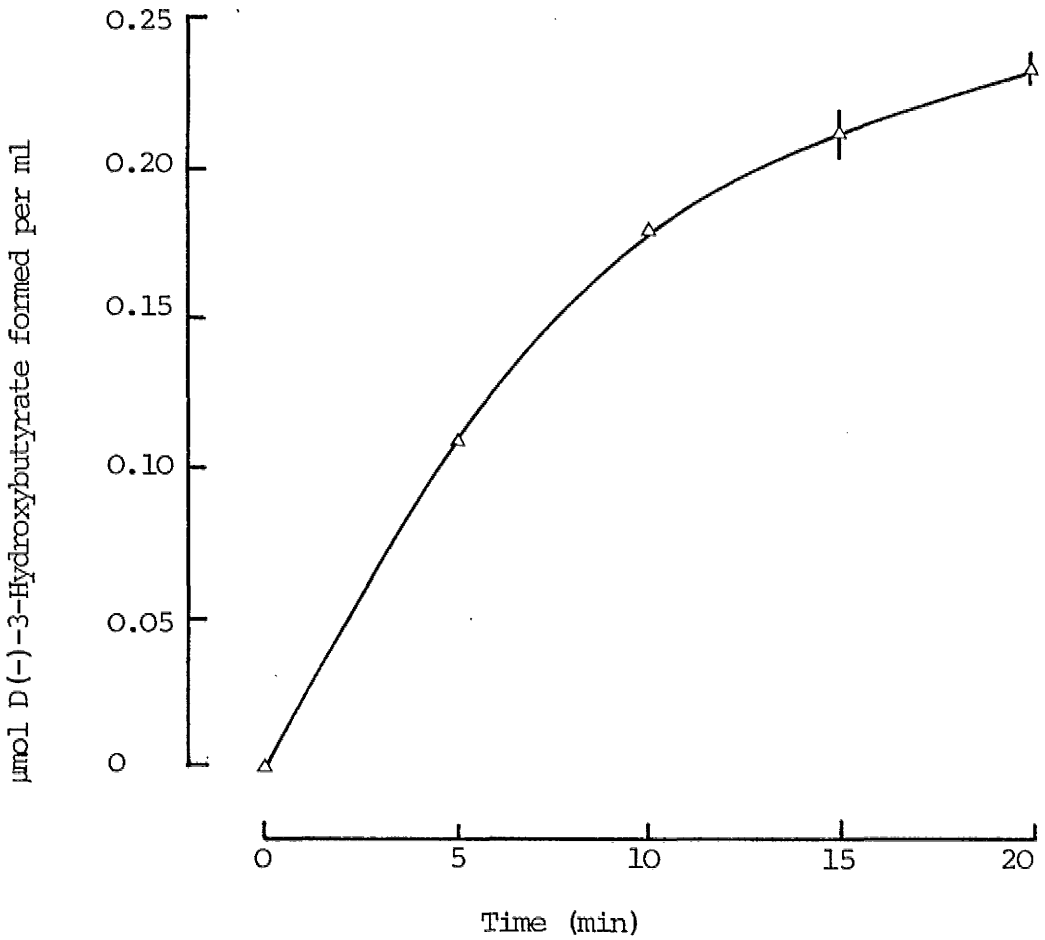


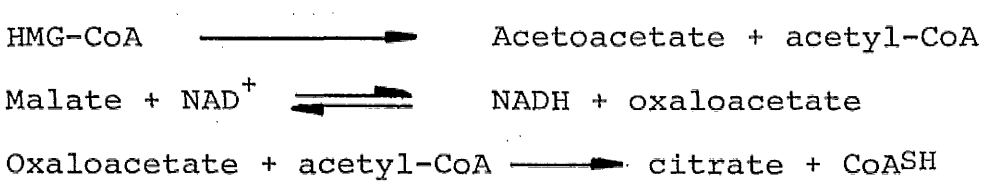
Fig. 2.3. Linearity of D(-)-3-Hydroxybutyrate Dehydrogenase Assay

Rumen epithelial homogenate samples (25µl) were assayed for D(-)-3-hydroxybutyrate dehydrogenase activity, as described in section 2.17.9. Results are means ± S.D. for triplicate incubations.

neutralised with 2M-KOH/0.5M-TEA (pH 7.4) and recentrifuged; the solution was analysed for D(-)-3-hydroxybutyrate content (section 2.18.9). The amount of this metabolite formed, over the incubation period, was considered to be a measure of D(-)-3-hydroxybutyrate dehydrogenase activity. Blanks containing either no extract or acetoacetate were carried out.

2.17.10. 3-(S)-Hydroxymethylglutaryl-CoA lyase (EC 4.1.3.4)

HMG-CoA lyase activity was assayed by the method outlined by Stegnik and Coon (1968) and the modifications made by Kramer and Miziorko (1980) were also included. In this system acetyl-CoA, produced upon cleavage of HMG-CoA, was coupled to the citrate synthase assay of Ochoa *et al.* (1951), according to the reactions:



The rate of NAD^+ reduction was proportional to the amount of enzyme added, to a limiting value, and was linear until a net absorbance increase of 0.4, at 340nm, had occurred.

However, citrate production and NAD^+ reduction are not equivalent in this system because the oxaloacetate concentration is not low, with respect to that of NADH (due to the malate dehydrogenase equilibrium; Buckel and Eggerer, 1965), before the addition of HMG-CoA to the otherwise complete system (Pearson, 1965).

Barth (1978) compared published methods for measuring HMG-CoA lyase activity and reported that HMG-CoA cleavage was higher than NADH formation by a factor of 1.33. When HMG-CoA lyase is assayed by the optical method activity can only be expressed in apparent units, unless this factor is taken into account, as units of activity are based on NADH formation.

The complete reaction mixture contained in 1ml:

150mM	Tris HCl buffer, pH 8.2.
10mM	$MgCl_2$
5mM	Dithiothreitol
2mM	NAD^+
0.05mM	NADH
2.5mM	Na Malate
20µg	Malate dehydrogenase (pig heart mitochondrial)
20µg	Citrate synthase (pig heart)
0.4mM	3-(S)-HMG-CoA

Enzyme and all other reagents, except HMG-CoA, are incubated for 10 min before addition of HMG-CoA to start the reaction. Suitable controls were carried out by incubation of extract with all reagents except either HMG-CoA or sodium malate.

2.17.11. 3-(S)-Hydroxymethylglutaryl-CoA Synthase (EC 4.1.3.5, Method 1)

The optical assay system described by Clinkenbeard *et al.* (1975), for crude extracts, was utilised to measure the enzymatic activity of 3-HMG-CoA synthase. Acetoacetyl-CoA was added to a final concentration of 0.02mM, as higher levels may inhibit synthase activity (Dashti and Ontko, 1979). The medium contained, in a final volume of 0.5ml:

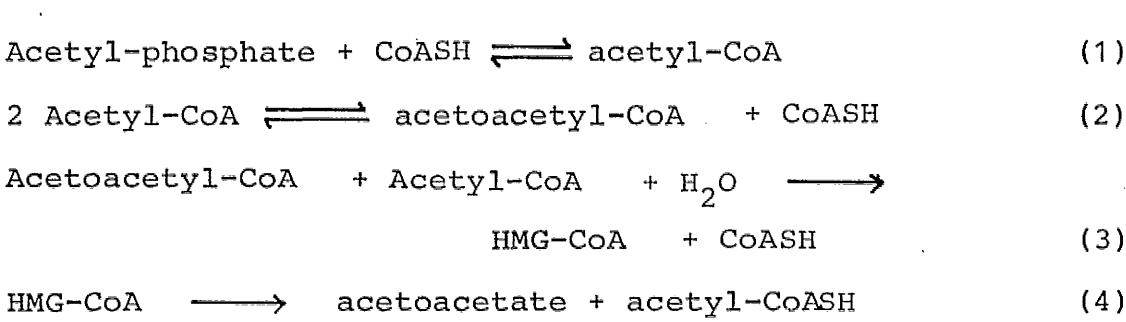
150mM	Tris HCl buffer, pH 8.2
5mM	$MgCl_2$
0.02mM	Li-Acetoacetyl-CoA
0.2mM	Acetyl-CoA
	Enzyme plus H_2O

Enzyme plus all reagents, except acetyl-CoA, were incubated for 3min before addition of acetyl-CoA to begin the assay.

HMG-CoA synthase activity was calculated as equivalent to half the measured rate. This correction is recommended by Clinkenbeard *et al.* (1975) to account for the CoASH-dependent consumption of acetoacetyl-CoA, catalysed by acetoacetyl-CoA thiolase. Furthermore, the use of this factor in calculations was supported by work carried out by Patel and Clark (1978). The molar absorbance coefficient of acetoacetyl-CoA at 303nm was taken as 13.8×10^3 litre.mol^{-1}cm^{-1} (Dashti and Ontko, 1979).

2.17.12. Hydroxymethylglutaryl-CoA Synthase (Method 2; Williamson et al., 1968).

In this assay system it was assumed that synthase activity was rate limiting in a series of four reactions;



Excess activities of acetoacetyl-CoA thiolase and HMG-CoA lyase are required to complete the reaction (the source of these enzymes was the extract). The incubation mixture (0.7ml) consisted of:

50mM	Tris-HCl buffer, pH 8.5
10mM	Acetyl-phosphate
0.86mM	CoASH
3.4units/ml	Phosphate acetyltransferase (EC 2.3.1.8)
	Extract plus H_2O

Samples (0.2ml) of the assay mixture were removed at 0, 8 and 16 min and mixed with 0.02ml 20% (v/v)-$HClO_4$. After removal of protein, by centrifugation, the supernatant fluid was neutralised with 0.5M-TEA/2M-KOH (pH 7.0). The $KClO_4$ precipitate was removed by centrifugation and the supernatant was assayed for acetoacetate (section 2.18.1). The production of acetoacetate was linear for at least 16 min (see Fig. 2.4).

2.17.13. Lactate Dehydrogenase (EC 1.1.1.27; Longshaw et al., 1972)

The assay medium contained in a total volume of 1ml:

100 mM	TEA/KOH buffer, pH 7.4
0.15 mM	NADH
0.86 mM	Na pyruvate (freshly prepared)
	Extract and H_2O

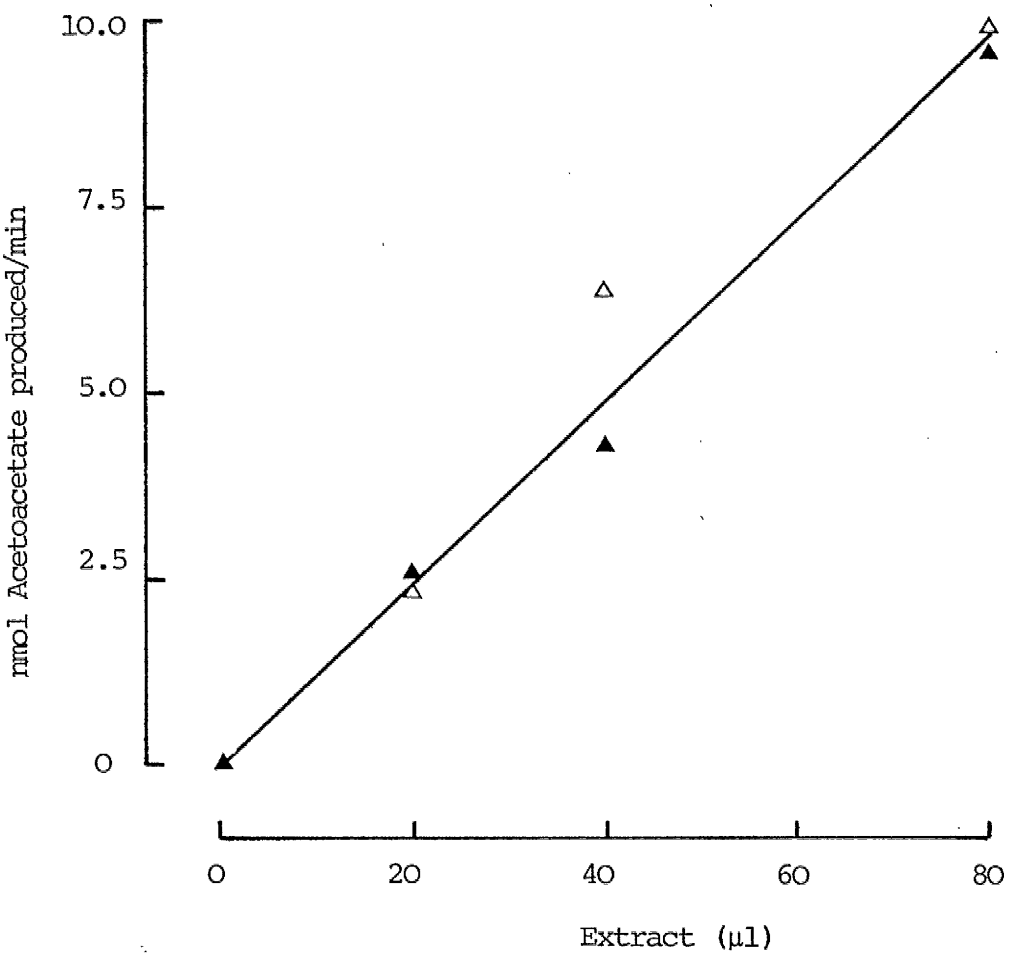


Fig. 2.4. Dependence of Hydroxymethylglutaryl-CoA Synthetic Rate on Enzyme Concentration

Rumen epithelial mitochondrial extract was assayed for hydroxymethylglutaryl-CoA synthase activity as described in section 2.17.12. The rate of acetoacetate production was assumed to be a measure of synthase activity. The 8 min (△) and 16 min (▲) time points are included.

All reagents, minus pyruvate, and enzyme were incubated for 2 min before addition of pyruvate to start the reaction. Lactate dehydrogenase activity was calculated from the decreased rate of absorbance, at 340nm, less any blank value.

2.17.14. *$NADP^+$-'Malic' Enzyme (EC 1.1.1.40)*

'Malic' enzyme was assayed as described by Stanley (1980). The reagents contained in the reaction medium (1ml) were as follows:

50mM	Tris HCl, pH 7.4
100mM	KCl
1mM	$MnCl_2.4H_2O$
0.2mM	$NADP^+$
1mM	K Malate
	Enzyme and H_2O

All reagents, except malate, and extract were preincubated for 5 min before initiation of the reaction by addition of malate to the cuvette. After subtracting the blank rate the activity of $NADP^+$-'Malic' enzyme activity was calculated from the increased rate of absorbance at 340nm.

2.17.15. Phosphoenolpyruvate Carboxykinase (EC 4.1.1.32)

Phosphoenol pyruvate carboxykinase (PEPCK) activity was measured in the 'forward' (physiological) direction by the method of Seubert and Huth (1965), as modified by Pogson and Smith (1975). This stopped assay requires enzymatic determination of phosphoenol pyruvate (PEP) after completion of the reaction.

Oxaloacetate, a substrate in the assay, undergoes spontaneous decarboxylation to pyruvate, which would give an overestimation of PEP content (see section 2.18.11). Therefore any pyruvate and excess oxaloacetate present were reduced to their corresponding hydroxyacids with $NaBH_4$. NaF was present to inhibit any enolase activity (usually found in crude tissue extracts).

The reaction mixture consisted of:

100mM	Tris HCl, pH 8.1
20mM	$MgCl_2$
15mM	NaF
6mM	NaITP
1mM	GSH
4.5mM	Na_2 Oxaloacetate (freshly prepared).

The reaction was started by addition of extract, after a 2 min preincubation of medium at 37°C, and allowed to proceed for 10 min; the incubation was stopped by addition of 0.1ml $NaBH_4$ (50mg/ml in 1mM NaOH).

After addition of 0.01ml 20% (v/v)-octan-2-ol in absolute ethanol (as an antifoaming agent), samples were chilled on ice for 2-3 min. Excess $NaBH_4$ was then destroyed with 0.2ml 20% (w.v)-PCA, the precipitated protein was removed by centrifugation (12 000g). A sample of the supernatant was removed and neutralised with 0.5M-TEA/ 2M-KOH (pH 7.4), with vigorous mixing to prevent hydrolysis of PEP by alkali.

Precipitated $KClO_4$ was removed by centrifugation (12 000g) and a portion of supernatant was assayed enzymatically for PEP (section 2.18.11). Blanks in which either extract or substrate were omitted were carried out throughout the entire assay procedure. The reaction was linear over the incubation period (see Fig. 2.5).

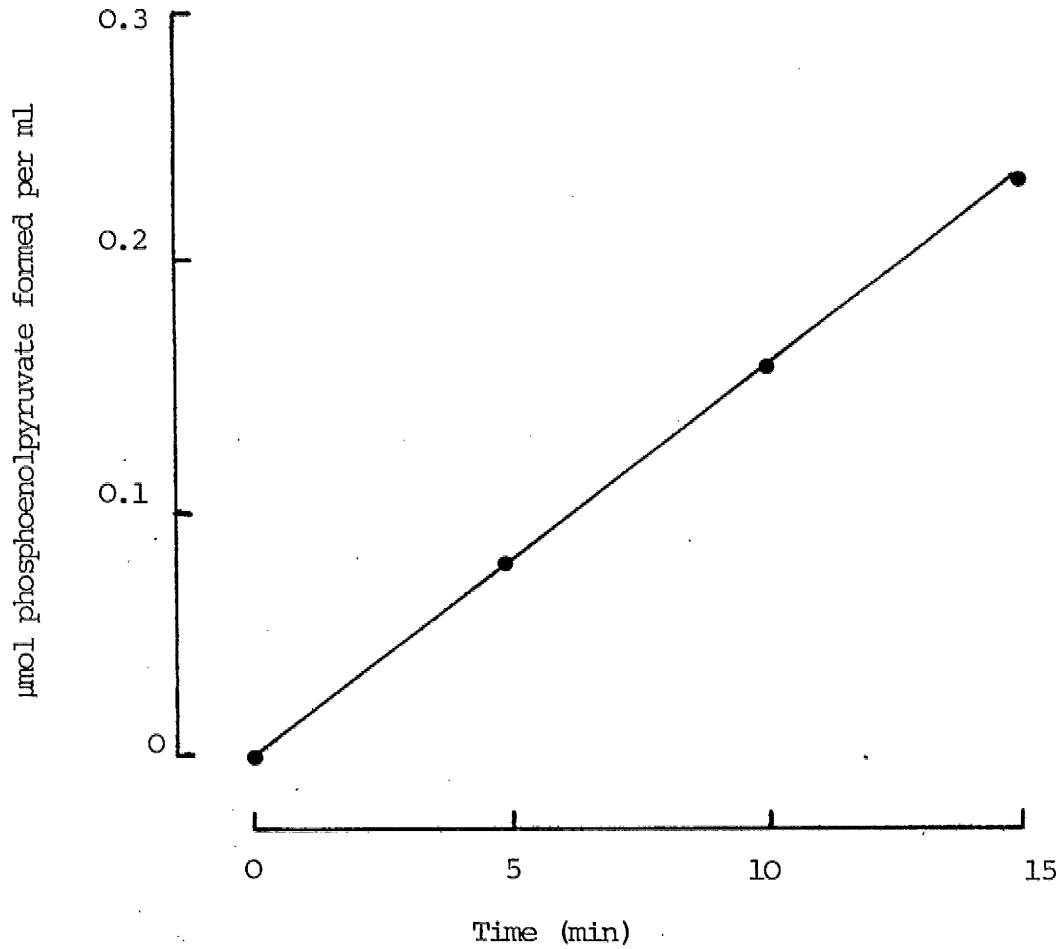


Fig. 2.5. Time Course of Phosphoenolpyruvate Carboxykinase Assay

Rumen epithelial extracts were assayed (in triplicate) for phosphoenolpyruvate carboxykinase activity as described in section 2.17.15. Standard deviations were less than 2% of mean values.

2.18. *Metabolite Assays*

All spectrophotometric determinations were carried out on a Gilford 250 spectrophotometer, equipped with a digital read-out. Assays requiring end point determinations were followed on a chart recorder (with a representative sample) until the reaction was completed. If 'drift' was observed, three sample readings were taken every 10 min and the final absorbance was obtained by extrapolation of the readings to zero time.

2.18.1. *Assays from a Standard Reference Source*

The following assays were carried out essentially as described by the authors in Bergmeyer (1974). Although the total volume of the medium (given in parenthesis) was altered in some assays, the final concentrations of substrates remained unchanged. Where assays were modified or obtained from different sources these methods are described in the text.

(1) ADP and AMP (Jaworek *et al.*, 1974b; 1ml)

(2) ATP (Jaworek *et al.*, 1974a; 1ml)

(3) L-Alanine (Grassl, 1974; 0.6ml)

(4) Citrate (Dagley 1974; 1ml)

(5) L-Glutamine (Lund, 1974; 1ml).

2.18.2. *Acetoacetate (Mellanby and Williamson, 1974)*

The assay medium contained:

100mM	TEA/KOH buffer, pH 7.4
0.15mM	NADH
50mUnits	D(-)-3-hydroxybutyrate dehydrogenase (from *Rhodopseudomonas sphaeroides*)

Samples were added to give a final volume of 0.5ml. The decrease in absorbance at 340nm was measured, after addition of enzyme, 60 min later.

2.18.3 *Acetone (Plus other Carbonyl Compounds)*

Acetone was measured as described by Armadzadeh and Harker (1974). This method involved the utilisation of 2, 4-dinitrophenylhydrazine (DNPH), which reacts specifically with carbonyl groups (of the type R_1-CO-R_2) forming a Schiff's base. The assay is sensitive over the range of 0.001-0.04 mg/ml.

1ml DNPH reagent was added to test solution (1ml) and the mixture was left to react for 30 min; alcoholic KOH (5ml) (10g KOH in 80% (v/v)-alcohol) was added and the absorbance was measured, within 5 min, at 435nm. An acetone standard curve was completed with each assay.

DNPH reagent : 1ml of boiling HCl was added to 0.06g 2,4-dinitrophenylhydrazine (AR); absolute alcohol (80ml) was added and the solution was warmed to 50°C for 3h. The solution was cooled before being diluted to 100ml with absolute alcohol and was stored in a dark place, where it was stable for 7 days.

This assay was used to estimate the extent of spontaneous decarboxylation of acetoacetate in incubation medium.

2.18.4. ATP (Stanley and Williams, 1969)

ATP was determined using a luciferase based assay. Three separate solutions containing: 80mM $MgSO_4$, 10mM Na_2 $HAsO_4$ and 10mM KH_2PO_4 were combined in equal proportions, before use, to give a final pH of 7.4; 3ml of this mixture was pipetted into acid/alkali washed 20ml glass scintillation vials.

The luciferase solution was prepared by homogenising 50mg luciferase extract in 20ml distilled water with 6-8 passes of a tight fitting teflon pestle. The solution was left for 1h before centrifugation (12000g for 2 min, three times) and the supernatant was stored at -20°C until required.

A standard curve in the range 0-140 pmol , was constructed from an ATP solution (standardised as described in section 2.18.1). Samples, and standards, were added to 3ml of buffer and placed in the dark for 30 minutes before assay of luminescence after addition of 0.05ml luciferase. Light production was measured in a Packard 3385 Scintillation Counter. All standards and samples were assayed in duplicate.

2.18.5. Ammonia and Urea (Fawcett and Scott, 1960)

To 0.1ml sample (pH 7.0), containing up to 100nmol urea or 200nmol ammonia, was added 0.05ml urease (EC 3.5.1.5; 100 Units/ml in 50mM-Na phosphate buffer, pH 6.5). The tubes were covered and incubated at 37°C for 20 min.

Equal volumes (1.25ml) of phenol reagent (1.106M phenol plus 0.17mM Na nitroprusside) and hypochlorite reagent (11mM NaHOCl plus 0.125M NaOH) were then added; followed by vigorous mixing and further incubation at 37°C for 30 min. The absorbance was measured at 635nm; urea or ammonia concentrations were determined from suitable standards assayed with the same reagents.

Blanks were included with all samples measured. Ammonia was determined in a similar manner but with omission of urease from the incubation medium.

2.18.6. *L-Aspartate (Bergmeyer et al., 1974a)*

Waarde and Heneganven (1982) recommended using phosphate buffer in place of tris HCl. The assay system contained:

67mM	Na,K Phosphate buffer, pH 7.2
0.15mM	NADH
3.1mM	2-Oxoglutarate
8 Units	Malate dehydrogenase
1.25 Units	Aspartate aminotransferase (EC 2.6.1.1)

Malate dehydrogenase was added to all reagents plus sample (up to 0.15mM aspartate), in a final volume of 1ml, and the decrease in absorbance at 340nm was measured 30 min later. Appropriate blanks were included.

2.18.7. *L-Citrulline (Archibald, 1944)*

This method is sensitive over a range of 0-400 nmole of citrulline in a final volume of 1ml. To the test solution was added 5ml of citrulline acid reagent (50ml conc. H_2SO_4 plus 150ml 85% (v/v) - H_3PO_4; diluted to 500ml with distilled water and 0.1ml of 1% (w/v) - $CuSO_4$ was added to this solution) and 0.25ml of 3% (w/v)-2,3-butanedione monoxime.

Samples were thoroughly mixed, stoppled with metal caps and finally covered with foil to exclude light. They were placed in a water bath at 100°C for 30 min and then the absorbances at 490nm were measured.

Because of the non-linearity of the standard curve, especially at low concentrations of citrulline, a known amount of standard solution (100nmole) was added to samples thus bringing them on to the linear part of the standard curve.

2.18.8. *D-Glucose (Krebs et al., 1963)*

The assay medium prepared just before use, contained:

100ml	0.5M Na Phosphate Buffer, pH 7.0
0.5ml	1% (w/v)-*O*-dianisidine (in methanol)
10mg	Glucose oxidase (EC 1.1.3.4)
10mg	Peroxidase (EC 1.11.1.7)

1ml of sample or standard (up to 400nmole) was mixed with 2ml assay medium and incubated at 37°C for 1h. The absorbance at 437nm was read after the incubation period. Appropriate blanks were tested simultaneously.

2.18.9. *L-Glutamate (Brent and Bergmeyer, 1974)*

This metabolite was assayed in medium similar to that used for measuring D(-)-3-hydroxybutyrate (section 2.18.9), except that 5.37mg ADP was added to the stock solution.

The increase in absorbance was measured 90 min after addition of 4.5 Units glutamate dehydrogenase. Appropriate blanks were included.

2.18.10. D(-)-3-Hydroxybutyrate (Williamson and Mellanby, 1974)

Some modifications were made to this assay based on the recommendations of Engel and Jones (1978). The assay mixture was taken from the following stock solution:

0.50g	Hydrazine sulphate (AR)
0.10ml	1M-$MgSO_4$
0.4ml	0.25M-EDTA (pH 7.0)
10mg	NAD^+

After mixing these reagents the solution was made up to 10ml with 2M-Tris, to give a final pH 9.0.

Samples (up to 200nmole) plus assay mixture were added to the cuvette to give a final volume of 0.5ml. The increase in absorbance at 340nm, following addition of 50 mUnits D(-)-3-hydroxybutyrate dehydrogenase (free of any malate dehydrogenase contamination), was measured 90 min later. Appropriate blanks were included.

2.18.11. L-Lactate and Malate

These two metabolites were assayed in the same assay system used for determination of D(-)-3-hydroxybutyrate (see above). The increase in absorbance at 340nm was determined 90 min after addition of either 2 Units malate dehydrogenase or 2 Units lactate dehydrogenase. Appropriate blanks were included.

2.18.12. *Pyruvate and Phosphoenolpyruvate (Czok and Lamprecht, 1974)*

Pyruvate and phosphoenolpyruvate were assayed sequentially in the same cuvette in a medium containing:

75mM	TEA/KOH buffer, pH 7.4
4.5mM	$MgSO_4$
0.15mM	NADH
0.5mM	ADP

The final volume was 0.5ml. Pyruvate was determined first, by measuring the decrease in absorbance at 340nm after addition of 1 Unit lactate dehydrogenase.

Phosphoenolpyruvate was calculated from the decrease in absorbance 5 min after addition of 1 Unit pyruvate kinase. Appropriate blanks were included.

2.19. *Statistical Methods*

In experiments where enzyme activities, metabolite concentrations or rates of metabolite fluxes were measured, results are expressed, unless otherwise stated in the form: mean value ± standard error of the mean (S.E.M.)

The S.E.M. was calculated from the following formula:

$$S.E.M. = \sqrt{\frac{\Sigma (m-x)^2}{n(n-1)}}$$

where 'x' represents an observed parameter, 'm' is the arithmetic mean of the observed values, 'n' is the number of observations; 'n-1' is the number of degrees of freedom.

The significance of the differences between means was determined by the use of the Student's 't' test, using the formula:

$$t = \frac{m_1 - m_2}{S\sqrt{1/n_1 + 1/n_2}}$$

Where 'm_1' and 'm_2' are the means of the two sets of observations, and 'n_1' and 'n_2' are the numbers of observations in each set. Values of 'S' are obtained by taking the square root of the mean square deviation of the two groups (S^2);

$$S^2 = \frac{\Sigma (m_1 - x_1)^2 + \Sigma (m_2 - x_2)^2}{n_1 + n_2 - 2}$$

where $n_1 = n_2$; 't' was calculated from the formula:

$$t = \frac{|m_1 - m_2|}{\sqrt{(SEM_1)^2 + (SEM_2)^2}}$$

The probability p, that the difference between any two means was significant, was obtained from the relevant value of 't' and the number of degrees of freedom ($n_1 + n_2 - 2$) by reference to appropriate probability tables. The difference between two means was considered significant when $p < 0.05$.

CHAPTER 3

SUBCELLULAR DISTRIBUTION OF ENZYMES OF KETOGENESIS AND PROPIONATE METABOLISM IN SHEEP RUMEN EPITHELIUM

3.1. *Introduction*

Three reported sites for ketogenesis in ruminant animals are the liver, the omasal mucosa and the rumen epithelium (Pennington, 1952; Hird and Symons, 1959; Hird and Weidemann, 1964; Baird *et al.*, 1979); in the latter organ generation of ketone bodies is quantitatively more significant in the fed state (Leng and West, 1969; Baird *et al.*, 1979).

There has been speculation that a cytoplasmic ketogenic pathway operates in livers of fed rats (Brunengraber *et al.*, 1978; Williamson *et al.*, 1968), guinea pigs (Sauer and Erfle, 1966) and chickens (Allred, 1973). More systematic studies have cast some doubts on this proposal (Endermann *et al.*, 1982) and support the view that hepatic ketogenesis is restricted to the mitochondrial compartment (Chapman *et al.*, 1973). Nonetheless, the possibility of a cytoplasmic pathway operating in rumen epithelium was investigated.

Also, although significant amounts of propionate are converted to L-lactate by rumen mucosa *in vitro* (Weekes, 1974) the metabolic pathway has not been absolutely defined. The subcellular distribution of some enzymes which are involved in propionate metabolism were also localised.

3.2.1. *Materials and Methods*

Procedures for transportation and homogenisation of rumen epithelium are described in Chapter 2, enyzme assay methods are also given in this chapter.

3.2.2. *Method for Measuring Incorporation of ^{14}C-labelled Propionate into Glucose*

Rumen papillae were incubated with 0.5mM-[1-^{14}C] propionate (0.5µCi per flask) with or without glucose (5mM) for 1h, at 39°C. Control flasks contained 5mM-[U-^{14}C] glucose plus $HClO_4$ (0.37M). Neutralised samples of incubation medium were analysed for ^{14}C-labelled glucose as follows: Sample (0.2ml) was loaded on to a CG_{400} column (acetate washed; 2.5ml bed volume); the column was washed with 2ml H_2O. Preliminary experiments showed that [1-^{14}C] propionate was bound tightly to this column. 1ml of wash was added to 1ml of buffer, pH 7.5 (0.2M-Tris HCl, 0.1M-KCl and 0.03M-$MgSO_4$); 2 Units of hexokinase (EC 2.7.1.1) and 0.01ml 200mM-ATP solution were also added to this mixture. After mixing, this solution was incubated for 45 min at 20°C before 0.1ml was removed and loaded on to a second CG_{400} column. This was washed with 4ml H_2O. 1M-HCl (0.5ml) was added and left to equilibrate in the column for 20 min before collection. A second volume of 1M-HCl (0.5ml) was added to elute any residual [^{14}C]glucose-6-phosphate. 0.1ml of this wash was added to 2ml PCS and counted for ^{14}C radioactivity.

Calculations were corrected for % recovery of [U-^{14}C]glucose (which averaged 62%) through the whole procedure.

3.3 *Calculation for Determination of the Percentage Distribution of Enzymes between Subcellular Compartments*

The distribution of the cytoplasmic (lactate dehydrogenase, L) and mitochondrial (glutamate dehydrogenase, G) marker enzymes were determined in each subcellular fractionation experiment. The percentage activities of any enzyme measured in the particulate pellets constituted the mitochondrial fraction ($\underline{x}$), whereas the percentage activity measured in the supernatant was assumed to be cytoplasmic (y).

By solving simultaneous linear equations, as shown below, an estimation of the subcellular location of rumen epithelial enzymes was made:

$$L_m \times C + G_m \times M = \underline{x}$$

$$L_c \times C + G_c \times M = y$$

where M and C represent the corrected percentage distribution of any rumen epithelial enzyme in the mitochondrial and cytoplasmic fractions, respectively. L_m, L_c, G_m and G_c are the percentage recoveries of the marker enzymes in the two subcellular fractions.

3.4 *Results and Discussion*

3.4.1. *Extraction of Enzymes from Sheep Rumen Mucosa*

In preliminary experiments tissue from rumen epithelium was dispersed by treatment with the following: digitonin; ultrasonic disruption; the Ystral homogeniser ('Polytron®-type); and the Potter-Elvehjem homogeniser. The results revealed different degrees of disruption of tissue and subsequent release of intracellular enzymes. Highest enzyme activities (and those closest to published values) were found with extracts prepared using the Ystral homogeniser, followed by solubilisation of suborganelle membranes with a combination of detergent and cycles of freeze-thawing. The detergent used was Triton-X100 as this is reported to increase the effectiveness of extraction of mitochondrial enzymes from rumen mucosa (Weekes, 1972).

Similar observations have recently been reported by Bush (1982) who employed a variety of techniques for extraction of enzymes from fresh and frozen bovine rumen papillae. Bush (1982) reported that Polytron extraction released the most protein and total enzyme activity into the extraction medium.

The total amounts of enzyme activities recovered from the nuclear, mitochondrial and cytoplasmic fractions were generally greater than 90% (Table 3.1 and Table 3.2). Lower percentage recoveries were recorded for hydroxy-

methylglutaryl-CoA (HMG-CoA) synthase and lyase. This observation may reflect the reported labile nature of these enzymes (Stegink and Coon, 1968; Dashti and Ontko, 1979).

The use of marker enzymes to indicate the extent of cross-contamination between subcellular fractions is common practice in enzyme localisation studies. Weekes (1972) reported that high lactate dehydrogenase activity is present in ovine rumen epithelium. Although glutamate dehydrogenase activity is much lower than lactate dehydrogenase activity, on a wet wt. basis, it is present in measurable amounts in sheep rumen mucosa (Chalupa *et al.*, 1970a; Watson & Lindsay, 1972; Weekes, 1972).

The suitability of glutamate dehydrogenase as a marker enzyme was evaluated by determination of the partition of citrate synthase amongst subcellular compartments. Citrate synthase is located in the mitochondrial matrix. It was found that most of the synthase activity was associated with the mitochondrial fraction, after correction for percentage distribution using glutamate dehydrogenase as the marker enzyme (see Table 3.3). The release of glutamate dehydrogenase activity into the cytoplasmic fraction averaged about 10% of the enzyme activity measured in the homogenate (see Table 3.1). Baird *et al.* (1970) reported a higher value (58%) for the percentage release of glutamate dehydrogenase after homogenisation of bovine rumen papillae.

TABLE 3.1. The Distribution and Activities of Some Enzymes Extracted from Sheep Rumen Epithelium

Homogenisation and fractionation of rumen epithelial papillae was as described in sections 2.6 and 2.7. Enzyme activities were measured at 30°C (except phosphoenolpyruvate carboxykinase, 37°C) and results are reported as means ± S.E.M. for the number of observations shown in parentheses. All results are reported in terms of μmol/min per g wet wt.

The percentage recovery is given as the sum of enzyme activities in the three subcellular fractions divided by the activity measured in the homogenate x 100.

Fractions	Glutamate Dehydrogenase (15)	Lactate Dehydrogenase (15)	ATP-Citrate Lyase (5)	Phosphoenolpyruvate carboxykinase (3)	NADP-'Malic' enzyme (1)	Citrate Synthase (3)
Homogenate	0.85 ± 0.08	56.7 ± 4.6	0.12 ± 0.01	0.46 ± 0.10	2.16	2.36 ± 0.54
Nuclear Fraction	0.21 ± 0.02	4.3 ± 0.4	0.02 ± 0.00	0.16 ± 0.03	0.50	0.69 ± 0.15
Mitochondrial Fraction	0.48 ± 0.05	3.2 ± 0.5	0.01 ± 0.00	0.25 ± 0.03	0.05	1.40 ± 0.51
Cytoplasmic Fraction	0.08 ± 0.01	45.2 ± 3.7	0.09 ± 0.01	0.02 ± 0.02	1.62	0.54 ± 0.15
% Recovery	90.6	92.9	100.0	93.4	100.4	111.4

TABLE 3.2. Subcellular Location of Ketogenic Enzymes in Sheep Rumen Epithelium

Procedures and expression of results were as described in the legend to Table 3.1.

Hydroxymethylglutaryl-CoA lyase activity was corrected by multiplying the measured activity by 1.33 as recommended by Barth (1978) (see section 2.17.10). Hydroxymethylglutaryl-CoA synthase activity has been multiplied by 0.5 as suggested by Clinkenbeard *et al.* (1975). Use of this factor for crude preparations, to correct for acetoacetyl-CoA thiolase activity, was verified by Patel and Clark (1978).

There was no detectable acetoacetyl-CoA hydrolase activity above the rate of spontaneous hydrolysis of acetoacetyl-CoA (see section 2.17.2). Initially acetoacetyl-CoA thiolase activity was assayed by the method described by Williamson *et al.* (1968) (Method A). This was re-assayed because of possible erroneous results in measuring acetoacetyl-CoA thiolase activity in the absence of a fixed K^+ concentration. The activity reported in this table (Method B) was measured in the presence of 50mM-KCl.

FRACTIONS	Acetoacetyl-CoA thiolase (A)	Acetoacetyl-CoA thiolase (B)	3-Ketoacid CoA-Transferase	HMG-CoA Synthase	HMG-CoA Lyase	D(-)3-Hydroxy-butyrate Dehydrogenase (A)	D(-)-3-Hydroxy butyrate Dehydrogenase (B)
	(3)	(3)	(3)	(3)	(3)	(3)	(3)
Homogenate	3.18±0.59	2.84±0.30	0.40±0.11	0.22±0.04	0.88±0.19	0.10±0.02	1.61±0.28
Nuclear Fraction	1.26±0.50	-	0.11±0.07	0.05±0.03	0.20±0.05	0.03±0.01	0.37±0.12
Mitochondrial Fraction	1.31±0.23	-	0.24±0.04	0.08±0.03	0.49±0.04	0.05±0.02	1.07±0.21
Cytoplasmic Fraction	0.95±0.15	-	0.11±0.02	0.07±0.02	0.04±0.01	0.02±0.01	0.04±0.03
% Recovery	92.1	-	111.4	86.4	81.7	100.0	92.0

TABLE 3.3. Percentage Distribution of Enzymes Extracted from Sheep Rumen Epithelium in Mitochondrial and Cytoplasmic Fractions

The percentage distribution of each enzyme measured was corrected for cross-contamination using marker enzymes (section 3.2.3). Results are given as means ± S.E.M. for at least 3 extractions from separate rumen epithelium.

Acetoacetyl-CoA thiolase (Method B) was assayed in the presence of either 50mM-Na^+ or 50mM-K^+ and the mitochondrial and cytoplasmic activities were estimated by the formulae given by Middleton (1973) (see section 2.17.4). D(-)-3-hydroxybutyrate dehydrogenase activity was measured using two different assays (see sections 2.17.8 and 2.17.9).

Enzymes	% of total activity in Mitochondrial fraction	% of total activity in Cytoplasmic fraction
Lactate dehydrogenase	(0)	(100)
Glutamate dehydrogenase	(100)	(0)
Citrate synthase	104.4 ± 4.7	5.0 ± 2.5
Acetoacetyl-CoA thiolase (A)	75.7 ± 5.5	27.0 ± 7.3
Acetoacetyl-CoA thiolase (B)	65.5 ± 6.9	34.1 ± 7.0
3-Keto acid CoA-transferase	88.4 ± 1.9	11.8 ± 1.1
3-HMG-CoA synthase	58.1 ± 13.0	44.2 ± 13.2
3-HMG-CoA lyase	103.0 ± 10.7	4.7 ± 1.7
Hydroxybutyrate (A)	110.0 ± 10.5	6.3 ± 0.9
Dehydrogenase (B)	103.8 ± 3.6	4.8 ± 0.8
ATP-citrate lyase	9.3 ± 2.7	90.3 ± 2.8
Phosphoenolpyruvate Carboxykinase	107.0 ± 2.9	6.9 ± 2.9
NADP-'Malic' enzyme	2.0	102.0

Some bacterial contamination of extracts prepared from rumen epithelium is possible for instance, a measurable urease activity, originating from microbial sources, is associated with sheep rumen wall (Michnova *et al.*, 1979). Lenartova *et al.* (1981) reported that glutamate dehydrogenase activities originating from prokaryotic and eukaryotic sources was identifiable in rumen mucosal extracts. The activity obtained from the tissue comprised more than 95% of the total glutamate dehydrogenase activity measured.

Semi-purified glutamate dehydrogenase, isolated from bovine rumen epithelium, is reported to have similar kinetic properties to the dehydrogenase isolated from hepatic tissues of other animals (Holavska *et al.*, 1981).

3.4.2. *Subcellular location of Ketogenic Enzymes*

It is proposed that butyrate is metabolised as a C_4 molecule by rumen epithelium, through enzymic reactions as far as acetoacetyl-CoA (see Chapter 1). Acetoacetyl-CoA is then converted to either acetoacetate or acetyl-CoA by one or more of the enzymatic steps illustrated on Fig. 3.1. In this study the activities of enzymes associated with all four pathways were measured.

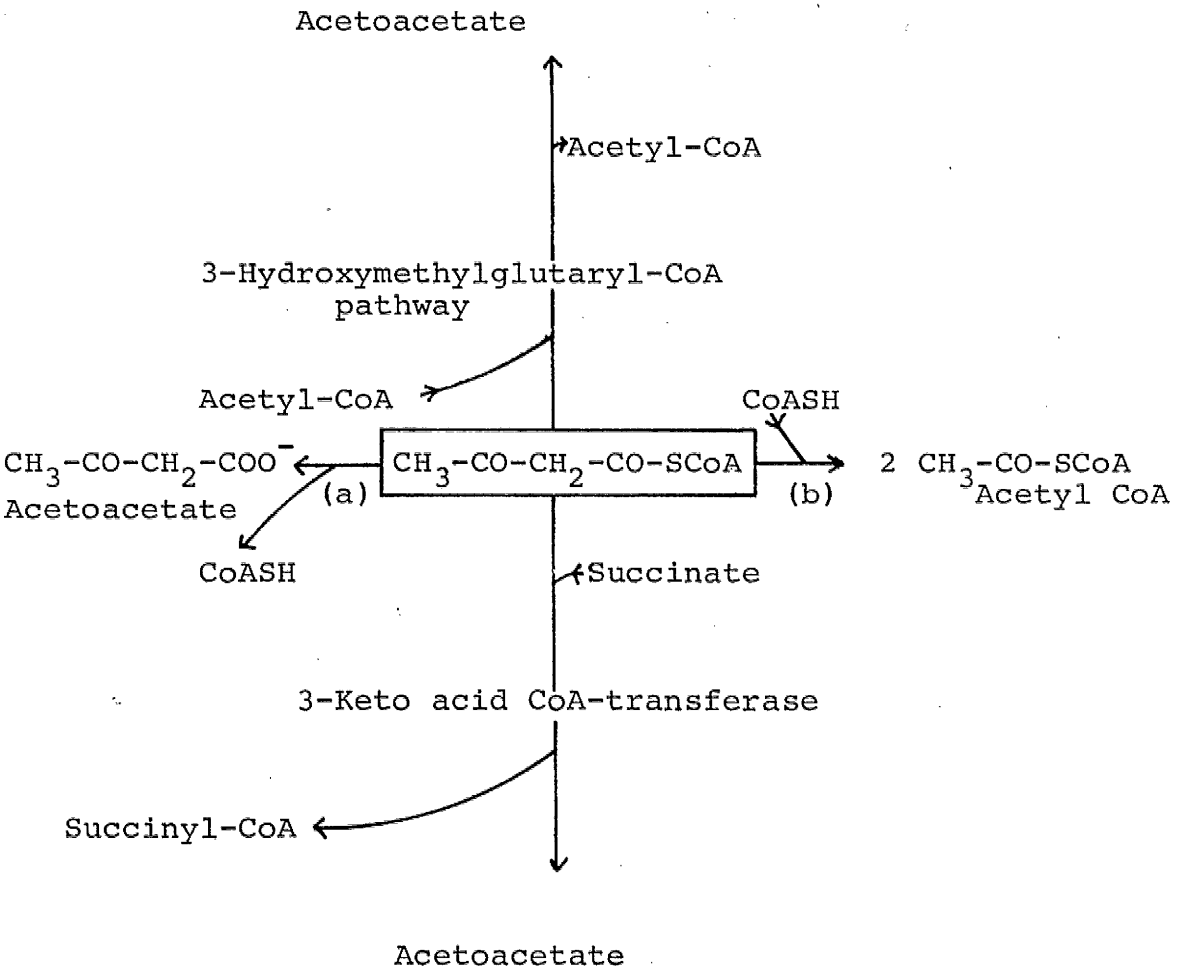


Fig. 3.1. Routes of Acetoacetyl-CoA Breakdown

(a) acetoacetyl-CoA hydrolase, (b) acetoacetyl-CoA thiolase.

There was no acetoacetyl-CoA hydrolase activity in homogenates or subcellular fractions prepared from sheep rumen epithelium (see Legend to Fig. 3.2). Baird *et al.* (1970) have reported that only slight acetoacetyl-CoA hydrolase activity is found in bovine mucosal extracts.

Henning and Hird (1972) observed that both rabbit caecal and colonic epithelia produced significant quantities of ketone bodies from butyrate *in vitro*. Butyrate-dependent ketogenesis has also been reported to occur in a number of other fermentative organs such as mucosa of Kangaroo stomach (Henning and Hird, 1970), rat colon mucosa (Roediger, 1980a) and human colonic epithelium (Roediger, 1982). It is found that the acetoacetyl-CoA hydrolase activity from rabbit caecal and colon epithelium is low, approximately 12 times less than HMG-CoA synthase activity measured in extracts from the same tissues.

The existence of 3-keto acid CoA-transferase activity was first reported by Bush and Milligan (1971). Addition of succinate to rumen epithelial extracts containing acetoacetyl-CoA resulted in a stimulation of the breakdown of this compound. An accumulation of succinyl-CoA was also identified. Recently, Emmanuel and Milligan (1983) have measured transferase activity from bovine rumen mucosa and reported this to be 0.43 Units per g wet wt. This value is approximately the same as the activity

found in the corresponding ovine tissue. Also 3-keto acid CoA-transferase activity was confined largely to the mitochondrial space (see Table 3.3).

Transfer of the CoA moiety, from acetoacetyl-CoA to acetate, propionate, and butyrate is also catalysed by an enzyme present in bovine rumen epithelial cell-free extracts (Emmanuel and Milligan, 1983). This CoA-transferase activity has originally been measured in homogenates of lysine-fermenting bacteria (Barker *et al.*, 1978). However, the assay system used to estimate the VFA: acetoacetyl-CoA transferase activity contained a non-physiological concentration of acetoacetyl-CoA (0.17mM) and so it remains to be seen if this enzyme catalyses a functional pathway in rumen epithelium.

Acetoacetyl-CoA thiolase activity was high, in relation to other ketogenic enzyme activities. The thiolase activity found in this study compared favourably to reported activities of 5.1 and 4.6 units per g of fresh wt. in rumen mucosa samples from healthy and ketotic cows, respectively (Baird *et al.*, 1970). Not only is thiolase activity important for the operation of the HMG-CoA pathway but this enzyme is required for the catalytic breakdown of acetoacetyl-CoA to acetyl-CoA, a major metabolic fuel for the tricarboxylic acid cycle.

Acetoacetyl-CoA thiolase extracted from rat liver mitochondrial fractions was identified as three isoenzymes (namely A_1, A_2 and B) by chromatography on cellulose phosphate columns (Middleton, 1978). A_1 and A_2 might, however, be interconvertible. All three peaks had properties typical of mitochondrial acetoacetyl-CoA thiolase activity, i.e. showing no activity with 3-oxohexanoyl-CoA as substrate and being activated by K^+. Two other thiolase enzymes exist (one cytoplasmic, the other mitochondrial) which are also active with acetoacetate as substrate, but are unaffected by K^+ (Middleton, 1973).

Middleton (1978) observed an association of isoenzyme A with those tissues (liver, kidney) capable of ketogenesis through an operational HMG-CoA pathway. From this observation it is proposed that the role of isoenzyme A is in net synthesis of mitochondrial acetoacetyl-CoA from acetyl-CoA. Conversely, isoenzyme B may preferentially catalyse the net breakdown of acetoacetyl-CoA. The apparent K_m for acetyl-CoA in the direction of acetoacetyl-CoA formation is around 200μM for all B isoenzymes extracted from different tissues, while liver A form has an apparent K_m of 50μM (Middleton, 1978).

Acetoacetyl-CoA thiolase isoenzyme A and B may also be interconvertible. Huth (1981) reported that thiolase B can be transformed into isoenzyme A by covalent

binding of CoA to the former enzyme. The amounts of the different forms of acetoacetyl-CoA thiolase may vary with the level of CoA in the cell.

It was found that acetoacetyl-CoA thiolase from rumen epithelium was activated by K^+, the activity from the mitochondrial fraction being stimulated the most (see section 2.17.4). Rumen epithelium may contain similar distributions of mitochondrial isoenzymes as rat liver and kidney, two non-fermentative tissues capable of ketogenesis.

Distribution of acetoacetyl-CoA between oxidation and ketogenesis may depend on the association of acetoacetyl-CoA thiolase with HMG-CoA synthase. There has been speculation that an intramitochondrial thiolase-synthase binary complex, with direct 'chanelling' of acetoacetyl-CoA between these two enzymes, may exist (Greville and Tubbs, 1968). If this complex does exist it is readily disrupted (Page and Tubbs, 1978).

HMG-CoA synthase was observed to have the lowest activity of all the measured ketogenic enzymes. This finding may agree with the theory that this is the rate limiting enzyme of ketone body production (Williamson *et al.*, 1968; Baird *et al.*, 1970; Chapman *et al.*, 1973; Dashiti and Ontko, 1979). HMG-CoA synthase, purified from avian liver, is found to be acetylated, by acetyl-CoA, at (cysteinyl-SH) giving rise to acetyl-S-enzyme (Miziorko and Lane, 1977).

It is proposed that this is the rate limiting step of 3-HMG-CoA synthesis. Page and Tubbs (1978) found that the kinetic behaviour of synthase purified from ox liver complies with the proposed reaction pathway involving condensation of an acetyl-enzyme complex with acetoacetyl-CoA.

Published values for HMG-CoA synthase activity in extracts prepared from bovine rumen epithelium range from 0.43 to 0.06μmoles/min per g wet wt. (Baird *et al.*, 1970; Bush and Milligan, 1971). The lower activity reported by Bush and Milligan (1971) could be because of a decreased Mg^{2+} concentration (0.2mM) in the assay system without lowering the absorbance coefficient of acetoacetyl-CoA accordingly (Stern, 1956; see below).

Baird's group used the assay system described by Williamson *et al.* (1968) for measuring synthase activity. Differing assay procedures could have been responsible for the discrepancy in activity values for HMG-CoA synthase extracted from cow and sheep rumen epithelium. It was found that when synthase activity, in sheep rumen mucosal extracts, was measured by the method described by Williamson *et al.*(1968) the activity was similar in magnitude to the original estimate (0.15μmoles/min per g wet wt; range 0.19 to 0.11 for three separate preparations). HMG-CoA synthase activities from proximal colon and caecal mucosae are also low, with values of 0.10 and 0.12μmol/min per g wet wt., respectively (Henning and Hird, 1972).

Chicken liver mitochondrial HMG-CoA synthase is reported to be strongly inhibited by Mg^{2+} (Reed *et al.*, 1975). Unfortunately, Mg^{2+} is required in the optical assay to increase the absorbance coefficient of acetoacetyl-CoA. Therefore, the effect of Mg^{2+} on rumen epithelial mitochondrial HMG-CoA synthase activity was investigated using the assay system recommended by Williamson *et al.* (1968). No inhibition of synthase activity was observed (see Fig. 3.2). This observation concurs with Allred's (1973) report that synthase activity, extracted from avian liver, was unaffected by additional Mg^{2+} in the assay mixture.

The affinity of HMG-CoA synthase, and acetoacetyl-CoA thiolase, for acetyl-CoA is decreased when the concentration of acetoacetyl-CoA is increased above 20μM (Huth *et al.*, 1975; Reed *et al.*, 1975). A suggestion for this substrate inhibition effect is that acetoacetyl-CoA binds to un-acetylated HMG-CoA synthase, with reasonably high affinity, and thus hinders binding of acetyl-CoA to the enzyme (Miziorko *et al.*, 1979). A 50% inhibition of mitochondrial HMG-CoA synthase activity is produced by 0.2mM-succinyl-CoA (Reed *et al.*, 1975).

The apparent K_m value of mitochondrial HMG-CoA synthase for acetyl-CoA ranges from 200μM, in the presence of 10μM acetoacetyl-CoA (Reed *et al.*, 1975), to 9μM in the absence of this substrate (Miziorko *et al.*, 1979). Ox liver synthase has a very low K_m for acetoacetyl-CoA (<1μM) at pH 7.8 (Page and Tubbs, 1978). Similar observations have been made by Menehan *et al.* (1981) for rat liver mitochondrial

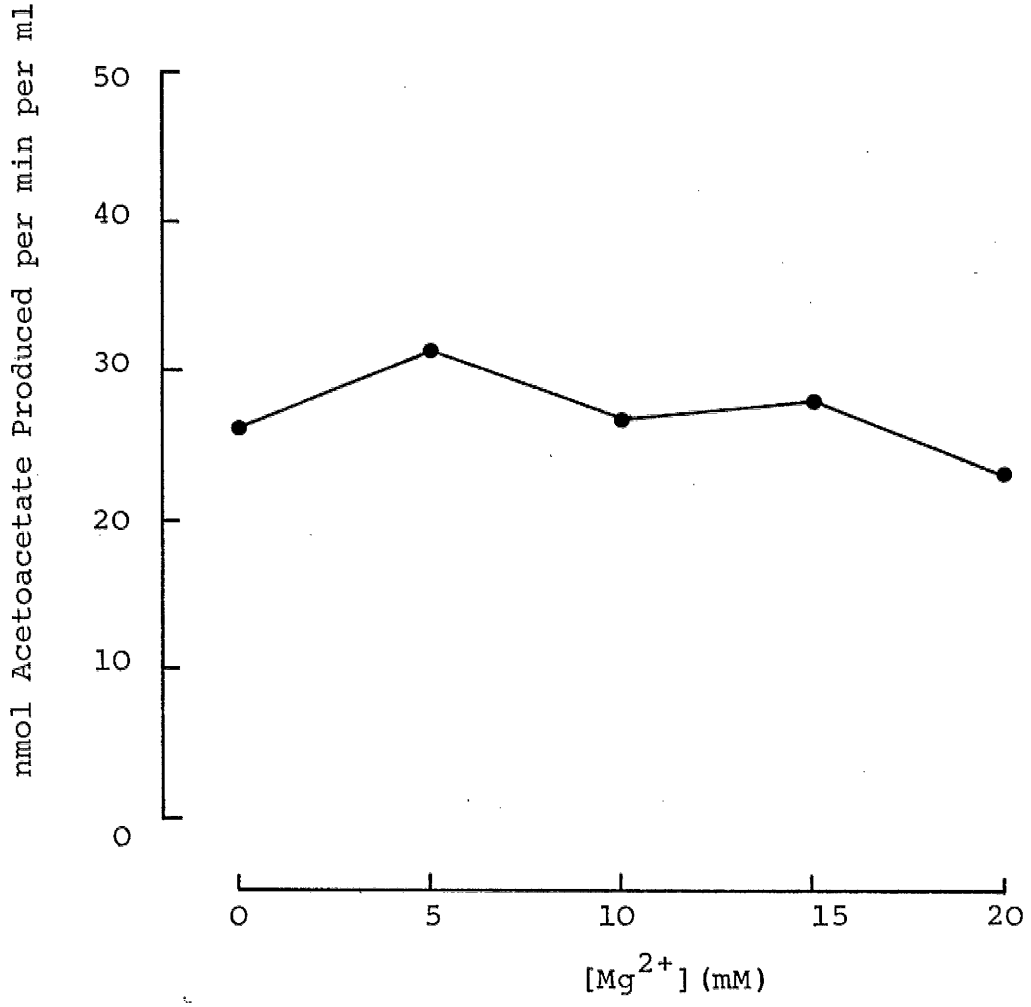


Fig. 3.2. Effect of Mg^{2+} on Mitochondrial Hydroxymethylglutaryl-CoA Synthase Activity

Rumen epithelial mitochondrial extract was assayed for hydroxymethylglutaryl-CoA synthase activity, as described by Williamson *et al.* (1968), in the presence of a range of $MgCl_2$ concentrations.

HMG-CoA synthase. Menehan *et al.* (1981) used a highly sensitive assay for acetoacetyl-CoA determination (Hron *et al.*, 1981) to estimate an apparent K_m for acetoacetyl-CoA of 0.35μM at pH 8.2. Mitochondrial concentrations of acetoacetyl-CoA may be in the range of 10^{-6} to 10^{-5}M and one theory is that flux through the synthase-catalysed reaction may be directly controlled by the concentration of this metabolite in the mitochondria (Menehan *et al.*, 1981).

In this present study HMG-CoA lyase activity was approximately five-fold less than the activity reported for cow rumen epithelium (4.46 μmoles/min per g wet wt.; Baird *et al.*, 1971). If the HMG-CoA pathway is the major route of ketone body synthesis then the presence of HMG-CoA lyase is an absolute requirement. Lyase activity from ovine rumen epithelium appeared to be predominantly, if not exclusively, mitochondrial (see Table 3.3 and the citrate synthase distribution). In sheep liver HMG-CoA lyase activity is also reported to be associated entirely with the mitochondrial fraction (Zammit, 1980).

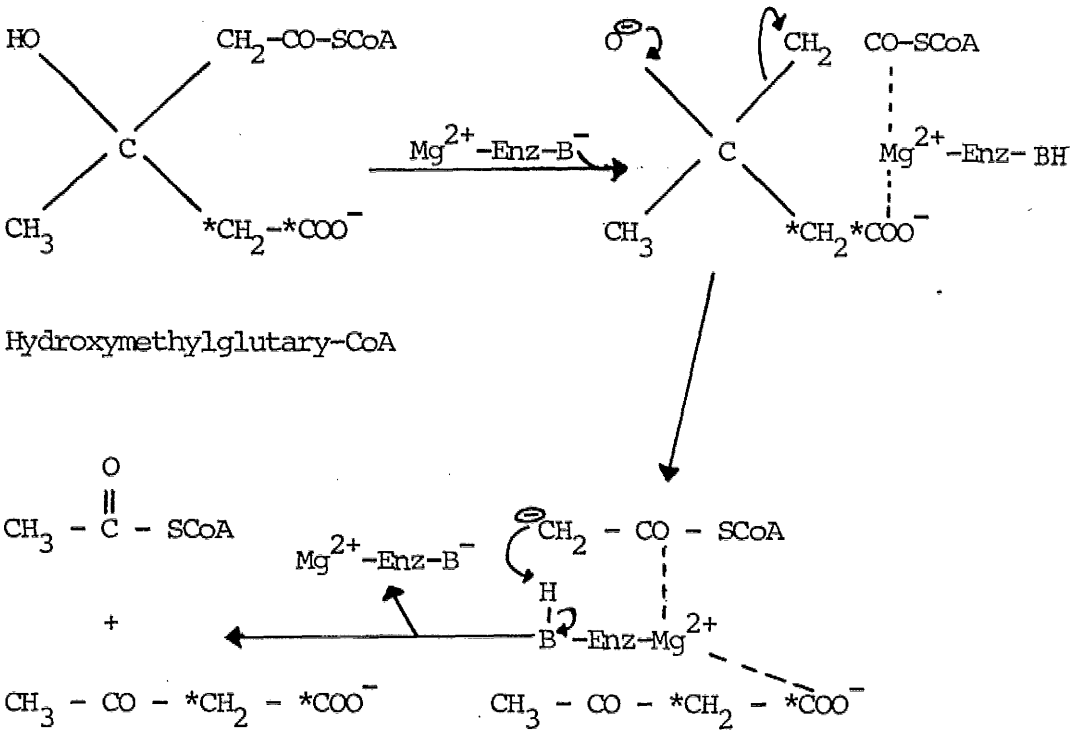
HMG-CoA lyase exhibits an absolute requirement for a divalent metal ion (see Fig. 3.3) and a thiol to reach maximum activity (Stegink and Coony, 1968; Dashti and Ontko, 1979). Co^{2+} and Mg^{2+} appear to be the most effective at increasing lyase activity (Stegink and Coon, 1968).

3-HMG-CoA binds to the lyase with an apparent K_m of 8μM for avian (Kramer and Miziorko, 1980) and beef liver (Stegink and Coon, 1968), although a lower value (3.5μM) has been reported for bovine liver enzyme (Higgins *et al.*, 1972).

The participation of HMG-CoA lyase in regulation of ketogenesis cannot be completely ruled out as this enzyme has a high turnover rate (Kramer and Miziorko, 1980). Modification of the turn over rate could possibly affect flux through the HMG-CoA pathway. Rat liver mitochondrial HMG-CoA lyase is reported to be inhibited by increased succinyl-CoA concentrations (Deana *et al.*, 1979).

The distribution of D(-)-3-hydroxybutyrate dehydrogenase was similar to the pattern of HMG-CoA lyase distribution (Table 3.3). This was further evidence to support the hypothesis that ketogenesis in rumen epithelium is confined solely to the mitochondrial compartment.

D(-)-3-hydroxybutyrate dehydrogenase activity is required for the last step in production, and first step in utilisation, of 3-hydroxybutyrate. Katz and Bergman (1969) reported that D(-)-3-hydroxybutyrate was produced in fed sheep, mainly by the tissues of the alimentary tract (especially the rumen epithelium). Coupled with the observation that the blood ratio of [D(-)-3-hydroxybutyrate]/[acetoacetate] was 4.8, for fed sheep (Chandrasena *et al.*, 1979), this implies that D(-)-3-hydroxybutyrate dehydrogenase plays an important role in maintaining high plasma concentrations of the reduced ketone body.



Acetyl-CoA + Acetoacetate.

Figure 3.3. Proposed Mechanism for Enzymatic Cleavage of Hydroxymethylglutaryl-CoA

The reaction mechanism was adapted from a diagram reported by Stegnik and Coon (1968). The fate of the carbon atoms from acetyl-CoA (*) are also illustrated. Mg^{2+}-Enz-B^- is hydroxymethyl-glutaryl-CoA lyase (EC 4.1.3.4).

The importance of this is because D(-)-3-hydroxybutyrate, *per se*, seems to be non-toxic, but high concentrations of acetoacetate and acetone can produce nervous symptoms and possibly contribute to clinical signs in ailing ruminants (Sollman, 1948). Acetoacetate by itself is in unstable compound and forms acetone and CO_2 by an irreversible and non-enzymatic process (Williamson, 1978b). However, rumen epithelium is reported to have an acetoacetate-decarboxylating enzyme (Seto *et al.*, 1964). The rate of spontaneous decarboxylation of acetoacetate is estimated to be about 5% per h at 37°C (Bergman *et al.*, 1963). In this study similar values were found for breakdown of acetoacetate during incubations *in vitro*.

An indication that acetone formation is by a non-enzymatic process is provided by the entry rates of acetone into normal (<1mg/min) and ketoanaemic sheep (2-6 mg/min) (Lindsay and Brown, 1966). Bergman *et al.* (1963) and Bergman and Kon (1964) have determined the entry rates of acetoacetic acid in normal (0.6-0.9 g/h) and ketoanaemic sheep (3.4 - 10.4 g/h). The entry rate of acetone is about 5% of the entry rate of acetoacetate. This is in good agreement with the rate of spontaneous decarboxylation of acetoacetate.

Initially D(-)-3-hydroxybutyrate dehydrogenase activity was assayed from soluble extracts prepared from rumen epithelium homogenates. It was found, however, that the activity was extremely low. Watson and Lindsay (1972) measured the enzyme activity in the direction of

D(-)-3-hydroxybutyrate formation and reported a value of 0.12 Units per g wet wt. The enzyme was also measured in the opposite direction by Weekes (1974), and a higher activity was recorded (1.0 Unit per g fresh wt.). As Weekes's (1974) assay system contained DL-3-hydroxybutyrate part of the increased activity could be explained by a contribution from L(+)-3-hydroxybutyrate dehydrogenase. Koundakjian and Snoswell (1970) reported that a 'cytosolic 3-hydroxybutyrate dehydrogenase' exists in a number of sheep tissues. It was subsequently shown that the 'cytosolic-enzyme' from sheep kidney reacted with the L(+)-isomer of hydroxybutyrate (Williamson and Kuenzel, 1970).

An explanation for the low activity of the 'soluble' enzyme may be the presence of a specific inhibitor in the extract. A substance has been reported to occur in extracts from rat gastric mucosa (Hanson and Carrington, 1981) that rapidly inactivated D(-)-3-hydroxybutyrate dehydrogenase extracted from other tissues.

A more likely interpretation is that the requirement of D(-)-3-hydroxybutyrate dehydrogenase for mitochondrial membrane phospholipids (Phelps and Hatefi, 1981; Cortese *et al.*, 1982; Eibl *et al.*, 1982) was not met under conditions where the enzyme is solubilised. In the second assay, used for determining D(-)-3-hydroxybutyrate dehydrogenase activity (Method B), the higher value may be a direct result of not centrifuging the enzyme sample before

measuring activity. Using this procedure the necessary phospholipids needed for expression of full enzymatic activity may be included in the sample used for the assay.

Although a large proportion of HMG-CoA synthase and acetoacetyl-CoA thiolase activity appears in the mitochondrial compartment there seemed to be a significant percentage of both these enzymes associated with the cytoplasmic fraction (table 3.3). The inference made from this observation is that rumen mucosa possesses an extra mitochondrial pathway capable of generating some acetoacetyl-CoA and HMG-CoA. Cytoplasmic HMG-CoA lyase is commonly found in tissues which produce cholesterol (Clinkenbeard *et al.*, 1975b).

3.4.3. *Other Enzymes Present in Rumen Epithelium*

In rumen mucosa transfer of acetyl-CoA from the mitochondrial space to the cytoplasm is required as rumen epithelial acetyl-CoA synthetase activity is reported to be confined to the former compartment (Cook *et al.*, 1969). The permeability of the mitochondrial membrane to CoA and its derivatives is very small and it is generally accepted that acetyl-CoA is generated in the cytosol by operation of the following pathway: in the mitochondria acetyl-CoA and oxaloacetate condense to form citrate, this reaction being catalysed by citrate synthase. Citrate is translocated across the mitochondrial membrane where it is cleaved by pro-(S)-ATP: citrate lyase to yield acetyl-CoA and oxaloacetate in the cytoplasm. Pro-(S)-ATP:

citrate lyase is considered to be located in the cytosol (Srere, 1961) but recent evidence suggests that it is associated with mitochondrial membranes (Janski and Cornell, 1980; 1982).

If ketogenesis were to take place in the cytoplasm then maintenance of an acetyl-CoA pool in this space must be achieved through this pathway. Experiments with (-)-hydroxycitrate, an inhibitor of pro-(S)-ATP: citrate lyase (Sullivan *et al.*, 1977), revealed no decrease in butyrate-dependent ketogenesis in rumen epithelium when (-)-hydroxycitrate was added (see Table 3.4).

The relatively low activity of pro-(S)-ATP: citrate lyase would also curtail the operation of a cytoplasmic ketogenic route. Young *et al.* (1969) reported that lyase activity was low in rumen epithelium taken from steers (range, 0.1 to 0.3 Units per g wet wt.). Neither starvation for 8 days for feeding the cattle on a concentrate diet, for three months prior to slaughtering, produced a significant change in lyase activity.

Metabolism of propionate, through the methyl malonyl-CoA pathway, will result in formation of succinyl-CoA as an intermediary metabolite. Synthesis of lactate from this metabolite may take place through one, or more, of the following routes (see Fig. 3.4); decarboxylation of malate catalysed by NADP-'Malic' enzyme, conversion of oxaloacetate to phosphoenol pyruvate and subsequently to pyruvate, or spontaneous decarboxylation of oxaloacetate to pyruvate. In each case the pyruvate formed is reduced to lactate.

	Ketone Body Synthesis	[D-(-)-3-hydroxybutyrate] /[acetoacetate] Ratio
Control	23.2 (2.0)	0.17 (0.07)
Butyrate	219.1 (9.4)	0.33 (0.2)
Butyrate (-)-Hydroxycitrate	220.3 (0.2)	0.42 (0.26)

Table 3.4. Effect of (-)-Hydroxycitrate on Ketogenesis in Rumen Epithelium

Strips of sheep rumen epithelium were incubated with 12mM-n-butyrate for 2h at 39°C. Additional flasks contained (-)-hydroxycitrate (2mM). Acetoacetate and D(-)-3-hydroxybutyrate released into the incubation medium were measured as described in Sections 2.18.2 and 2.18.9. Results are given as average values of means from two separate experiments with the range between the means given in parentheses. Ketone body synthesis is reported in terms of µmol/g dry wt. per 2h.

Young *et al.* (1969) have assayed phosphoenolpyruvate carboxykinase (PEPCK) activity and NADP-linked 'malic' enzyme activity in homogenates prepared from bovine rumen mucosa. The latter enzyme was first demonstrated to be present in bovine rumen epithelium in a histochemical study carried out by de Lahunta (1965).

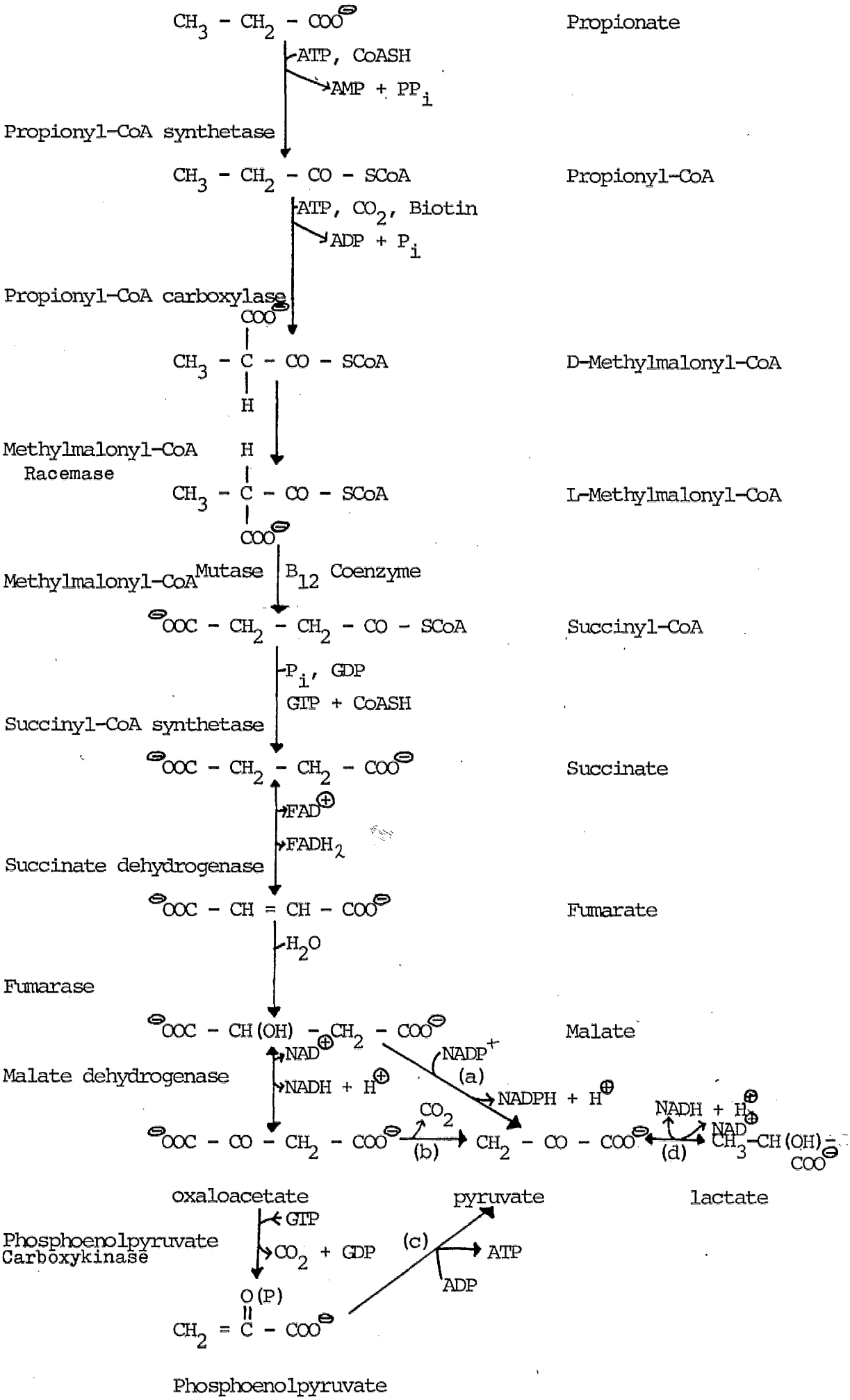
It is proposed that NADP-'malic' enzyme plays a major role in conversion of propionate to lactate in rumen epithelium. This suggestion is based on findings that homogenates of this tissue contain activities of NADP 'malic' enzyme about seven-fold higher than PEPCK activities.

It was found that the enzyme activities differed by about five-fold for ovine rumen mucosa, but PEPCK activity appeared to be confined mainly to the mitochondrial space. This compartmentation of enzymes may play a role in determining the pathway of propionate metabolism. Therefore proposals for metabolic pathways based on ratios of enzyme activities must be treated with caution.

Most of the rat intestinal mucosa PEPCK activity was associated with the particulate fraction (89%), with very little activity being found in the cytoplasm (Anderson, 1970). A suggestion for this activity is that during the neonatal period gluconeogenesis may occur in the small intestine epithelial tissues because PEPCK activity was observed to be high in enteric tissues of suckling rats and mice (Hahn and Smale, 1982). PEPCK activity decreased to a low value after weaning.

FIG. 3.4. Possible Pathways of Propionate Metabolism to L-Lactate

a, NADP 'malic'-enzyme; b, spontaneous decarboxylation; c, pyruvate kinase; d, lactate dehydrogenase.


CH3 - CH2 - COO⊖
Propionate
ATP, CoASH
AMP + PPi
Propionyl-CoA synthetase
CH3 - CH2 - CO - SCoA
Propionyl-CoA
ATP, CO2, Biotin
ADP + Pi
Propionyl-CoA carboxylase
COO⊖
CH3 - C - CO - SCoA
H
D-Methylmalonyl-CoA
Methylmalonyl-CoA Racemase
H
CH3 - C - CO - SCoA
COO⊖
L-Methylmalonyl-CoA
Methylmalonyl-CoA Mutase
B12 Coenzyme
⊖OOC - CH2 - CH2 - CO - SCoA
Succinyl-CoA
Pi, GDP
GTP + CoASH
Succinyl-CoA synthetase
⊖OOC - CH2 - CH2 - COO⊖
Succinate
FAD⊕
FADH2
Succinate dehydrogenase
⊖OOC - CH = CH - COO⊖
Fumarate
H2O
Fumarase
⊖OOC - CH(OH) - CH2 - COO⊖
Malate
NAD⊕
NADH + H⊕
Malate dehydrogenase
NADP+
(a)
NADPH + H⊕
⊖OOC - CO - CH2 - COO⊖
CO2
(b)
CH2 - CO - COO⊖
NADH + H⊕
NAD⊕
(d)
CH3-CH(OH)-COO⊖
oxaloacetate
pyruvate
lactate
GTP
CO2 + GDP
Phosphoenolpyruvate Carboxykinase
(c)
ATP
ADP
O(P)
CH2 = C - COO⊖
Phosphoenolpyruvate

It is a possibility that gluconeogenesis occurs in rumen epithelium of neonatal and newborn lambs. Glucose synthesised could have been stored as glycogen in this tissue. For example, the rumen epithelium glycogen contents of a three month old foetus, one week old lamb and adult sheep were 15.5, 10.8 and 0.14mg/g wet wt., respectively. Glycogen content in the liver of adult sheep are about 46mg/g wet wt. (Emmanuel, 1980). The observed PEPCK activity in rumen epithelium of adult animals, observed in this study, could be assigned as residual activity from when the enzyme was more active in the neonatal period.

The absence of a gluconeogenic pathway in rumen epithelium from fully developed animals is supported by the observation that the key enzymes fructose-1,6-bis-phosphatase (EC 3.1.2.11) has a very low activity in this tissue (Bush, 1982). In this study it was found that less than 0.2% of ^{14}C-labelled propionate was incorporated into the glucose fraction when isolated rumen papillae were incubated in the presence of [1-^{14}C] propionate with and without glucose (see section 3.3.3).

3.5. *SUMMARY*

A method for the fractionation of ovine rumen epithelium with limited mitochondrial breakage is described. Using marker enzymes, 3-hydroxy-3-methylglutaryl-CoA lyase and D(-)-3-hydroxybutyrate dehydrogenase are shown to be located exclusively in the mitochondrial fraction, indicating that ruminal ketogenesis is exclusively mitochondrial too. The failure of (-)-hydroxycitrate to block ketogenic flux supports this view. D(-)-3-hydroxybutyrate dehydrogenase activity is largely associated with particulate material in the mitochondrial fraction. Appreciable acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase activity is found in the cytoplasmic fraction.

ATP citrate lyase activity, although low, is measurable and confined to the cytoplasmic compartment. $NADP^+$ 'malic' enzyme activity is about five times greater than phosphoenolpyruvate carboxykinase activity. However, these two enzymes are found in the cytoplasmic and mitochondrial fractions respectively.

C H A P T E R 4

INFLUENCES ON RUMEN MUCOSAL KETOGENESIS

4.1. *Introduction*

The presence of acetoacetate (Gerhardt, 1865), D(-)-3-hydroxybutyrate (Kulz, 1884) and acetone (Lusk, 1909) in the urine of diabetic patients was discovered over 70 years ago. It was subsequently demonstrated that the liver is the major organ for ketone body synthesis (Fischler and Kassow, 1913). This demonstration and other evidence led to the proposal that the liver produced ketone bodies to supply alternative, but readily usable, metabolic fuels for extra hepatic tissues (MacKay, 1943). In many extrahepatic tissues ketone bodies are used preferentially to glucose. Ketone bodies are also recognised as lipogenic precursors and as metabolic regulators.

Ruminants have a second primary site for ketogenesis, namely the rumen epithelium. The glucose sparing effect produced in ruminant tissues that utilise ketone bodies helps to compensate for the lack of any significant glucose uptake from the alimentary tract *in vivo*.

Butyrate has deleterious effects in many biological systems both *in vivo* and *in vitro* (see Chapter 1). However, ruminal butyrate is extensively metabolised on passage through the rumen wall, the major end products of this metabolism are ketone bodies. It is clear, therefore, that metabolism of butyrate in rumen epithelium makes a significant contribution to ruminant whole body metabolism.

In this chapter experimental results are reported on the effects of physiological substrates used by rumen epithelium, potential inhibitors of ketogenesis and hormones on butyrate-dependent ketogenesis in isolated rumen papillae.

4.2. Materials and Methods

Procedures for incubation of rumen epithelium *in vitro* and details of metabolite assays are described in Chapter 2. Initially metabolites were determined with respect to the dry wt. of the tissue but estimation of this variable for each incubation proved to be time-consuming. For convenience, DNA content in the tissue was measured in later experiments. DNA content was also estimated in relation to the dry wt. of rumen epithelium and a mean value (± S.E.M.) of 27.6 (± 2.6) mg DNA per g dry wt. was obtained for 13 separate determinations. Throughout the rest of this thesis results are reported in terms of either mg DNA or g dry wt.

4.3.1. Ketone Body Synthesis in Rumen Epithelium

The apparent K_m values for ketone body production from butyrate and acetate were determined before studying any aspects of rumen epithelial ketogenesis. Using a titration of butyrate from 0.5 to 20mM (the physiological range found in rumen liquor) the apparent K_m was estimated to be 12mM (see Fig. 4.1). Although acetate was a poor precursor, when compared to butyrate, for ketogenesis in rumen mucosa an

apparent K_m value of 50mM was determined (see Fig. 4.2). The much lower rate of ketogenesis from acetate is in keeping with the observation that butyrate uptake into rumen epithelium is 5-9 times greater than acetate uptake (Pennington, 1952).

Ketone body synthesis decreased significantly as the chain-length (C4 or greater) of added fatty acids increased (see Table 4.1). In separate experiments rates of ketone body production were low with palmitate as the sole substrate.

Illustrated on Fig. 4.3 is the time course of ketogenesis in rumen epithelium, with butyrate as the exogenous substrate. After an initial lag phase the time course was linear over a 2h incubation period. Routinely, experiments were carried out over periods of 2h or less. The ketogenic rate varied for different preparations or rumen wall but normally the rates were within the range of 110-140 μmol/g dry wt. per h. Metabolism of butyrate resulted in greater acetoacetate formation (see Fig. 4.3).

Isolated sheets of rumen epithelium, when bathed with a VFA mixture on the papillae side, gave a linear time course for ketone body release into the buffer on the blood side (see Fig. 4.4). However, there was no obvious preferential accumulation of ketone bodies on either side (see Fig. 4.5). Similar observations have been reported by Stevens and Stettler (1966). It was also found that the pH of the lumen buffer (papillae side) increased during the incubation period. The magnitude of this rise ranged from 0.1-0.3 pH units and this observation was made in several experiments.

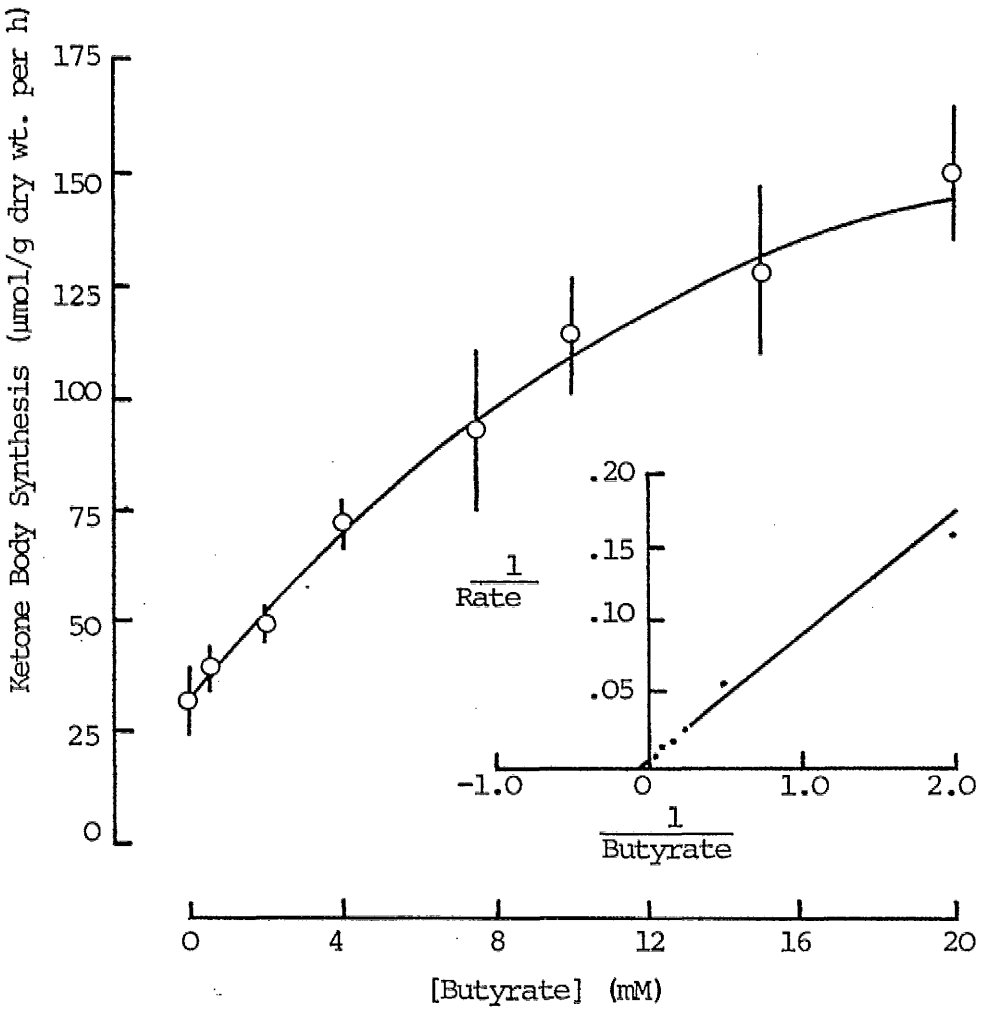


Fig. 4.1. Determination of Ketogenic Apparent K_m value for Butyrate

Rumen epithelial strips were incubated, as described in Section 2.9, with butyrate at different concentrations. The tissue was preincubated for 10 min before addition of the volatile fatty acid. The totals (O) of acetoacetate and D(-)-3-hydroxybutyrate produced by rumen epithelium in the presence of different butyrate concentrations are given in terms of means ± S.D. The insert represents a Lineweaver-Burk plot of the mean values against the reciprocal of the rate of ketone body synthesis. V_{max} and K_m values were 160 µmol/g dry wt. per h. and 12mM, respectively.

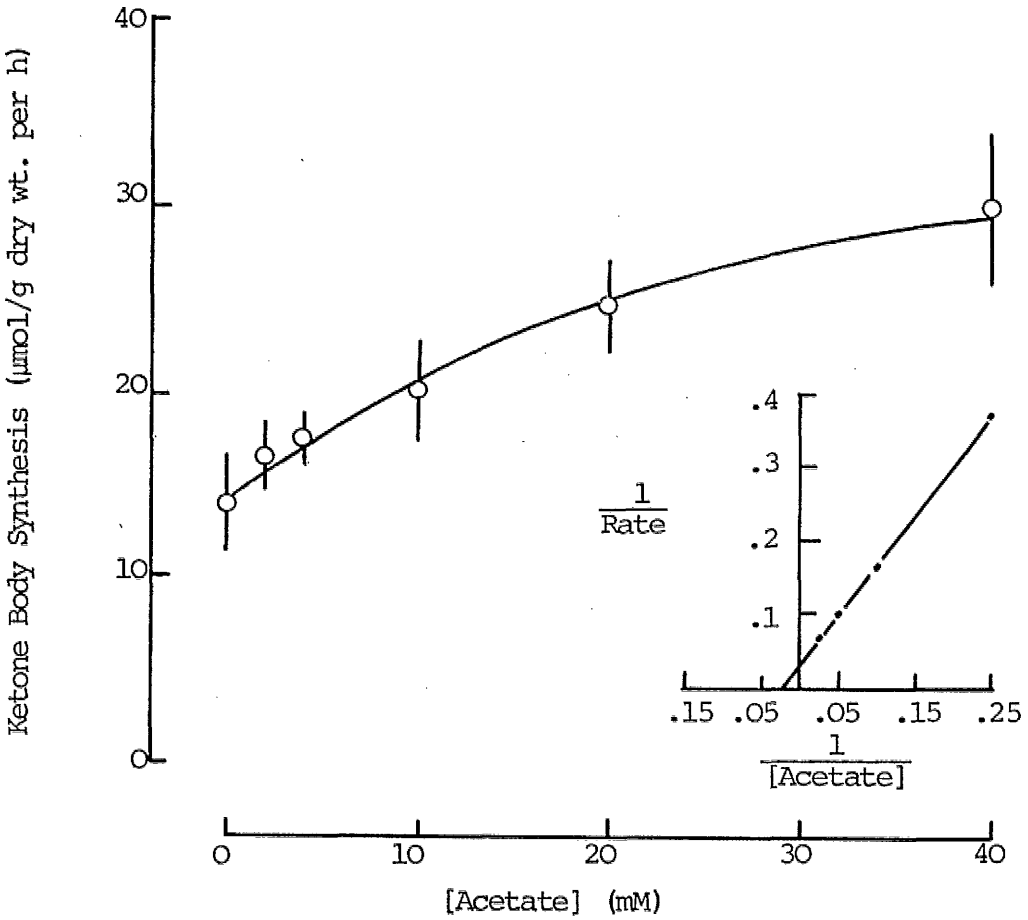


Fig. 4.2. Determination of Ketogenic Apparent K_m value for Acetate

Rumen epithelial strips were incubated and ketone body production was measured, with acetate as the added substrate, as described in the legend to Fig. 4.1. The sums (O) of acetoacetate plus D(-)-3-hydroxybutyrate synthesised by rumen papillae are given as means ± S.D. The insert represents a Lineweaver-Burk plot of the mean values against the reciprocal of the rate of ketone body production. V_{max} and K_m values were 80 µmol/g dry wt. per h and 50mM, respectively.

	Ketone Body Synthesis (μmol/g dry wt. per 2h)	[D(-)-3-hydroxybutyrate]/ [Acetoacetate] Ratio
Butyrate (12mM)	160.8 ± 9.8	0.17 ± 0.05
Octanoate (1mM) plus BSA (0.6%; w/v)	82.0 ± 30.5	0.11 ± 0.05
Oleate (1mM) plus BSA (0.6%; w/v)	44.2 ± 22.3	0.13 ± 0.06
Control	30.4 ± 5.0	0.10 ± 0.04
BSA (0.6%; w/v)	31.3 ± 8.2	0.46 ± 0.10

Table 4.1. Production of Ketone Bodies from Long-Chain Fatty Acid by Rumen Papillae

Rumen epithelium was incubated with long-chain fatty acids (bound to bovine serum albumin (BSA); Garland and Randle, 1964). Ketone bodies released by the tissue were measured as described in Section 2.17. The results are means ± S.D. for triplicate incubations.

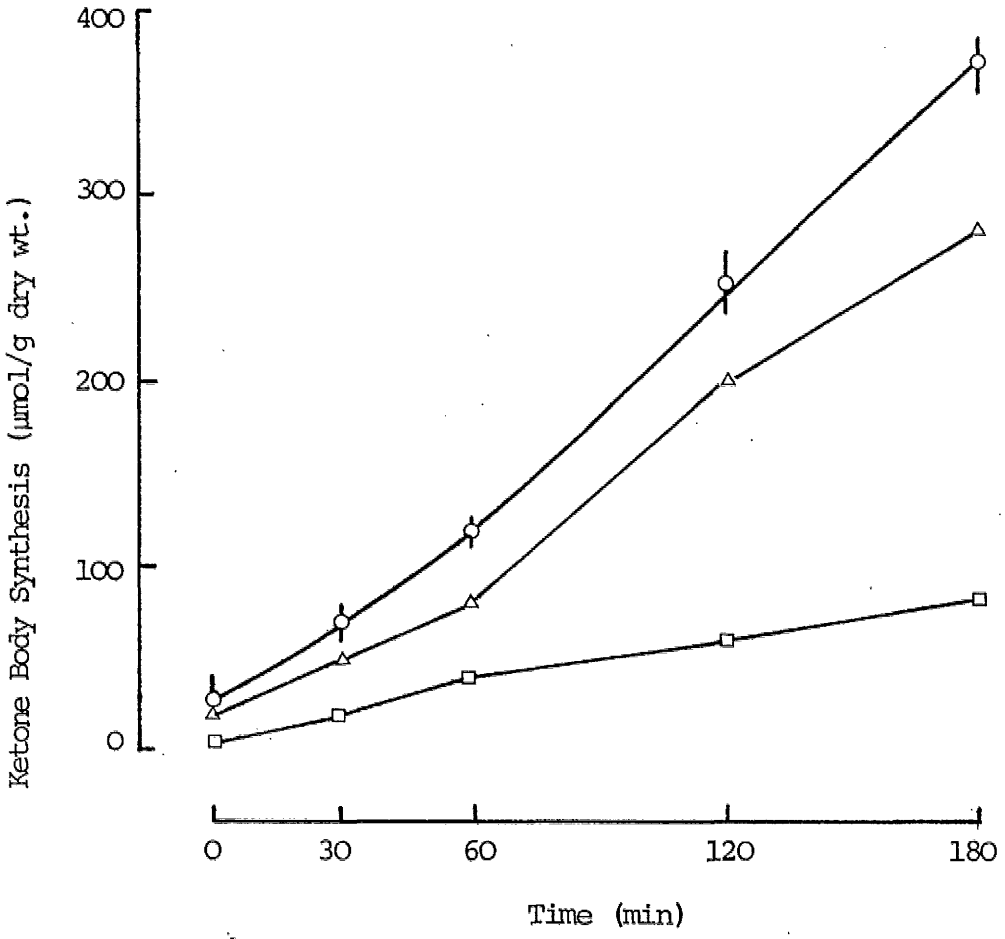


Fig. 4.3. Time Course of Rumen Epithelial Ketogenesis

Rumen epithelium was incubated with butyrate (12mM) as described in section 2.9. At the indicated times incubations were terminated by addition of 0.37M-$HClO_4$. A sample of the neutralised medium was assayed for acetoacetate (△) and D(-)-3-hydroxybutyrate (□). Mean values (○) ± S.E.M. of the totals for ketone body synthesis for four separate experiments are also given.

Fig. 4.4. Ketone Body Accumulation in the Solution Bathing and Blood Side

These are results from a representative experiment whereby strips of rumen epithelium were fixed on to one end of a test tube (open at both ends), with the papillae facing outwards into the buffer in the incubation flask (see Fig. 2.2). Modified-Krebs buffer was placed in the test tube and the incubation was started. Samples were taken from the test tube during the incubation and acetoacetate (O) and D(-)-3-hydroxybutyrate (●) released from the tissue were assayed as described in section 2.18. The incubation buffer (pH 7.0) contained acetate (65mM) propionate (20mM) and butyrate (15mM). Results are means ± S.D. for triplicate incubations

Fig. 4.5. Percentage Ketone Body Accumulation in Both Bathing Solutions

This diagram represents the percentage distribution of acetoacetate and D(-)-3-hydroxybutyrate in the blood side (△ and □ , respectively) and lumen side (▲ and ■, respectively) buffers. Values are percentage results of the total ketone body concentrations in both buffers at the end of the experiment (150 min). Results are means ± S.D. for triplicate incubations.

FIG. 4.4

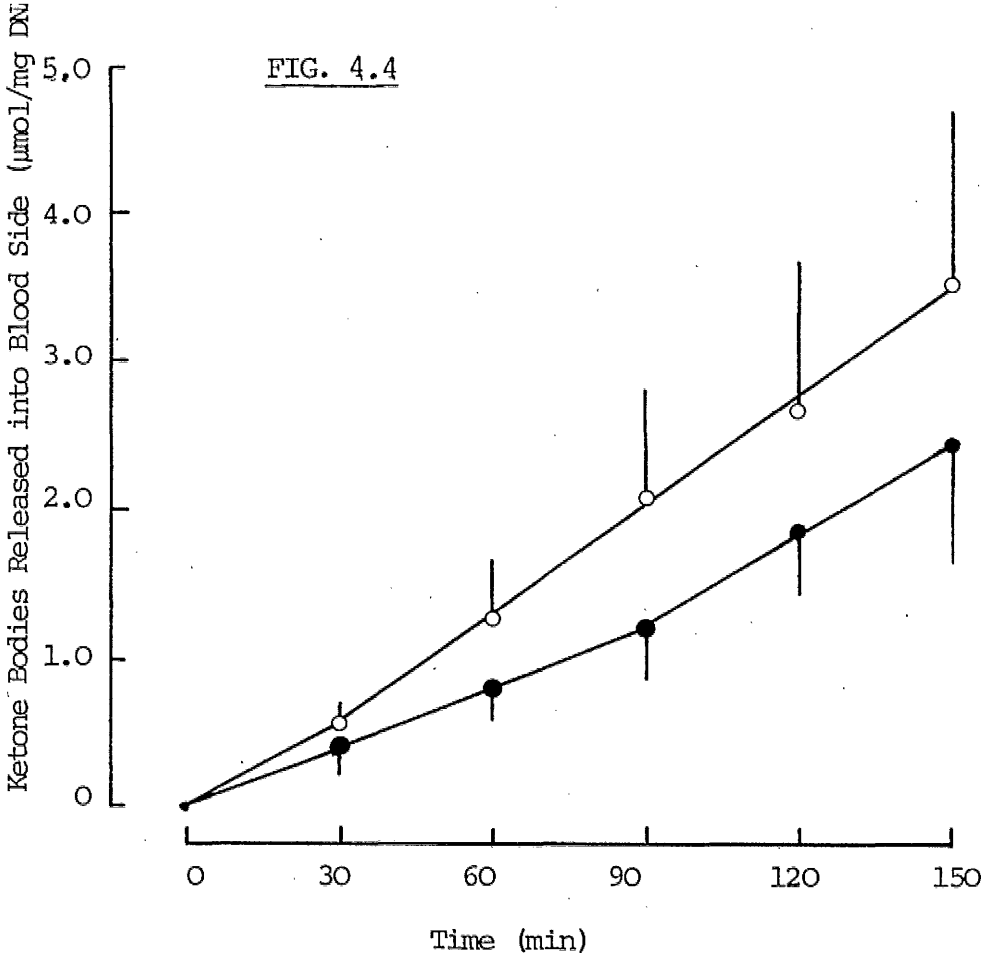
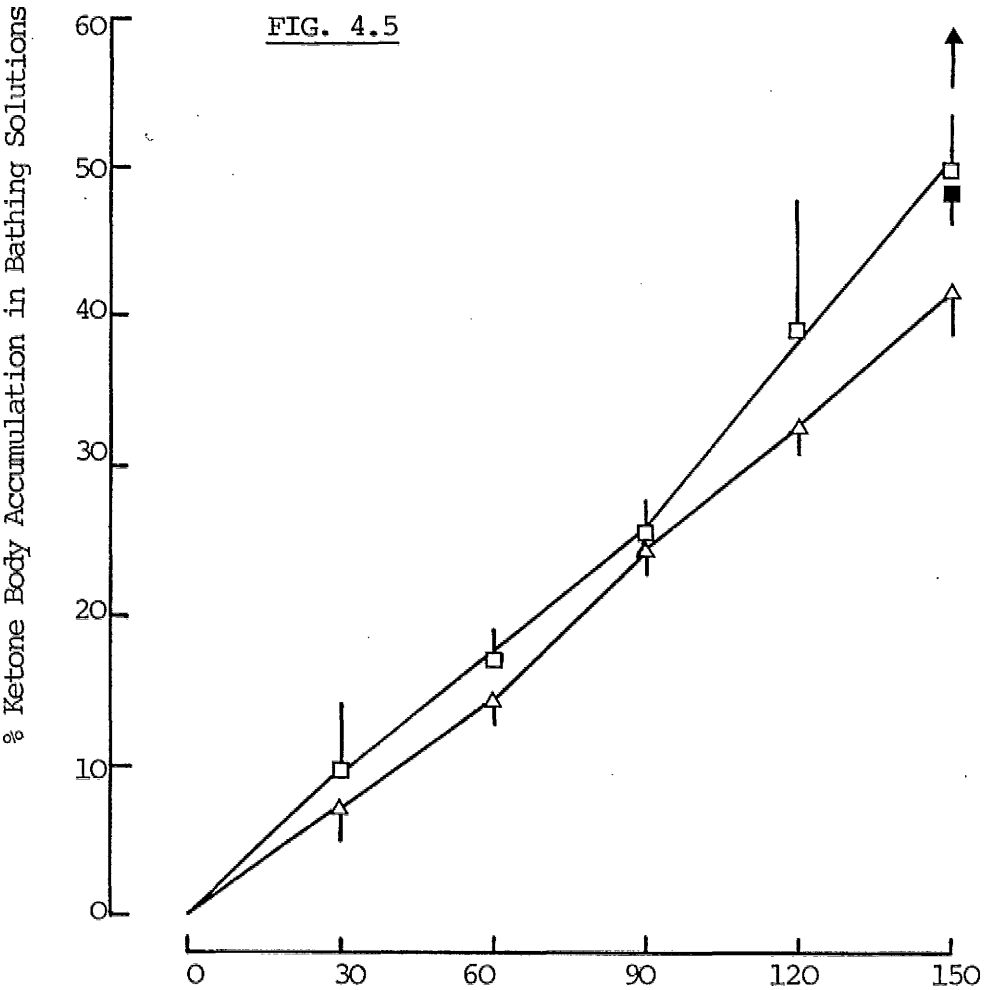

Ketone Bodies Released into Blood Side (µmol/mg DW
5.0
4.0
3.0
2.0
1.0
0
0
30
60
90
120
150
Time (min)

FIG. 4.5


% Ketone Body Accumulation in Bathing Solutions
60
50
40
30
20
10
0
0
30
60
90
120
150

4.3.2. *Cell Viability During Incubations In Vitro*

For other cell preparations ATP content is used as an indicator of viability during incubations *in vitro* (Dickson and Pogson, 1977). Rumen papillae incubated with either butyrate or propionate maintained their ATP content over a 2h incubation period. Acetate produced a decrease in rumen epithelial ATP content over the same period (see Table 4.2 and Fig. 4.6). In separate experiments sodium acetate replaced an equivalent amount of NaCl in the buffer but the ATP content was lowered by about the same magnitude observed in the original experiment (results not shown).

4.3.3. *Influences on Ketogenesis in Rumen Epithelium*

4.3.3.1. *Effect of Calcium Ions*

Ontko and Otto (1978) have shown that Ca^{2+} produced an increase in ketone body synthesis in isolated liver cells. Also, the activity of the HMG-CoA pathway in rat-liver mitochondria is reported to be stimulated by Ca^{2+} (Mulder and Van den Bergh, 1981). However, no change in rumen epithelial ketogenesis was observed when rumen papillae were incubated in the presence of a range of Ca^{2+} concentrations (see Fig. 4.7). The [D(-)-3-hydroxybutyrate]/[acetoacetate] ratio (an indicator of the mitochondrial redox state) increased two-fold when Ca^{2+} concentrations in the medium were greater than 0.5mM. A similar increase in this ratio is observed in isolated rat hepatocytes incubated with 1mM-$CaCl_2$ (Ontko and Otto, 1978).

Time (min)	Cellular Adenosine Triphosphate Content (nmol/mg DNA)			
	CONTROL	ACETATE (60mM)	PROPIONATE (15mM)	BUTYRATE (12mM)
0	102 ± 22	-	-	-
30	126 ± 37	70 ± 25	97 ± 37	89 ± 29
60	99 ± 21	44 ± 15	100 ± 34	109 ± 28
120	110 ± 25	52 ± 13	113 ± 30	123 ± 31

TABLE 4.2. Effect of Volatile Fatty Acids on Rumen Epithelial Adenosine Triphosphate Concentrations

Incubation of rumen papillae with volatile fatty acids and measurement of ATP and DNA content, after homogenisation of the tissue, was as described in Chapter 2. The mean values ± S.E.M. for three separate experiments are given. The final concentrations of the volatile fatty acids are also indicated in parentheses.

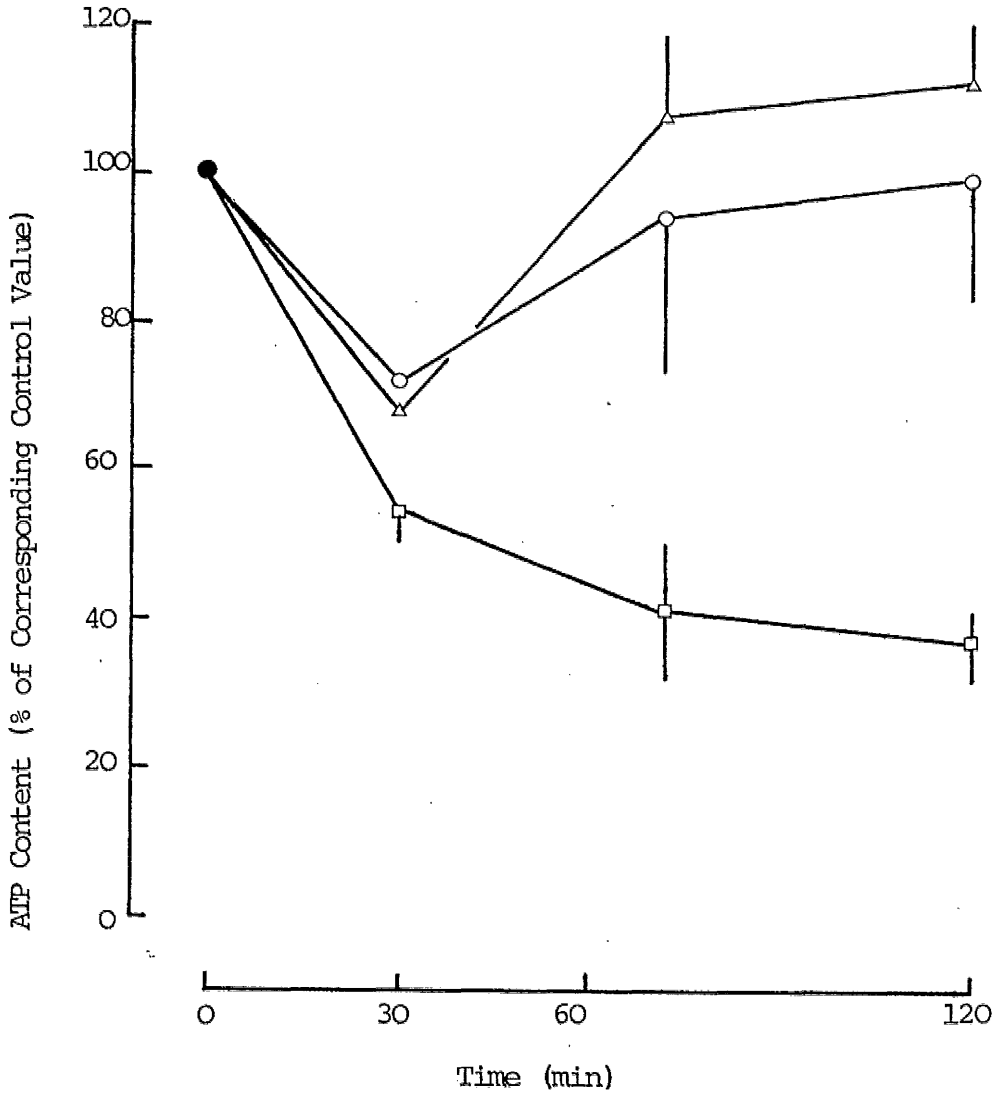


Fig. 4.6. Adenosine Triphosphate Content in Sheep Rumen Epithelium

The results from Table 4.2, are presented in terms of percentage values of the relative control time points. Values are given as means ± S.E.M. for incubations containing acetate (□ ; 60mM), propionate (o ; 15mM) or butyrate (△ ; 12mM).

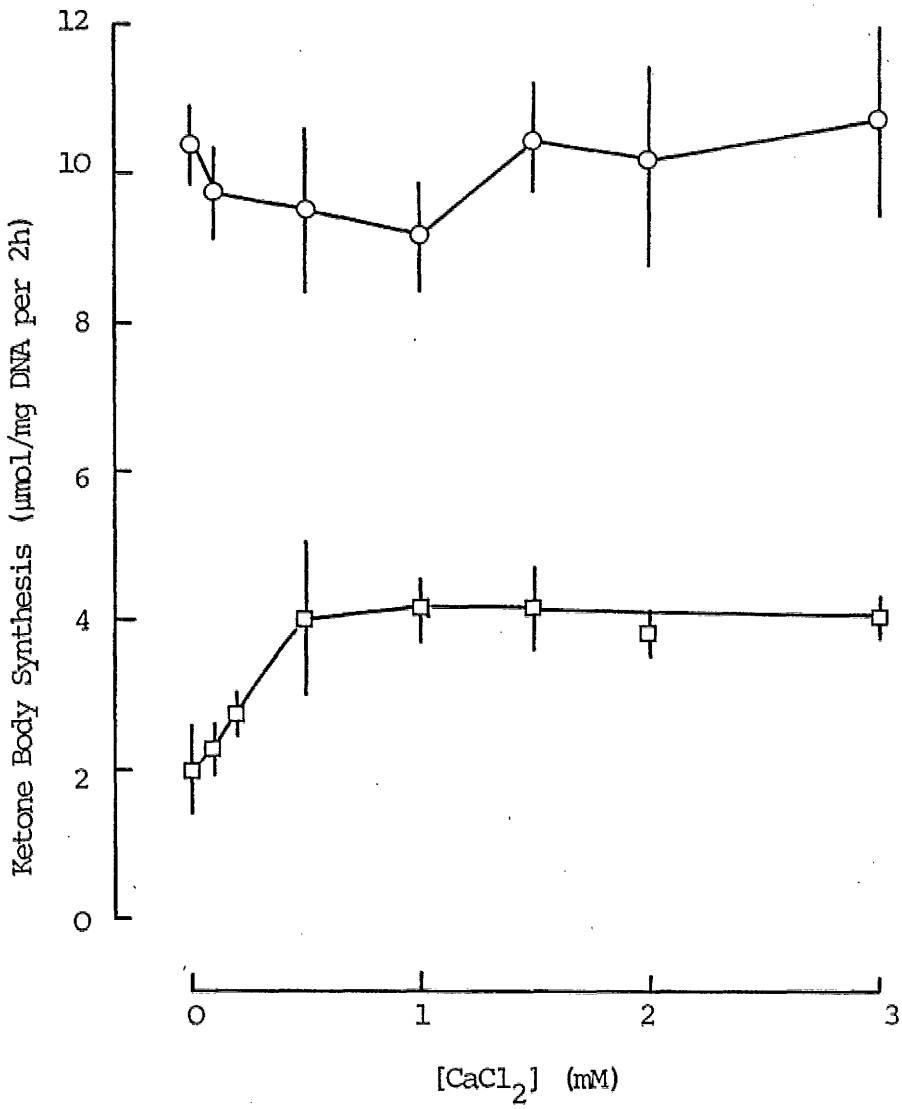


Fig. 4.7. Effect of Calcium on Ketogenesis

Isolated papillae were incubated in the presence of a range of Ca^{2+} concentrations. After a 2h incubation period the ketone bodies released into the medium (O) were measured as described in Section 2.17. The ratio of [D(-)-3-hydroxybutyrate]/[acetoacetate] is given too (□).

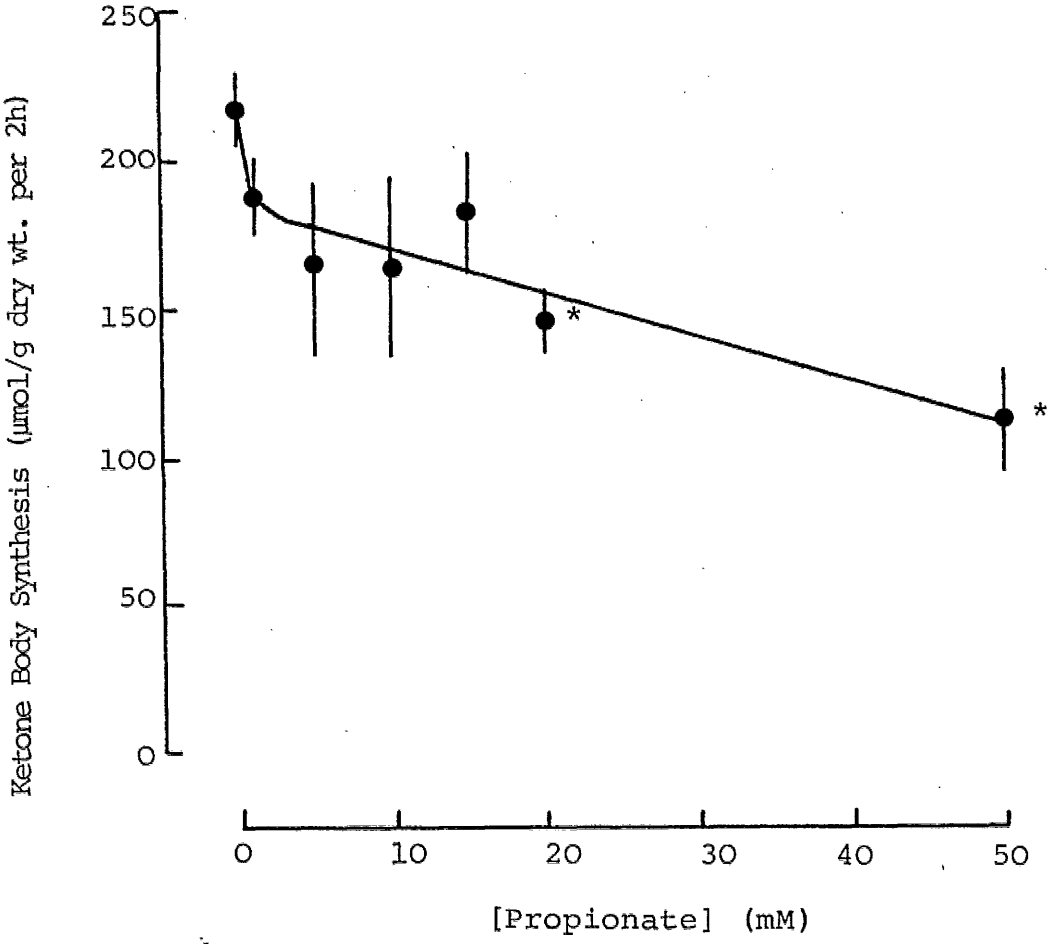


Fig. 4.8. Effect of Propionate on Ketone Body Production in Sheep Rumen Epithelium

Rumen epithelial strips were incubated with butyrate (12mM) plus various propionate concentrations. After a 2h experimental period the incubation medium was assayed for ketone bodies as described in section 2.17. The sums of acetoacetate and D(-)-3-hydroxybutyrate released (minus endogenous production) are given in terms of means ± S.E.M. for three independent experiments; *, $P < 0.05$.

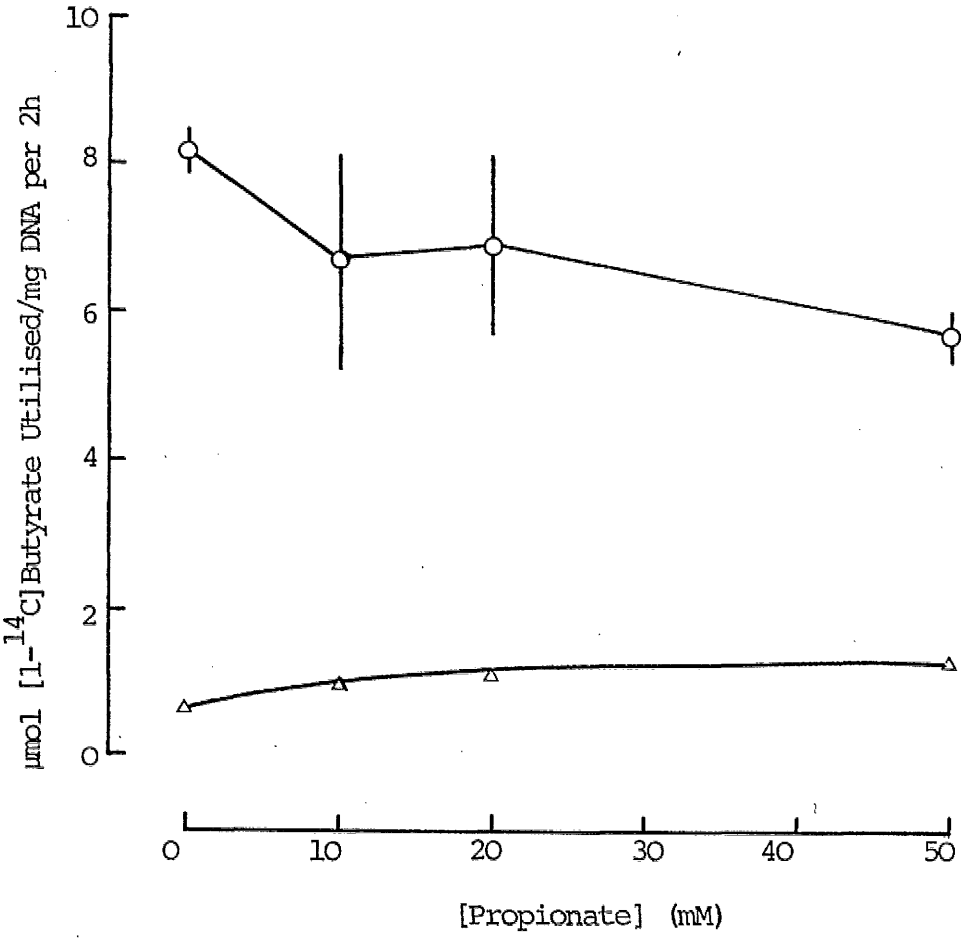


Fig. 4.9. Metabolism of [1-^{14}C]Butyrate. Effect of Propionate

Isolated rumen papillae were incubated with [1-^{14}C]butyrate (12mM) and ^{14}C-labelled acetoacetate produced by the tissue was measured as described in section 2.14. Assuming that acetoacetate and D(-)-3-hydroxybutyrate are in isotopic equilibrium, the radioactivity of ketone bodies (o) was calculated using the radioactivity in acetoacetate and the ratio of the content of D(-)-3-hydroxybutyrate and acetoacetate. The rates of [1-^{14}C]butyrate oxidation to $^{14}CO_2$ are also given (Δ). All results are presented as the average mean values of two separate experiments plus the range between mean values.

4.3.3.2. *Effects of Volatile Fatty Acids*

In preliminary experiments acetate and propionate produced opposite effects on butyrate-dependent ketogenesis. Ketone body synthesis increased slightly with acetate as the secondary substrate, though this was likely to be an additive effect as acetate is a ketogenic substrate (see Fig. 4.2). Endogenous and butyrate-dependent ketogenesis were both inhibited by propionate.

The propionate effect was investigated further as ketogenesis in bovine and ovine rumen epithelium is reported to be insensitive to propionate (Seto *et al.*, 1955; Pennington and Pfander, 1957; Bush *et al.*, 1970). These experiments confirmed that propionate was antiketogenic, especially when added to medium at concentrations about two-fold or higher than the butyrate concentration (see Fig. 4.8).

As propionate inhibited endogenous ketogenesis (which made an appreciable contribution to the butyrate-dependent ketogenesis) experiments were carried out using [1-^{14}C]butyrate as an exogenous substrate. As Fig. 4.9 shows, a similar pattern of inhibition was observed for the production of ^{14}C-labelled ketone bodies indicating that the butyrate-dependent ketogenesis was being inhibited. Propionate increased the rate of butyrate oxidation too (see Fig. 4.9).

A statistically significant reduction in the mitochondrial redox state was also produced by propionate (see Fig. 4.10), assuming that D(-)-3-hydroxybutyrate dehydrogenase shared a common mitochondrial NAD^+ pool (Siess *et al.*, 1978). Also, there appeared to be a good correlation between the mitochondrial redox state and the rate of lactate production. Lactate is a major end-product of propionate metabolism. Furthermore, because the pyruvate content of rumen papillae was usually low the lactate content was assumed to reflect the cytoplasmic redox state. The good correlation between the redox states in the two subcellular compartments may imply that very effective substrate shuttling systems exist in the mitochondrial membranes to transport reducing equivalents from the mitochondria to the cytoplasm or *vice versa*. Alternatively, metabolism of propionate in rumen epithelium may generate reducing equivalents in both subcellular compartments.

Rat liver mitochondrial ketogenesis is reported to be strongly influenced by the $NADH/NAD^+$ ratio (Huth *et al.*, 1978). Considering the effects that propionate has on this ratio and ketogenesis in rumen epithelium similar mechanisms may also operate in this tissue. To test this hypothesis methylene blue, which decreases the cellular $NADH/NAD^+$ ratio (Buse *et al.*, 1980), was added to rumen papillae incubated with propionate or butyrate. It was found that methylene blue decreased both the mitochondrial and cytoplasmic $NADH/NAD^+$ ratios but did not produce any obvious change in the inhibition of butyrate-dependent ketogenesis. [1-^{14}C]-

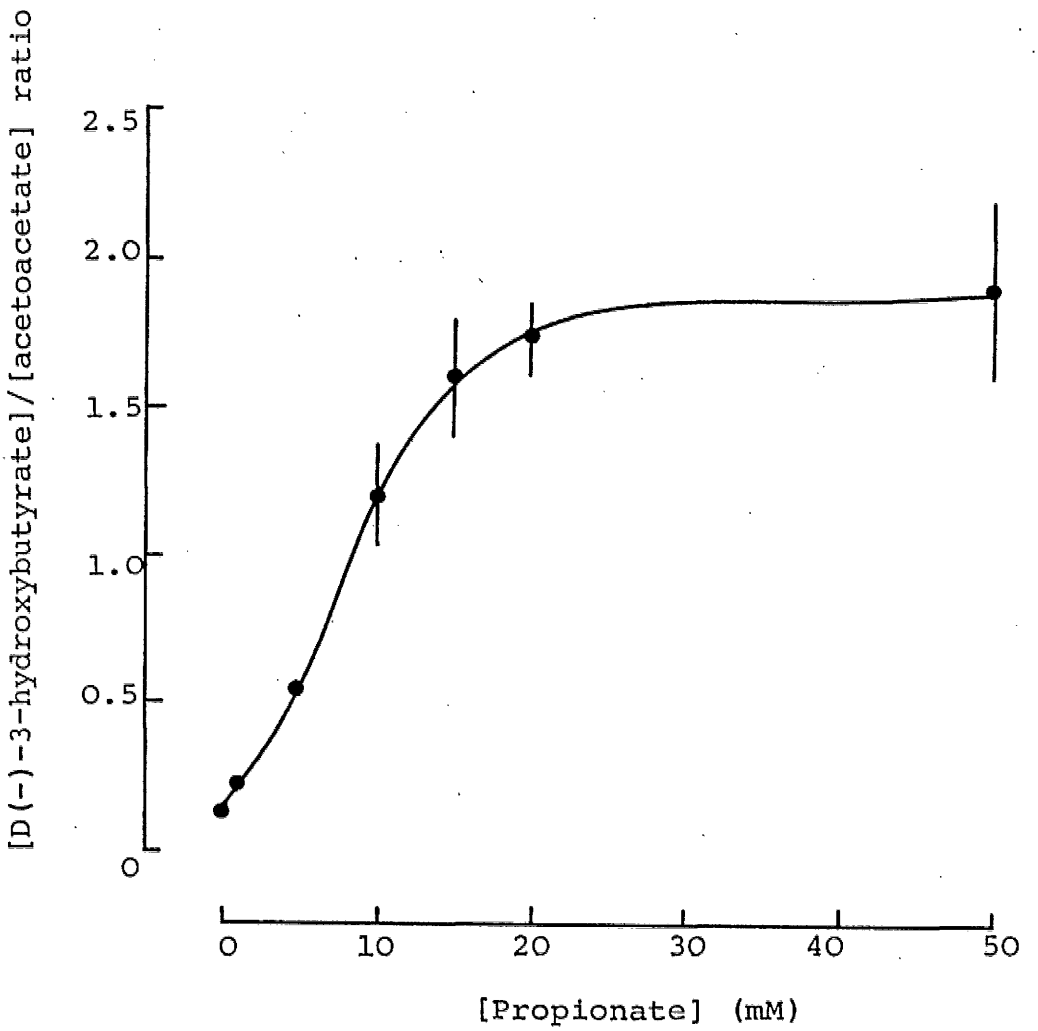


Fig. 4.10. Effect of Propionate on Rumen Epithelial Intramitochondrial Redox State

Details of incubation conditions and measurement of metabolites released by rumen papillae are given in the legend to Fig. 4.8. Ratios are expressed in terms of means ± S.E.M. for three separate experiments.

Additions	$^{14}CO_2$	[Lactate]/ [Pyruvate]	^{14}C-Labelled Ketone Bodies	[D(-)-3-Hydroxybutyrate]/ [Acetoacetate]
[1-^{14}C]Butyrate	413 (14)	10.8 (3.6)	3556 (49)	0.16 (0.03)
[1-^{14}C]Butyrate + Methylene Blue	362 (82)	9.0 (3.1)	3267 (148)	0.10 (0.01)
[1-^{14}C]Butyrate + Propionate	784 (137)	27.7 (13.1)	2154 (264)	0.84 (0.27)
[1-^{14}C]Butyrate, Propionate + Methylene Blue	839 (320)	18.5 (14.8)	1966 (188)	0.38 (0.08)

Table 4.3. Influence of Methylene Blue on Rumen Epithelial Ketogenesis

Rumen epithelium was incubated with [1-^{14}C]butyrate (5mM). The end products of metabolism, $^{14}CO_2$ and ^{14}C-labelled ketone bodies, were measured as described in sections 2.13 and 2.14. The effects of propionate (10mM) and methylene blue (0.2mM) on keotgenesis were also determined. Results are presented as average values between two separate experiments, with the range between means given in parentheses. $^{14}CO_2$ and ^{14}C-labelled ketone body production rates are given in terms of nmol/mg DNA per h.

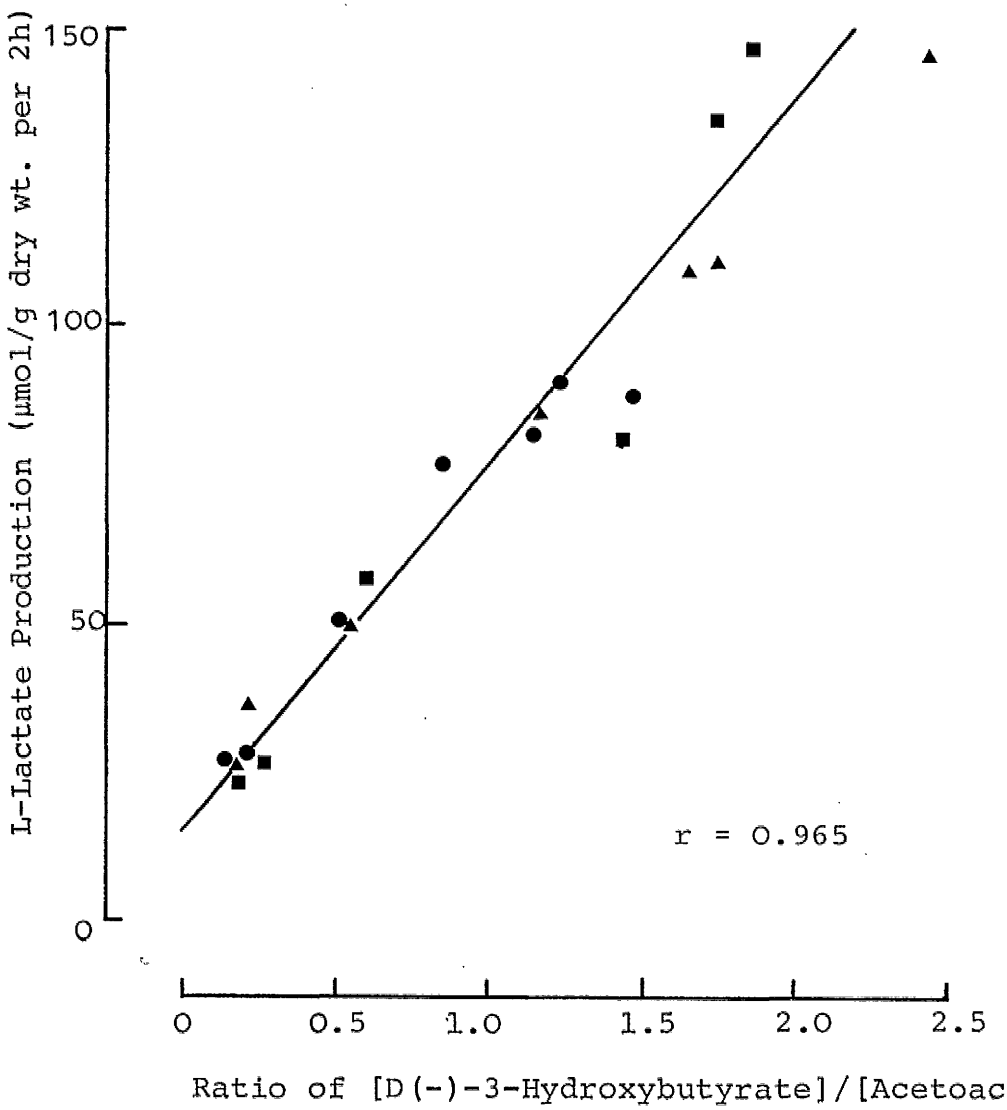


Fig. 4.11. A Plot of [D(-)-3-Hydroxybutyrate]/[Acetoacetate] Ratios versus Rates of L-Lactate Production

Rumen epithelium was incubated as described in the legend to Fig. 4.8. Metabolites were assayed as described in section 2.17. Each point is the mean value of triplicate incubations and the results are drawn from three separate experiments (● , ■ , ▲). The equation for the line is $Y = 54.5X + 18.6$.

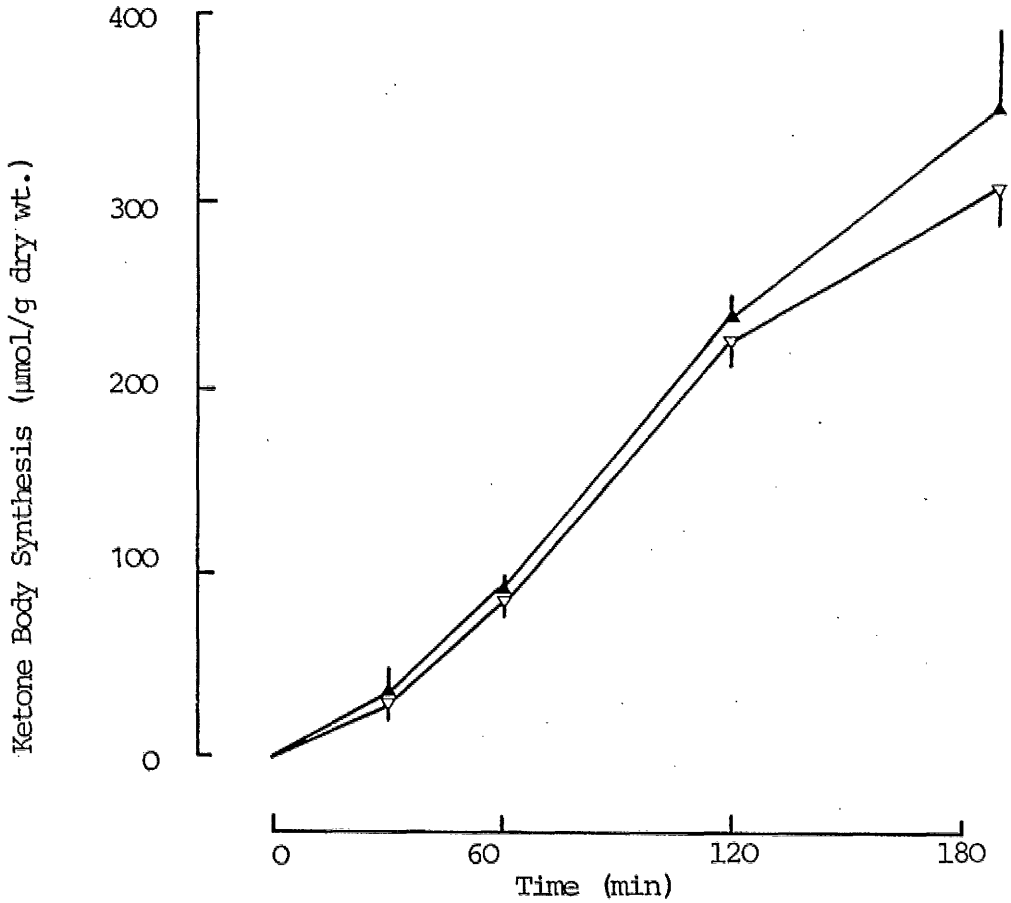
FIG. 4.12. Effect of Glucose on Ketogenesis in Rumen Epithelium

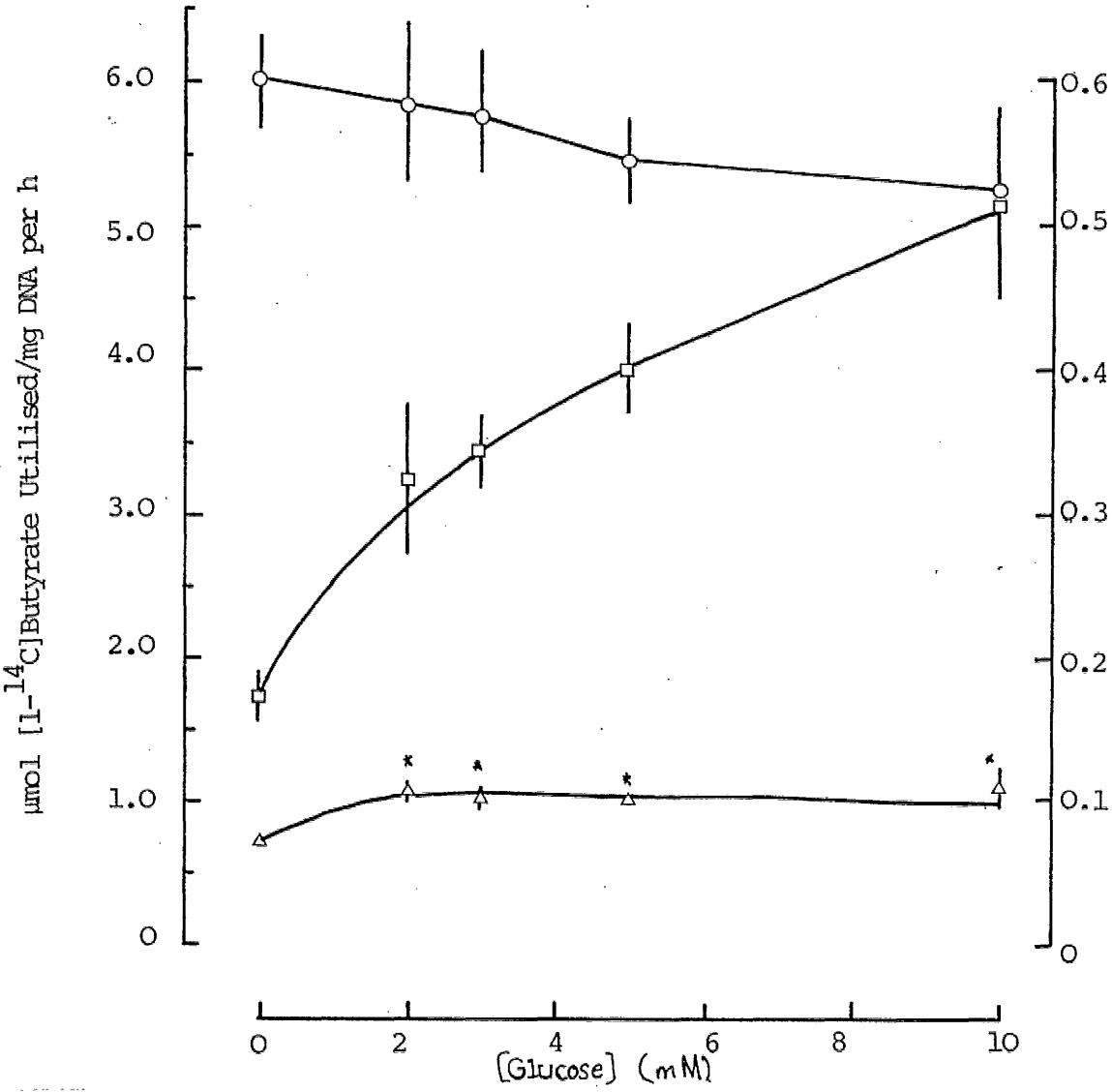
(a) Top - Time Course of Possible Glucose Effect on Ketogenesis in Rumen Mucosa

Strips of rumen epithelium were incubated with n-butyrate (12mM) (▲) plus glucose (5mM) (▽). Ketone bodies released into the incubation medium were measured as described in the legend to Fig. 4.7. Results are given as means ± S.D.

(b) Bottom - The Effect of Concentration of Glucose on ^{14}C-labelled Ketone Body Production

Isolated papillae were incubated with 5mM-[1-^{14}C] butyrate plus glucose (2-10 mM) before measuring $^{14}CO_2$ production (△) and accumulation of [^{14}C]-labelled ketone bodies (○) as described in section 2.13 and 2.14. The [D(-)-3-hydroxybutyrate]/[acetoacetate] ratios are also given (□). Each result is the mean ± S.D. of triplicate incubations and values that are significantly different, from the appropriate control values, are marked with an asterisk; * $P < 0.01$.


Ketone Body Synthesis (µmol/g dry wt.)
400
300
200
100
0
0
60
120
180
Time (min)


µmol [1-14C]Butyrate Utilised/mg DNA per h
6.0
5.0
4.0
3.0
2.0
1.0
0
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
[Glucose] (mM)

Butyrate oxidation, in the presence or absence of propionate, was unaffected by addition of methylene blue.

4.3.3.3. D-Glucose

Several investigators have reported an increase in butyrate-dependent ketogenesis in rumen epithelium *in vitro* with glucose as an added secondary substrate (Hird and Symons, 1959; Strangassinger *et al.*, 1979; Gieske *et al.*, 1979). However, I found no detectable change in the butyrate-dependent ketogenic rate when sheep rumen papillae were also inucbated with glucose (see Fig. 4.12). This observation agrees with results that are reported by Pennington and Pfander (1957).

Glucose produced a significant increase (38%) in the rate of [1-^{14}C]butyrate oxidation (see Fig. 4.12.b) and this rise was similar in magnitude to the stimulation of CO_2 production, from butyrate, in ovine omasal epithelium (Hird and Symons, 1959). The [D(-)-3-hydroxybutyrate]/[acetoacetate] ratio increased with a concomitant rise in medium glucose concentrations (see Fig. 4.12.b). Giescke *et al.* (1979) indicate that glucose inhibits butyrate oxidation in sheep rumen epithelium, thus directing more butyrate to ketone body formation. My results do not support this suggestion.

4.3.3.4. *Hormonal Effects on Rumen Epithelial Ketogenesis*

Butyrate-dependent ketogenesis in rumen epithelium was insensitive to insulin, glucagon and both α- and β-adrenergic agonists (see Table 4.4). Only dibutyryl cyclic AMP and vasopressin always produced increases in ketogenesis greater than control values and the increase was only significant in the case of vasopressin. In addition to this no changes were observed in either the mitochondrial redox state or the rate of $^{14}CO_2$ production from [1-^{14}C]butyrate in the presence of any of these agents.

Vasopressin inhibits oleate-stimulated ketogenesis in hepatocytes isolated from fed rats but has no effect with butyrate as an exogenous substrate (Williamson *et al.*, 1980). Rat liver glycolytic flux is increased by vasopressin (Williamson *et al.*, 1980) and this stimulation is attributed to activation of pyruvate dehydrogenase (Hems *et al.*, 1978). Vasopressin may have increased the endogenous epithelial glycolytic flux in rumen epithelial cells thus providing substrates for endogenous ketogenesis and cause the small observed rise (see section 6.3). Another suggestion is that vasopressin is mimicking the action of a gastrointestinal hormone which may act on rumen epithelial cells.

Additions	Rates of Ketone Body Synthesis µmol/mg DNA per 30 min		Additions
Control	3.25 ± 0.14	3.38 ± 0.21	Control
Insulin (10^{-7}M)	3.37 ± 0.16	3.70 ± 0.18	Adrenaline (10^{-5}M)
Glucagon (10^{-7}M)	3.30 ± 0.08	3.47 ± 0.13	Isoproterenol (10^{-6}M)
Dibutyryl cyclic AMP (10^{-5}M)	3.62 ± 0.33	3.56 ± 0.17	Phenylephrine (2 x 10^{-5}M)
Noradrenaline (10^{-5}M)	3.41 ± 0.13	*3.85 ± 0.15	Vasopressin (10^{-7}M)
Endogenous	0.64 ± 0.19	0.57 ± 0.15	Endogenous

Table 4.4. Effect of Hormones on Rumen Epithelial Ketogenesis

Additions were made to incubation flasks containing isolated rumen papillae and n-butyrate (12mM). After a 30 min incubation period the acetoacetate and D(-)-3-hydroxybutyrate contents were measured. The endogenous ketogenic rates were not corrected with appropriate zero-time controls. All results are means ± S.E.M. for three separate experiments; *, $P < 0.05$.

4.3.3.5. Ketone Bodies

Assuming that acetoacetate is not catabolised by rumen epithelium, then the rate of acetoacetate formation, upon addition of D(-)-3-hydroxybutyrate, should equal the rate of D(-)-3-hydroxybutyrate utilisation. It was found, however, that there was a greater utilisation of D(-)-3-hydroxybutyrate than acetoacetate formation by rumen papillae (see Table 4.5). Similar observations were made for incubations containing either added propionate or butyrate, when the ketone bodies synthesised from these substrates were accounted for. This suggests that the rumen epithelium cannot only synthesise but also utilises ketone bodies.

Rat colonic mucosa, another fermentative organ, has many metabolic similarities with rumen epithelium, including the capacity to oxidise butyrate to ketone bodies (Roediger, 1980a). Roediger (1982) has also reported that ketone bodies are readily utilised in isolated rat colonocytes.

Furthermore, oxidation of [1-^{14}C]butyrate was significantly decreased in the presence of DL-3-hydroxybutyrate (see Fig. 4.13). This effect could be caused by dilution of the ^{14}C-labelled acetoacetyl-CoA pool (synthesised from [1-^{14}C]butyrate) by 'cold'-acetoacetyl-CoA formed from D(-)-3-hydroxybutyrate. As rumen epithelium contains measurable activities of enzymes of the D(-)-3-hydroxybutyrate dehydrogenase/3-ketoacid CoA-transferase pathway (see Table 3.2) this suggestion is

feasible. Another possibility is that metabolism of D(-)-3-hydroxybutyrate led to reduction of the mitochondrial redox state and thus a lowering of the mitochondrial free oxaloacetate concentration. This would decrease flux of ^{14}C-labelled acetyl-CoA, formed from [1-^{14}C]butyrate, through the citrate synthase step.

4.3.3.6. Metabolic Inhibitors of Butyrate-Dependent Ketogenesis

Usually metabolic inhibitors produced only small decreases in the rate of ketogenesis and because of differences in the absolute rates of ketogenesis between papillae preparations it was difficult determining if these effects were statistically significant. Nonetheless, although all the results are not given the findings from separate experiments are summarised below (table 4.6 gives representative values for one experiment).

The rate of butyrate-dependent ketogenesis was unaffected by malonate, 3-mercaptopicolinic acid (MPA), n-butylmalonate (BM) and aminooxyacetate (AOA) (see Table 4.6). In one experiment it was found that 5-(tetradecyloxy)-2-furoic acid (TOFA), an analogue of a long-chain fatty acid, significantly increased both 'cold' and ^{14}C-labelled ketone body synthesis by 52% and 31% respectively.

Additions	Acetoacetate Production	D(-)-3-Hydroxybutyrate Production	D(-)-3-Hydroxybutyrate Utilisation
Control	0.69 ± 0.19	0.24 ± 0.15	-
Butyrate (5mM)	5.70 ± 0.89	1.29 ± 0.15	-
Butyrate (5mM) + DL-3-Hydroxybutyrate (20mM)	7.29 ± 0.59	-	4.48 ± 0.64
DL-3-Hydroxybutyrate (20mM)	4.47 ± 0.69	-	7.22 ± 1.13
Propionate (5mM) + DL-3-Hydroxybutyrate (20mM)	2.30 [0.29]	-	5.57 [1.96]
Propionate (5mM)	0.17 [0.05]	0.35 [0.11]	

Table 4.5. Utilisation of DL-3-Hydroxybutyrate by Rumen Epithelium

Papillae were incubated with combinations of DL-3-hydroxybutyrate and either propionate or butyrate. D(-)-3-hydroxybutyrate utilisation was estimated by subtraction of the final D(-)-3-hydroxybutyrate content from appropriate zero-time control flasks. These values may include an unmeasured amount of D(-)-3-hydroxybutyrate produced by the tissue. Results are reported as means ± S.E.M. except for incubations containing propionate. In this case results are given as the average of means obtained from two separate experiments. The range between the means are given in the square brackets. Values are given in terms of μmol/mg DNA per h.

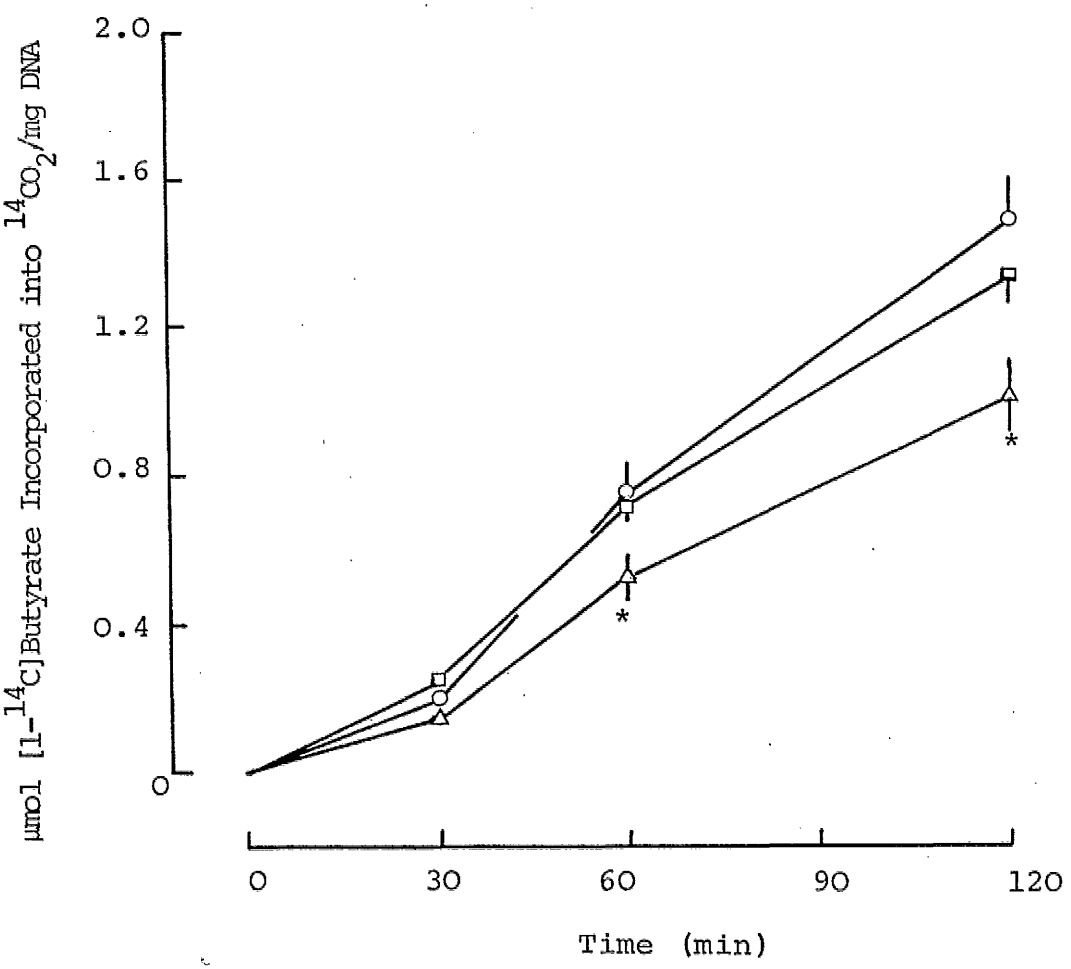


FIG. 4.13. Effect of Acetoacetate and DL-3-Hydroxybutyrate on [1-^{14}C]Butyrate Oxidation

Rumen papillae were incubated with [1-^{14}C]butyrate (5mM) and either DL-3-hydroxybutyrate (20mM) (△) or acetoacetate (10mM) (□). $^{14}CO_2$ was collected as described in section 2.13 and results are presented as means ± S.D. for triplicate incubations. Asterisks indicate values significantly different from incubations containing [1-^{14}C]butyrate alone (○) (*; $P < 0.01$).

Additions	Butyrate	Butyrate plus Propionate
None	241.8 ± 11.6 (0.11 ± 0.02)	218.7 ± 12.2 (1.13 ± 0.12)
Malonate	239.0 ± 18.6 (0.11 ± 0.03)	234.0 ± 9.5 (0.63 ± 0.06)
α-Cyannocinnamate	181.7 ± 12.6 (0.08 ± 0.02)	227.0 ± 15.9 (1.52 ± 0.08)
Mercaptopicolinic acid	221.2 ± 20.1 (0.16 ± 0.03)	204.2 ± 11.4 (1.12 ± 0.18)
Butylmalonate	236.7 ± 12.8 (0.11 ± 0.01)	215.6 ± 26.0 (0.96 ± 0.15)

Table 4.6. Effect of Metabolic Inhibitors on Rumen Epithelial Ketogenesis

Rumen papillae were incubated in the presence of butyrate (12mM), with or without propionate (15mM), and various metabolic inhibitors and the rate of release of ketone bodies into the incubation medium was determined (μmol/g dry wt. per 2h). The [D(-)-3-hydroxybutyrate]/[Acetoacetate] ratio is given in parentheses. Results are means ± S.D. for triplicate incubations and ketone body production for control flasks was 16.9 ± 2.6 μmol/g dry wt. per 2h.

TABLE 4.7. METABOLIC INHIBITORS

Incubation concn.	Inhibitors	Locus of Inhibition	References
(0.2mM)	Aminooxyacetate (AOA)	Pyridoxal-containing enzymes e.g. transaminases	Smith *et al.* (1977)
(5mM)	n-Butylmalonate (BM)	Blocks malate transport across mitochondrial membranes	Robinson & Chappell (1967)
(0.1mM)	2-Cyano-4-hydroxy-cinnamate (CHC)	Blocks pyruvate transport across mitochondrial membranes	Halestrap (1978)
(15mM)	Malonate	Inhibits succinate dehydrogenase enzyme	Weekes (1974)
(0.5mM)	3-mercaptopicolinic acid (MPA)	Inhibits phosphoenolpyruvate carboxykinase enzyme	Jomain-Baum *et al.* (1976)
(0.1mM)	5-(Tetradecyloxy)-2-furoic acid (TOAF)	Decreases cellular CoASH content	McCune & Harris (1979)

Inhibition of butyrate-dependent ketogenesis, produced by propionate, was relieved by malonate. This indicates that some metabolism of propionate does occur *in vitro*, with butyrate present in the medium. The inhibition of ketogenesis produced by propionate was not influenced by MPA or BM. MPA was added to block any decrease in oxaloacetate content (if it was generated from propionate) by metabolism through the phosphoenolpyruvate carboxykinase reaction. The lack of any effect by MPA may be related to the weak inhibition of the mitochondrial carboxykinase enzyme by MPA (Robinson and Oei, 1975) or to a lack of plasma membrane permeability to this compound.

Antiketogenic substrates may be effective in rat and sheep hepatocytes because they influence a possible site regulating ketogenesis; namely, by influencing the pathway of acetyl-CoA metabolism between ketogenesis or oxidation by affecting the concentration of free oxaloacetate (Sieśs *et al.*, 1982; Lomax *et al.*, 1983). Potential precursors of oxaloacetate (pyruvate, lactate, alanine, fructose and glutamine) did not influence butyrate-dependent ketogenesis. Pyruvate was the only substrate significantly to increase [1-^{14}C]butyrate oxidation. Ammonia was found to be a weak ketogenic effector, producing a stimulation of ketogenesis of about 15% of the butyrate-dependent ketogenic rate.

4.4. *Discussion*

The quantitative significance of rates of metabolic processes in rumen epithelium *in vitro*, for tissue sampled from abattoir-killed animals, has been investigated by Hird and Weidemann (1964). Although Hodson *et al.* (1967) demonstrated that rumen epithelium, from slaughter-house animals, still retained intact mitochondria after transportation to the laboratory Hird and Weidemann (1964) found that tissue excised from laboratory killed sheep is more active (60-70%). However, the patterns of metabolism are similar in rumen epithelium excised from laboratory- and abattoir -killed animals (Hird and Weidemann, 1964).

Extension of metabolic observations made *in vitro* to the metabolism carried out in the epithelium *in vivo* has obvious problems. For example, the tissue *in situ* utilises nutrients from the rumen liquor on one side and the blood serving the epithelium on the other. But in incubations the epithelial tissue has medium, supplemented with a limited number of substrates, on all sides. In this present study, where changes in metabolic fluxes (produced by effectors) occurs quantitative deductions should be made with caution.

Quantitatively, butyrate was the most significant substrate for ketogenesis, of all the fatty acids substrates metabolised. Metabolism of long-chain fatty acids in rumen mucosa has been reported to contribute both to transport of long-chain fatty acids across the epithelium (Hird and Weidemann, 1964; Hird *et al.*, 1966; Goosen, 1975) and to spontaneous ketosis in undernourished ruminants (Jackson *et al.*, 1964).

Rates of butyrate-dependent ketogenesis, found in this study, compare favourably with published values (Hird and Symons, 1959; Bush *et al.*, 1970 ; Strangassinger *et al.*, 1979). However, the ratio of D(-)-3-hydroxybutyrate to acetoacetate in incubations was 5-10 times lower than some reported ratios (Emmanuel, 1979; Bush *et al.*, 1970). Even so, all ratios obtained *in vitro* were always lower than the ratios (5-25) observed in the sheep portal blood *in vivo* (Hodson *et al.*, 1965; Weigand *et al.*, 1972).

The activity of HMG-CoA synthase measured in this study (see Table 3.2) was only just sufficient to account for the maximal rates of ketogenesis *in vitro*. Measureable activities of 3-ketoacid CoA-transferase in rumen epithelium extracts raises the possibilities that acetoacetate was formed from acetoacetyl-CoA through the reaction catalysed by this enzyme.

Activities of 3-ketoacid CoA-transferase have been measured in the livers of some fish (Phillips and Hird, 1977; Zammit *et al.*, 1979) where it has been suggested that this enzyme is involved in ketone body production rather than utilisation (Phillips and Hird, 1977). However, Zammit *et al.* (1979) have proposed that the role of 3-ketoacid CoA transferase is to produce a substrate cycle between acetoacetyl-CoA and acetoacetate as shown in Fig. 4.14.

Hird and Symons (1961) have proposed that operation of the HMG-CoA pathway is the main route for ketogenesis in rumen epithelium. They examined [1-^{14}C]butyrate metabolism in this tissue and found an unequal isotopic distribution between carbon one (81%) and carbon three (19%) of acetoacetate. Similar asymmetrical labelling patterns were found in this study (see Table 4.8).

Metabolism of [1-^{14}C]butyrate through the HMG-CoA pathway would result in the loss of all ^{14}C radioactivity from the molecule (see Fig. 3. . But, as shown in Table 4.8, there was greater ^{14}C activity of carbon one of acetoacetate than carbon three. For this to happen the acetyl-CoA pool must become highly radioactively labelled, probably through the acetoacetyl-CoA thiolase catalysed reaction. It has been shown that thiolase catalyses a rapid exchange between the carboxyl half of acetoacetyl-CoA and acetyl-CoA (Beinert and Stansly, 1953). This exchange is confirmed as a two step mechanism (Gehring *et al.*, 1968): Initially acetoacetyl-CoA is cleaved;

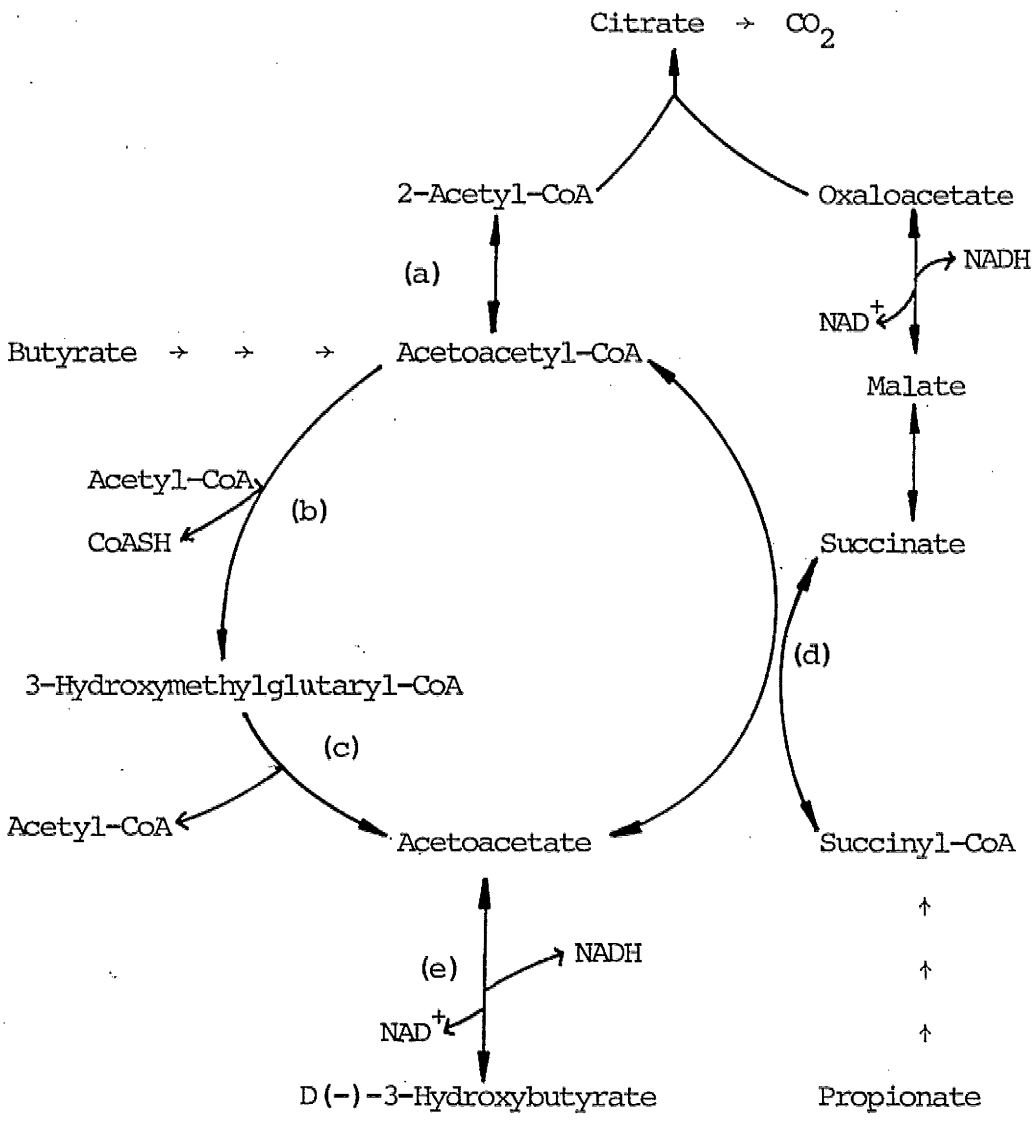


FIG. 4.14. Possible Involvement of 3-Keto acid CoA-Transferase in Rumen Epithelial Ketogenesis

The enzymes which catalyse the reactions are: a, acetoacetyl-CoA transferase; b, 3-hydroxymethylglutaryl-CoA synthase; c, 3-Hydroxy-methylglutaryl-CoA lyase; d, 3-keto acid CoA-transferase; e, D(-)-3-Hydroxybutyrate dehydrogenase.

Table 4.8. Distribution of ^{14}C in Ketone Bodies formed from [1-^{14}C]Butyrate

Rumen papillae were incubated with [1-^{14}C]butyrate (5mM) plus other substrates (concentration given in parentheses) as described in the legend to Fig. 4.9. ^{14}C-labelled acetoacetate released into the medium was decarboxylated with aniline citrate and the specific radioactivity of the C_1 and C_{2-4} moieties measured as described in section 2.14. Results are means ± S.D.

Additions	Ketone Bodies % of total ^{14}C in	
	Carbon one	Carbon three
[1-^{14}C]Butyrate	77.9 ± 2.0	22.1 ± 2.0
+ Propionate (10mM)	75.8 ± 1.4	24.2 ± 1.4
+ Glucose (10mM)	76.5 ± 1.8	23.5 ± 1.8
+ Lactate (10mM)	76.8 ± 1.2	21.2 ± 1.2
+ Pyruvate (10mM)	81.3 ± 3.2	18.8 ± 3.2
+ Fructose (10mM)	76.9 ± 1.2	23.1 ± 1.2
+ DL-3-Hydroxybutyrate	78.0 ± 1.9	22.0 ± 1.9
+ [1-^{14}C]Palmitate	65.4 ± 1.4	34.6 ± 1.4
+ [2-^{14}C]Pyruvate	49.9 ± 1.3	50.1 ± 1.3

acetyl-CoA is set free and the carbonyl moiety remains bound to thiolase. Then, this bound acetyl group is transferred to free CoASH. The first step is reversible and relatively fast compared to the second step.

Operation of the forward thiolase reaction is indicated by production of $^{14}CO_2$ from [1-^{14}C]butyrate (see Fig. 4.9). Relocation of ^{14}C from [1-^{14}C]butyrate during its conversion to acetoacetate and synthesis of ^{14}C-labelled ketone bodies from [2-^{14}C]pyruvate is evidence for operation of both the forward and reverse thiolase reaction. Also, Hird and Symons (1961) reported that 1.7-4.0 times as many carbon atoms one and two of butyrate as carbon atoms three and four appeared in CO_2, suggesting preferential removal of the former moieties by thiolase.

Perhaps a HMG-CoA synthase/acetoacetyl-CoAthiolase binary complex, as proposed by Greville and Tubbs (1968), exists in rumen epithelium. Butyrate would be metabolised as a C_4 unit as far as acetoacetyl-CoA (see Fig. 1.4) and here, at the binary complex, there may be chanelling of acetoacetyl-CoA between the two pathways. Of course, most of the flux would be through the HMG-CoA pathway because if ketone bodies originated from a common pool of acetyl-CoA then it would be expected that there would be an equal activity of ^{14}C in the carbonyl and carboxyl groups of acetoacetate (see [2-^{14}C]pyruvate distribution, Table 4.8).

Involvement of 3-keto acid CoA-transferase in production of ketone bodies could in theory result in the asymmetrical labelling patterns observed. But if transferase catalyses a near equilibrium reaction (Zammit *et al.*, 1979) then substrate concentrations could regulate enzyme activity. For instance, a high mitochondrial $NADH/NAD^+$ ratio will favour the formation of D(-)-3-hydroxybutyrate and reduce the concentration of acetoacetate, thus lowering the concentration of acetoacetyl-CoA and lead to an increase in ketogenesis (acetoacetyl-CoA is a substrate inhibitor of HMG-CoA synthase). However, rumen epithelial ketogenesis appears to be insensitive to the mitochondrial redox state (see section 4.3.3.2).

3-Keto acid CoA-transferase activity may be necessary for utilisation of acetacetate in rumen mucosa. As shown in Table 4.5, some oxidation of ketone bodies occurs in this tissue. Also, metabolism of propionate generates succinyl-CoA which will shift the thiolase equilibrium towards acetoacetyl-CoA formation, in the presence of high acetoacetate concentrations. The effect of propionate on acetoacetate and D(-)-3-hydroxybutyrate formation from butyrate is shown in Fig. 4.15. If a substrate increases the mitochondrial $NADH/NAD^+$ ratio then D(-)-3-hydroxy-butyrate formation should follow a negative reciprocal pattern to acetoacetate production; as was the case for glucose (see Fig. 4.16). However, the faster rate of acetoacetate disappearance than D(-)-3-hydroxybutyrate formation may be accounted for by metabolism through the 3-keto acid CoA-transferase reaction.

Fig. 4.15 (Data from Fig. 4.8)

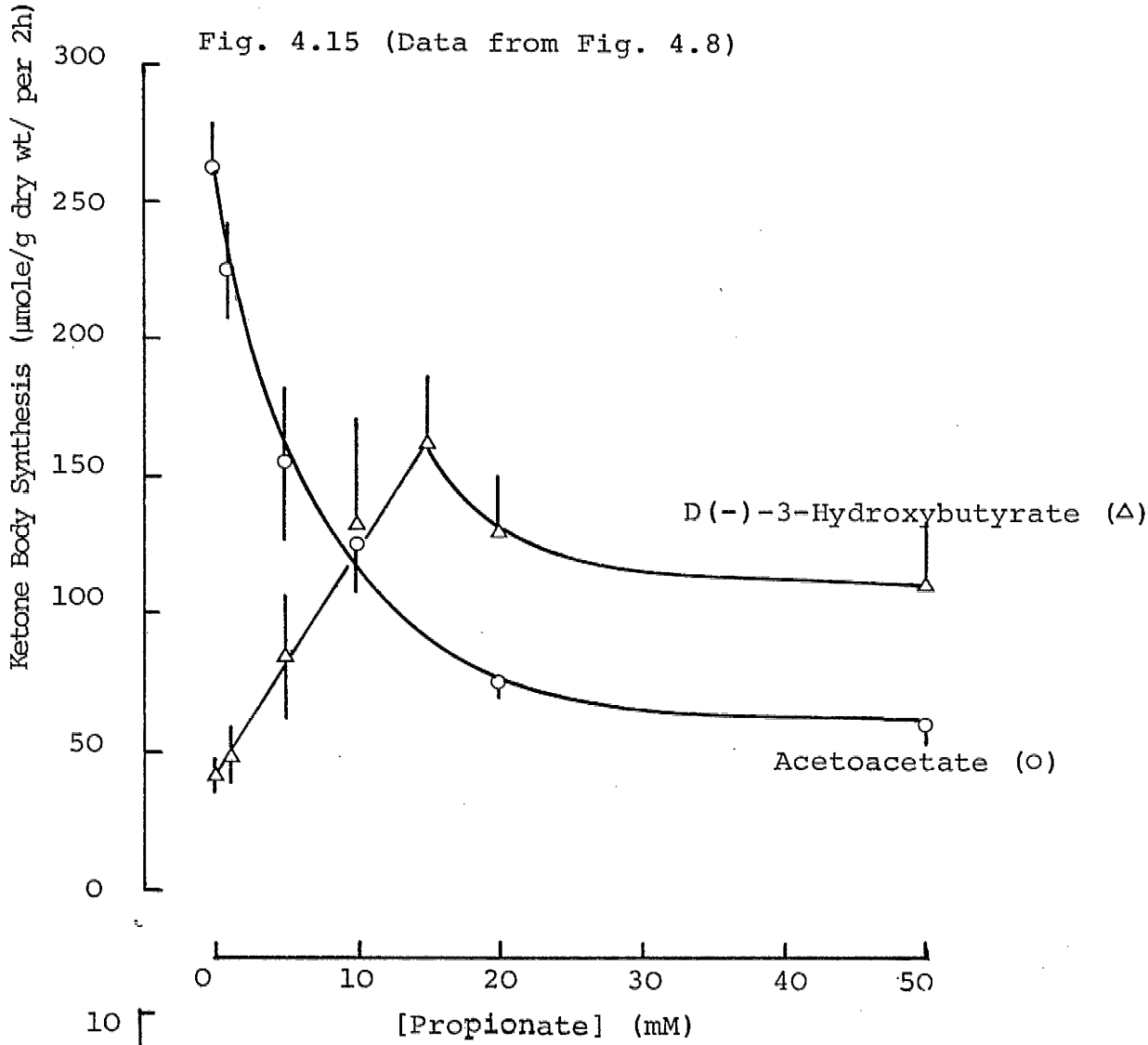
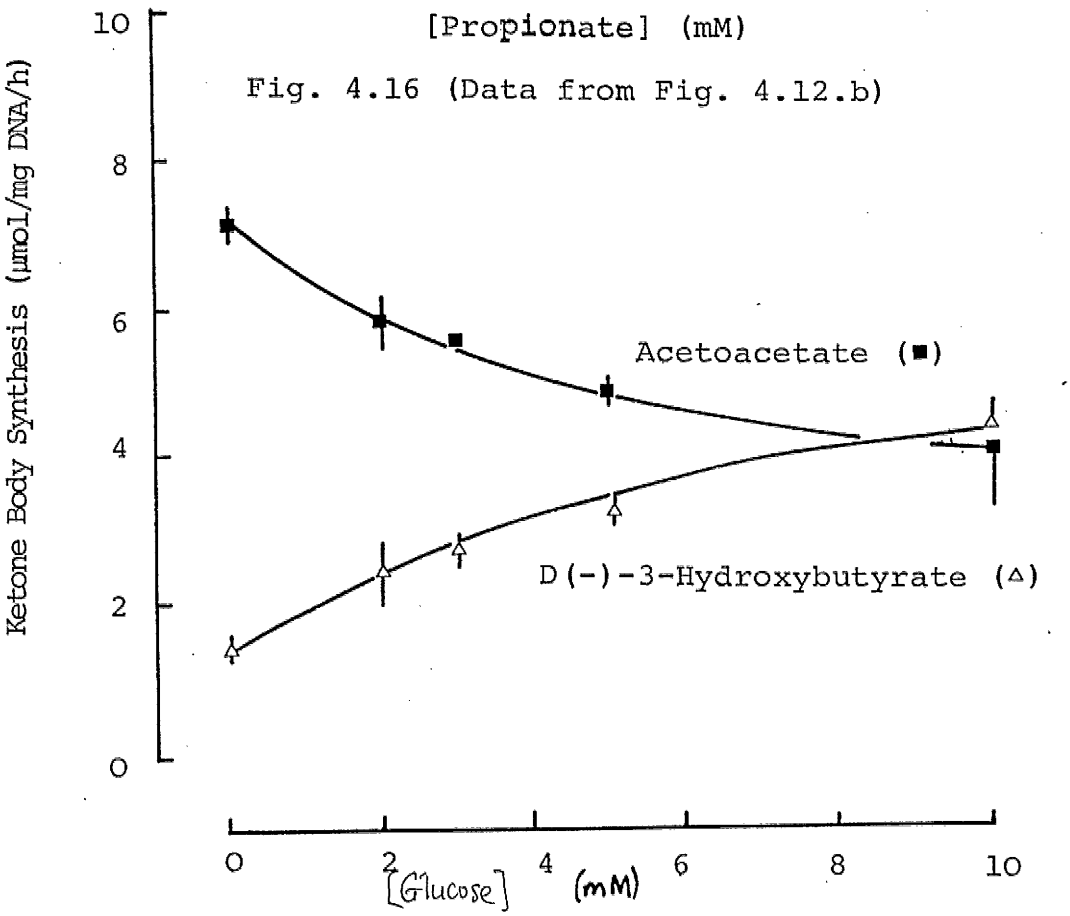


Fig. 4.16 (Data from Fig. 4.12.b)



Indirect evidence for this hypothesis is the small uptake of ketone bodies across the gut of starved cows (Baird *et al.*, 1979). Also, utilisation of acetoacetate by rumen epithelium may be responsible for the high portal blood [D(-)-3-hydroxybutyrate]/[acetoacetate] ratios observed *in vivo* (see page 140). Also, the appearance of acetate when propionate and butyrate are incubated with rumen papillae *in vitro* (Pennington and Pfander, 1957) could be the result of acetyl-CoA hydrolase activity on high intramitochondrial acetyl-CoA concentrations, caused by 'recycling' of aceoacetate formed from butyrate. Lastly, DL-3-hydroxybutyrate decreases the rate of lactate and $^{14}CO_2$ formation from [U-^{14}C] glucose. One explanation for this effect is that it is produced by an increase in mitochondrial acetyl-CoA, from 3-hydroxybutyrate, which inhibits pyruvate dehydrogenase activity (see section 4.3).

Utilisation of acetoacetate by rumen epithelium may not be the cause of the inhibition of butyrate-dependent ketogenesis produced by high medium propionate concentrations (see Fig. 4.8). There is evidence that activation of VFAs in rumen mucosa is controlled by autoregulation (Ash and Baird, 1973). Scaife and Tichivangana (1980) report that sheep rumen epithelium does not contain an acyl-CoA synthetase specific for propionate but two synthetases: one capable of activating all three VFAs and the other will only accept butyrate as substrate. Indeed, the K_i value for propionate,

acting as an inhibitor of butyrate (10mM) activation, was 38.0 mM. This may indicate that relatively high propionate levels are required for inhibition of butyrate activation.

In conclusion it would appear that butyrate-dependent ketogenesis in rumen epithelium is insensitive to change from a variety of effectors. This characteristic of rumen epithelial ketogenesis is advantageous for the animal because it means that butyrate will be, under most circumstances, extensively metabolised and will not enter the ruminant humoral system.

4.5 SUMMARY

The influences of various potential effectors on ketone body production from butyrate in sheep rumen epithelial papillae was studied. Ketogenesis is weakly stimulated by addition of acetate and NH_4^+. However, insulin, glucagon, dibutyryl-cyclic AMP, Ca^{2+} and both α- and β-agonists did not affect butyrate-dependent ketogenesis. Vasopressin produces about 14% increase in flux.

Glucose increases butyrate oxidation but did not influence ketone body production. Propionate also increases butyrate conversion to CO_2 and causes a significant decrease in butyrate-dependent ketogenesis at high concentrations. Results do not support the view that the oxaloacetate concentration plays a significant role in the regulation of epithelial ketone body synthesis. The site for the propionate inhibition may be the flux generating step, i.e. butyryl-CoA synthetase.

Evidence for utilisation of D(-)-3-hydroxybutyrate in papillae is given and a method for measuring uptake and transport of substrates across isolated sheets of rumen epithelium is described.

CHAPTER 5

SUBSTRATE OXIDATION, GLYCOLYSIS, LIPOGENESIS AND PROPIONATE CONVERSION TO L-LACTATE IN ISOLATED RUMEN PAPILLAE

5.1. *Introduction*

In this chapter, and the following chapters, aspects of other metabolic processes carried out in rumen epithelium are described. In many of the experiments emphasis was placed on the interactions of physiological substrates with the particular metabolic process being investigated.

5.2. *Substrate Oxidation in Isolated Rumen Papillae*

Rumen epithelium exhibits high endogenous rates of respiration *in vitro* (Smith *et al.*, 1961). Butyrate produced a significant increase in endogenous O_2 consumption (Pennington, 1954; Hird and Symons, 1959; Smith *et al.*, 1961; Goosen, 1976); although propionate and acetate metabolism did not result in any measurable rise in O_2 uptake (Smith *et al.*, 1961; Goosen, 1976). However, Pennington (1954) pointed out that CO_2 is required in the incubation atmosphere to observe any stimulatory effect of propionate on O_2 uptake. Incubation of rumen epithelium in flasks containing 100% O_2 may have accounted for an absence of a propionate effect in studies carried out by Smith *et al.* (1961) and Goosen (1970).

In this present study, oxidation of ^{14}C-labelled propionate or butyrate in rumen epithelium resulted in the highest rates of $^{14}CO_2$ formation, compared to other substrates (see table 5.1), whether the VFAs were added as the sole substrates or as constituents of a substrate mixture.

Substrate	$^{14}CO_2$ Production (μmol [^{14}C]Substrate Utilised per mg DNA per h)		
	(A)	%	(B)
[U-^{14}C]Acetate (15mM)	0.280 ± 0.051	9.0	0.119 [0.099]
[1-^{14}C]Propionate (5mM)	1.275 ± 0.153	41.0	0.677 [0.290]
[1-^{14}C]Butyrate (3mM)	1.050 ± 0.183	33.8	0.641 [0.200]
D-[U-^{14}C]Glucose (5mM)	0.160 ± 0.013	5.2	0.179 [0.068]
L-[U-^{14}C]Alanine (1mM)	0.010 ± 0.002	0.3	0.114 [0.060]
L-[U-^{14}C]Glutamine (1mM)	0.029 ± 0.003	0.9	0.084 [0.030]
L-[U-^{14}C]Lactate (1mM)	0.028 ± 0.002	0.9	0.334 [0.096]
All ^{14}C-labelled Substrates	3.102 ± 0.255	100.0	-

Table 5.1. Oxidation of ^{14}C-labelled Substrates in Rumen Epithelium

Isolated rumen papillae were incubated with ^{14}C-labelled substrates before evolved $^{14}CO_2$ was collected and measured as described in section 2.13. Each [^{14}C]substrate was added either alone (B), at a final medium concentration of 10mM, or as part of a mixture of substrates (A) (final concentrations given in parenthesis). Results in column (A) are means ± S.D. Values in column (B) are the average of means obtained from two separate experiments; the range between mean values is given in the square brackets.

The rate of butyrate conversion to CO_2 was similar to the value reported by Emmanuel (1980b) for ovine rumen epithelium (i.e. 2.76 µmol [1-^{14}C]butyrate utilised per h/g wet wt; this is converted to 0.60 µmol/mg DNA/h using a wet wt. to dry wt. ratio of 6 for rumen papillae and the DNA to dry wt. ratio given in section 4.2). This figure is misleading if it is taken to mean total butyrate oxidation since carbon atoms 1 and 2 of butyrate are oxidised at a greater rate than carbon atoms 3 and 4 (Hird and Symons, 1961).

In the presence of a mixture of substrates at physiologically-relevant concentrations (on a molar basis), glucose, alanine, glutamine and lactate contributed less than 8% to the total CO_2 formed (see column (A) Table 5.1). Alanine, glutamine and lactate were oxidised at greater rates when incubated as sole substrates (and at higher concentrations too) with isolated rumen papillae. Although the final concentrations of propionate and butyrate were lower in the substrate mixture the rates of CO_2 formation produced by the VFAs increased almost two-fold.

Rates of propionate and butyrate oxidation were unchanged upon addition of DL-carnitine (results not shown). In sheep liver L-carnitine is esterified to short-chain acyl-CoA, by carnitine acetyltransferase (EC 2.3.1.7; Fishlock *et al.*, 1982), to give short-chain acylcarnitine. This metabolism of carnitine modulates the CoASH/short-chain acyl-CoA ratio in the mitochondrial matrix (May *et al.*,

1980; Patel *et al.*, 1983). Pyruvate dehydrogenase enzyme is strongly influenced by this ratio (see section 5.3) and the absence of any effect of carnitine, especially on propionate oxidation, may indicate that rumen epithelium contains low carnitine acetyltransferase activity.

5.3 *Glycolysis*

The rate of glycolysis in rumen epithelium *in vitro* has been measured by several investigators (Pennington and Sutherland, 1956a; Weekes, 1974; Emmanuel, 1980b), although little work has concentrated on possible means of regulating the glycolytic flux. For instance, there has been a report of a synergistic increase in L-lactate and pyruvate formation when glucose and propionate are metabolised together in rumen epithelium (Weekes, 1974). However, it is not known if the end-products arise from increased glucose or propionate metabolism,and much less the mechanism to bring about this increase.

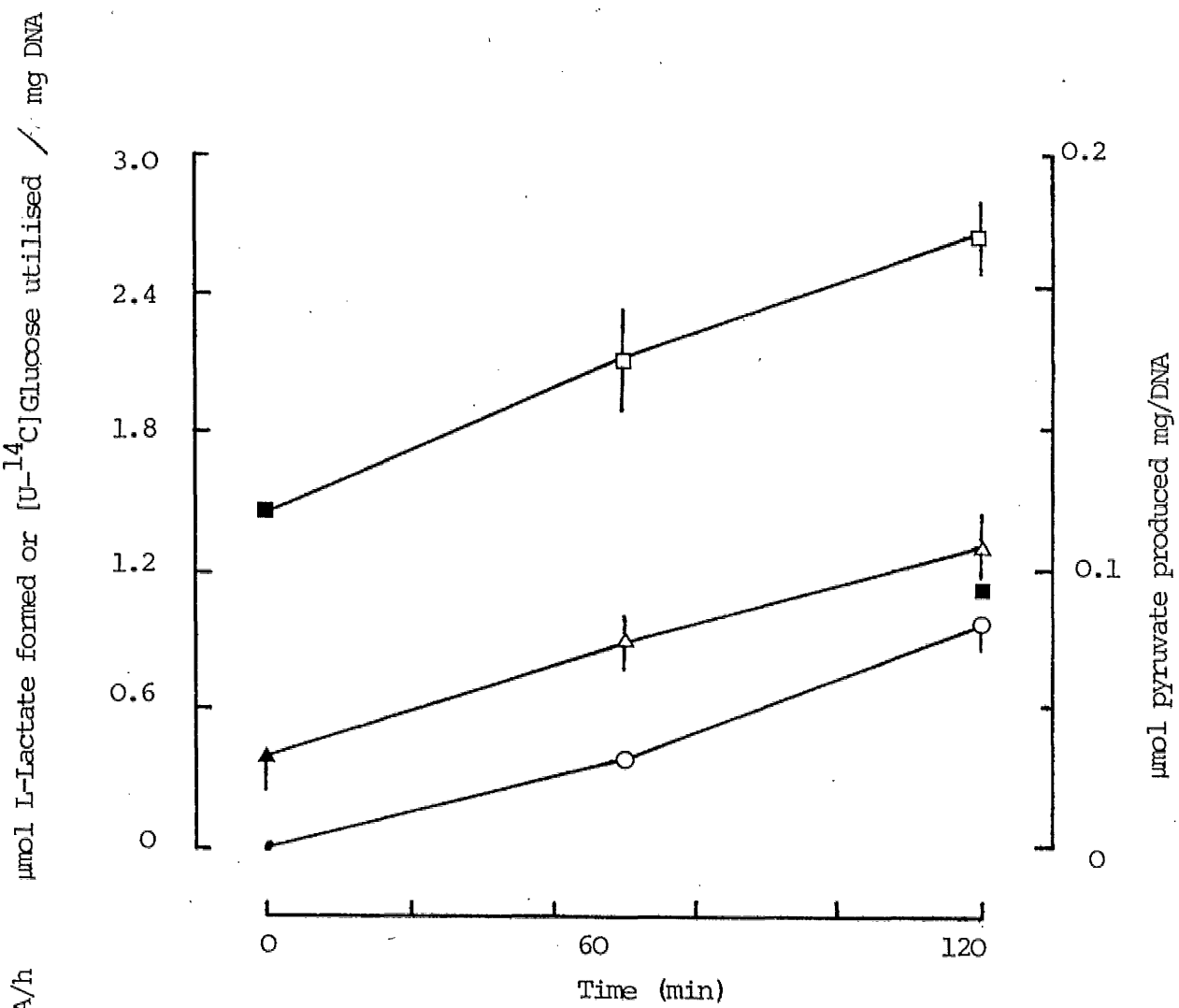
In isolated rumen papillae rates of lactate, pyruvate and $^{14}CO_2$ formation *in vitro* from [U-^{14}C]glucose were found to be 0.64, 0.05 and 0.38 µmol end-products formed or glucose oxidised per mg DNA per h (see Fig. 5.1a). These production rates are similar in magnitude to values reported by Emmanuel (1980b). (i.e. 0.90, 0.02 and 0.37 µmol end-products formed or glucose oxidised per mg DNA per h, respectively; values have been converted to mg DNA from g wet wt. using the conversion factors discussed in section 5.2). Also an apparent K_m value of 5.2mM was

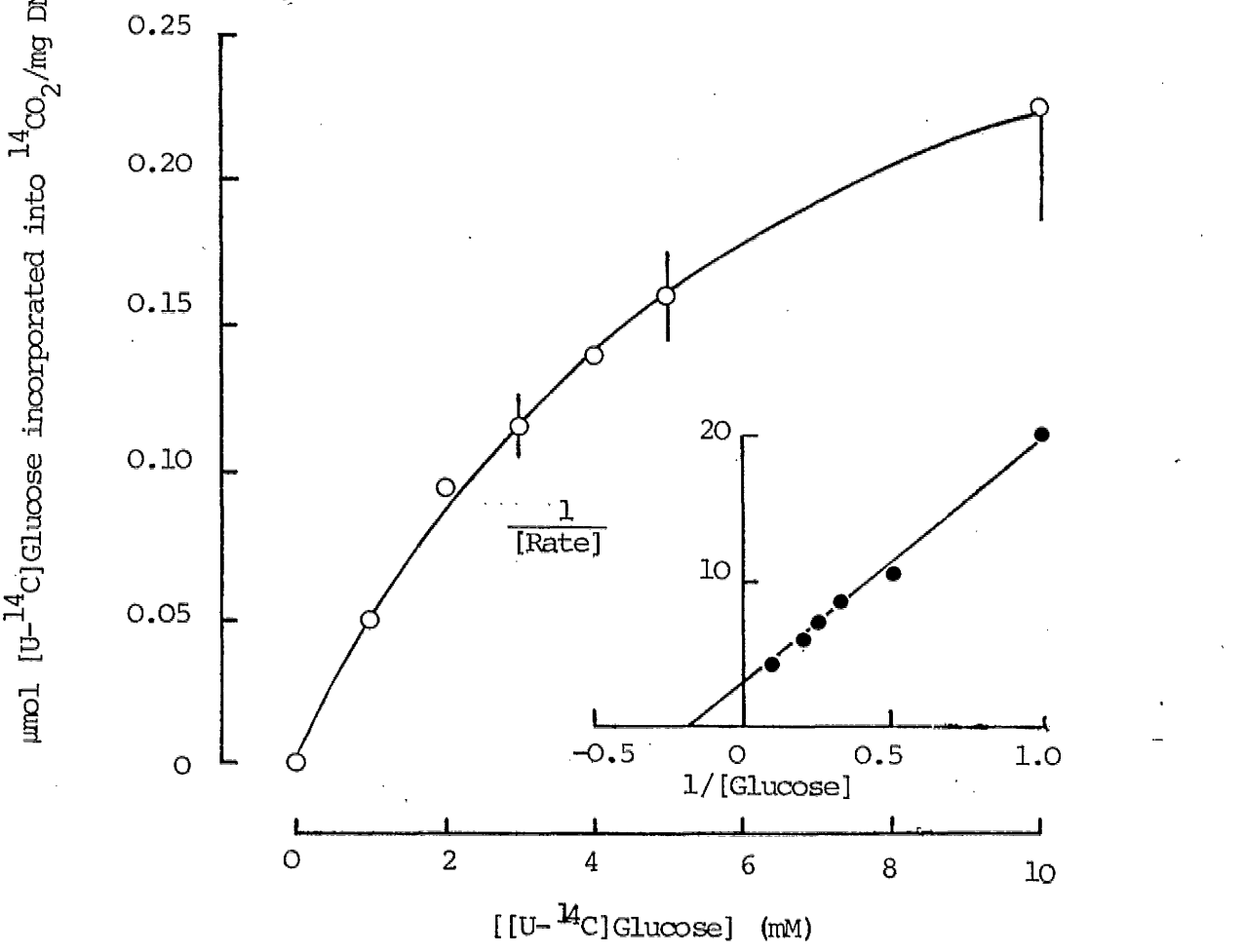
Fig. 5.1(a). Top. Time Course of [U-^{14}C]Glucose Metabolism in Rumen Epithelium

Isolated rumen papillae were incubated with [U-^{14}C] glucose (5mM) over a 2h incubation period. L-lactate (□), pyruvate (△) and $^{14}CO_2$ (○) released from the epithelial cells were measured by methods described in Chapter 2. Results are means ± S.D. Endogenous lactate production (■) was measured in control flasks at the start and end of the incubation.

Fig. 5.1(b). Bottom - The Effect of Medium Glucose Concentration on $^{14}CO_2$ Production

Rumen papillae were incubated with a range (1-10mm) of glucose concentrations over a 1h incubation period. A Lineweaver-Burk plot of the results gave an apparent K_m value and V_{max} value for glucose oxidation of 5.2mM and 0.322 μmol per mg DNA per h, respectively. Results are means ± S.D.


µmol L-Lactate formed or [U-14C]Glucose utilised / mg DNA
µmol pyruvate produced mg/DNA
3.0
2.4
1.8
1.2
0.6
0
0.2
0.1
0
0
60
120
Time (min)


µmol [U-14C]Glucose incorporated into 14CO2/mg DNA/h
0.25
0.20
0.15
0.10
0.05
0
1/[Rate]
20
10
-0.5
0
0.5
1.0
1/[Glucose]
0
2
4
6
8
10
[[U-14C]Glucose] (mM)

determined from $^{14}CO_2$ production rates after rumen papillae were incubated with a range of medium [U-^{14}C] glucose concentrations (see Fig. 5.1b). This K_m value is close to sheep plasma glucose concentrations (3-3.5 mM; Trenkle, 1970; Wilson *et al.*, 1981).

Glycolysis in sheep rumen epithelium *in vitro* is about 11 and 7 times more active than in sheep, liver or kidney, respectively (Emmanuel, 1980c; 1980e).

Acetate was the only substrate to produce a significant inhibition of glycolysis (see Table 5.2). A decrease of glycolytic flux was also observed at lower acetate and butyrate concentrations (see Fig. 5.2). Similar decreases in lactate production and $^{14}CO_2$ for mation, as produced by the two VFAs, were found when long-chain fatty acid were metabolised in rumen epithelium. The inhibition of glucose oxidation produced by octanoate was greater than the effect produced by oleate (see Table 5.3). This concurs with the observation that metabolism of fatty acids to ketone bodies in rumen epithelium decreased as the fatty acid chain length increased. (see section 4.3.1).

The observed effects of 2-cyano-4-hydroxycinnamate, a potent inhibitor of mitochondrial pyruvate transport (Clark *et al.*, 1979), were as expected. The decrease in $^{14}CO_2$ production could be accounted for by the significant increase in pyruvate released by rumen papillae (see table 5.2), but a greater inhibition in the rate of $^{14}CO_2$ formation was anticipated (see Lane *et al.*, 1981).

Additions	Pyruvate	Lactate	$^{14}CO_2$
None	0.04 ± 0.01	1.09 ± 0.04	-
[U-^{14}C]Glucose (5mM)	0.13 ± 0.02	2.63 ± 0.15	0.98 ± 0.01
+ Glucagon (10^{-4}M)	0.11 ± 0.05	1.89 ± 0.65	0.63 ± 0.29*
+ Dibutyryl cyclic AMP (10^{-5}M)	0.15 ± 0.01	2.48 ± 0.30	0.97 ± 0.08
+ Dibutyryl cyclic AMP (10^{-4}M)	0.14 ± 0.02	2.24 ± 0.23	0.92 ± 0.04
+ Glutamine (5mM)	0.09 ± 0.03	2.28 ± 0.23	0.85 ± 0.03*
+ Glutamine (10mM)	0.12 ± 0.02	2.18 ± 0.30	0.56 ± 0.10*
+ NH_4Cl (5mM)	0.15 ± 0.02	2.48 ± 0.21	0.62 ± 0.08*
+ NH_4Cl (10mM)	0.14 ± 0.01	2.45 ± 0.15	0.71 ± 0.04*
+ 2-cyanno-4-hydroxy cinnamate (0.5mM)	0.65 ± 0.24*	2.94 ± 0.50	0.53 ± 0.10*
+ Acetate (60mM)	0.12 ± 0.06	2.07 ± 0.23*	0.49 ± 0.08*
+ DL-3-Hydroxybutyrate (10mM)	0.08 ± 0.01	2.35 ± 0.29	0.58 ± 0.04*

Table 5.2. Effect of Various Substrates and Effectors on Rumen Epithelial Glycolysis

Isolated papillae were incubated with [U-^{14}C]glucose (5mM) plus other additives indicated on the table (final concentrations are given in parentheses). Rates of end-product formation or [U-^{14}C]glucose oxidation are all given as µmol per mg DNA per 2h; *$P < 0.05$. Results are means ± S.D.

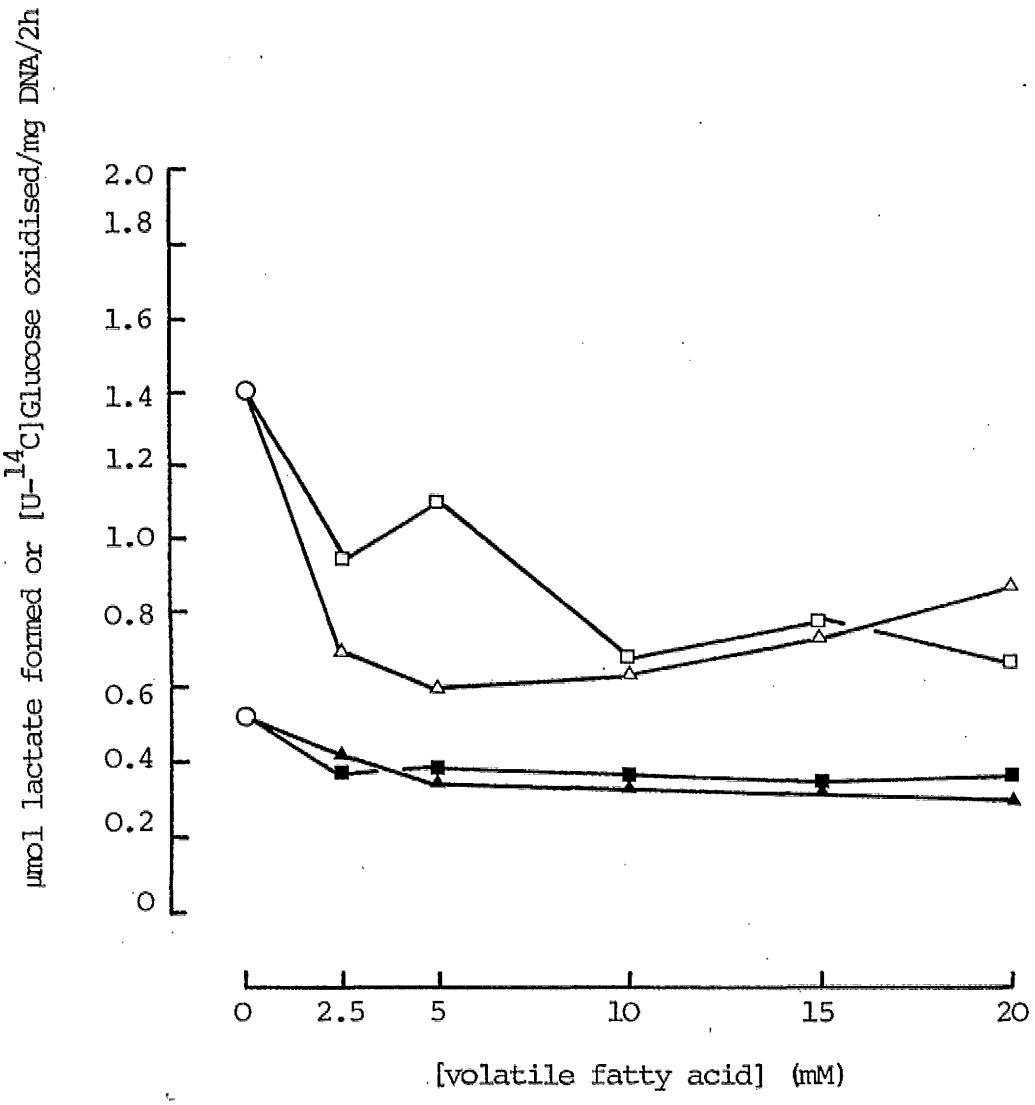


Fig. 5.2. Effect of Butyrate and Acetate on Glycolysis

Rumen papillae were incubated with [U-^{14}C]glucose (3mM) plus a range of acetate or butyrate concentrations. L-Lactate, corrected for endogenous production, and $^{14}CO_2$ formation were measured in the presence of acetate (□ and ■ , respectively) and butyrate (△ and ▲ , respectively). All results are means of triplicate incubations. Also, pyruvate formation from glucose (0.05 µmol/mg DNA/2h) was unchanged by either volatile fatty acid .

Additions	Pyruvate	Lactate	$^{14}CO_2$
None	0.04 ± 0.01	1.09 ± 0.04	-
[U-^{14}C]Glucose (5mM)	0.13 ± 0.02	2.63 ± 0.15	0.98 ± 0.01
Oleate (1mM)	0.09 ± 0.02	0.93 ± 0.04	-
Octanoate (1mM)	0.12 ± 0.05	0.89 ± 0.13	-
Glucose + Oleate (0.5mM)	0.12 ± 0.01	2.27 ± 0.26	0.83 ± 0.05*
Glucose + Oleate (1.0mM)	0.13 ± 0.02	2.45 ± 0.25	0.86 ± 0.05*
Glucose + Octanoate (0.5mM)	0.09 ± 0.02	2.05 ± 0.08	0.66 ± 0.03*
Glucose + Octanoate (1.0mM)	0.11 ± 0.03	2.58 ± 0.06	0.53 ± 0.08*

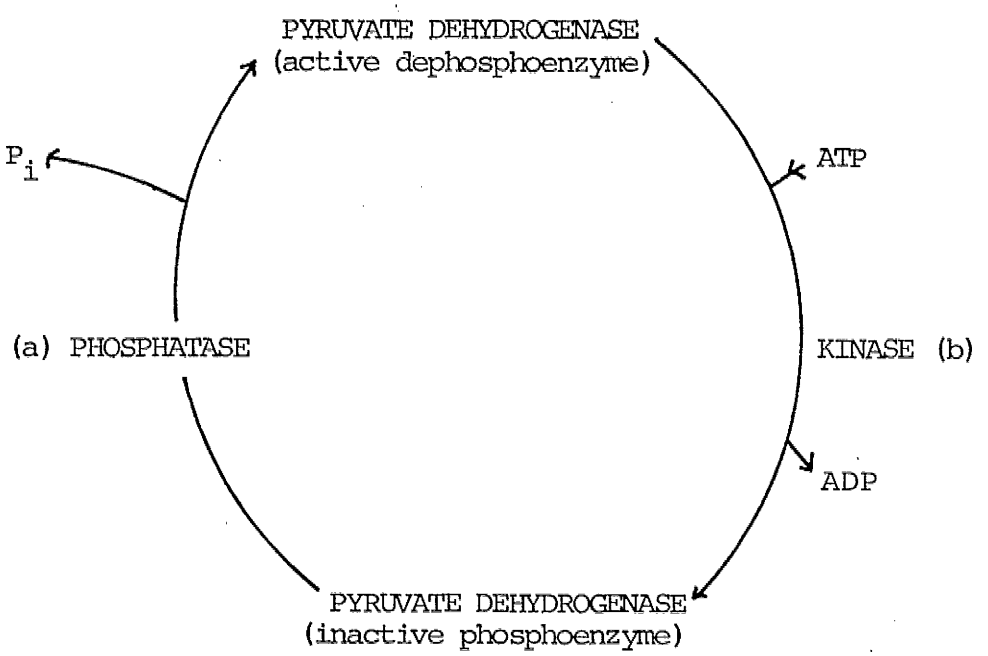
Table 5.3. Effect of Long-Chain Fatty Acids on Glycolysis

Octanoate and oleate, bound to defatted bovine serum albumin (0.6%, w/v) (Garland and Randle, 1964), were incubated with rumen papillae and [U-^{14}C]glucose. All results are means ± S.D. and are given in terms of μmol end-products formed or glucose oxidised per mg DNA per 2h; * $P < 0.05$.

One possible site which could control glucose oxidation (and glycolysis), as a direct result of fatty acid conversion, is the pyruvate dehydrogenase complex. This inner mitochondrial membrane enzyme (Denton *et al.*, 1981) catalyses the reaction whereby acetyl-CoA is formed from pyruvate. Pyruvate dehydrogenase is regulated by product inhibition of the active dephosphoenzyme form (Tsai *et al.*, 1973) and by interconversion of the active enzyme and inactive phosphoenzyme by a specific protein kinase/phosphoprotein phosphatase system (Linn *et al.*, 1969a; Linn *et al.*, 1969b). Agents which regulate the kinase/phosphatase system are shown on Fig. 5.3.

Oxidation of fatty acids in rumen epithelium may cause increased mitochondrial acetyl-CoA and citrate contents and decreased CoASH content. Increased concentration ratios of acetyl-CoA/CoASH inhibits pyruvate oxidation by activating the protein kinase, which converts pyruvate dehydrogenase to the inactive phosphoenzyme. However, Siess and Wieland (1982) suggest that it is CoASH that acts as the physiological modifier of pyruvate dehydrogenase kinase activity.

The increase in mitochondrial citrate content regulates phosphofructokinase activity, since citrate inhibits this enzyme (Garland *et al.*, 1963). Thus, regulation of phosphofructokinase leads to increased concentrations of glucose-6-phosphate, which in turn directly regulates hexokinase activity (Newsholme and Start, 1973). This hypothesis would explain why all the fatty acids inhibited CO_2 production and caused a decrease in lactate production.



Activators of (a)	Inhibitors and Activators of (b)	
Calcium ions	pyruvate	acetyl-CoA/CoA
magnesium ions	ADP	$NADH/NAD^+$
	calcium ions	ATP/ADP
	dichloroacetate	

Fig. 5.3. Regulation of the Pyruvate Dehydrogenase Kinase/Phosphatase System (Denton *et al.*, 1981; Patel *et al.*, 1983)

Dibutyryl-cyclic AMP inhibits glycolysis in chick liver cells and the site of inhibition is thought to be at the phosphofructokinase-catalysed reaction (Lane and Mooney, 1981). Glucagon and dibutyryl-cyclic AMP did decrease lactate and CO_2 formation, though this was found to be non-significant. This requires further work to determine if rumen epithelial phosphofructokinase is regulated in an analogous manner to the liver enzyme.

Glutamine and NH_4Cl decreased lactate formation and glucose oxidation by about the same magnitude to the effect produced by fatty acids. However, it was found that flux through the pyruvate dehydrogenase reaction, assessed by measurement of decarboxylation of [1-^{14}C]pyruvate (or oxidation of [6-^{14}C]glucose)was increased by NH_4^+ (see Table 5.4). The conflicting NH_4^+ effects, on the one hand inhibition of glucose oxidation and on the other hand increased flux through the pyruvate dehydrogenase step, maybe explained by diversion of carbon skeletons away from the glycolytic pathway towards non-essential amino acid synthesis. This may also be true for the effect produced by glutamine, as metabolism of this amino acid and propionate *in vitro* results in significant alanine synthesis (see Fig. 7.3).

Butyrate significantly inhibited pyruvate oxidation, though this effect was almost abolished upon addition of NH_4^+ (see Table 5.4). This may account for the weak ketogenic effect of NH_4^+ on butyrate-dependent ketogenesis. Insulin is reported to stimulate liver pyruvate dehydrogenase activity (Wieland *et al.*, 1972) but no effects of insulin on the rumen epithelial enzyme were observed.

Additions	$^{14}CO_2$ Formation (nmol [1-^{14}C]Pyruvate utilised/mg DNA/h)	%
[1-^{14}CPyruvate (3mM)	424.5 ± 25.8	100.0
+ Butyrate (10mM)	256.9 ± 21.9*	62.9
+ Propionate (10mM)	455.2 ± 141.6	107.2
+ NH_4Cl (10mM)	735.2 ± 156.2*	173.2
+ Butyrate (10mM) NH_4Cl (10mM)	358.3 ± 127.9	84.4
+ Propionate (10mM) NH_4Cl (10mM)	661.9 ± 170.5	155.9
+ Insulin (50μM)	380.0 ± 150.9	89.5

Table 5.4. Effect of NH_4Cl, Insulin and Volatile Fatty Acids on [1-^{14}C]Pyruvate Oxidation in Rumen Epithelium

Isolated rumen papillae were incubated with [1-^{14}C]pyruvate plus volatile fatty acids, NH_4Cl and bovine insulin (final concentrations given in parenthesis). Results for release of $^{14}CO_2$ are means ± S.D. for triplicate incubations. *, $P < 0.05$.

Propionyl-CoA has been demonstrated to inhibit purified pyruvate dehydrogenase activity from various sources (Bremer, 1969; Gregersen, 1981), and the enzyme complex in perfused rat-liver (Patel *et al.*, 1983), by competing with CoASH is a similar fashion to acetyl-CoA. Propionate decreased [6-^{14}C]glucose oxidation in rumen epithelium (see Fig. 5.4) but did not, as expected, decrease the flux through the pyruvate dehydrogenase-catalysed reaction (see Table 5.4).

The absence of a propionate effect on [1-^{14}C]pyruvate oxidation may not necessarily mean that propionate does not inhibit glucose metabolism *in vitro* but that propionate has no influence on the 'lower activity' form of the enzyme. For instance, dichloroacetate, a pyruvate dehydrogenase kinase inhibitor (Weinburg and Utter, 1980), stimulated [1-^{14}C]pyruvate oxidation in rumen epithelium (see Fig. 5.5) but did not affect [6-^{14}C]glucose oxidation (see Fig. 5.4), even when rumen papillae were preincubated in the presence of dichloroacetate for 1h (results not shown). In separate experiments the stimulation of [1-^{14}C]pyruvate oxidation by dichloroacetate was found to be equal to the rate of $^{14}CO_2$ formation from [6-^{14}C]glucose (results not shown). Perhaps the lack of an effect of dichloroacetate on glucose oxidation may mean that glucose, or one of its metabolites, can activate pyruvate dehydrogenase. Therefore, propionate possibly inhibits pyruvate dehydrogenase when it is in the activated form. Such an inhibition could contribute to the

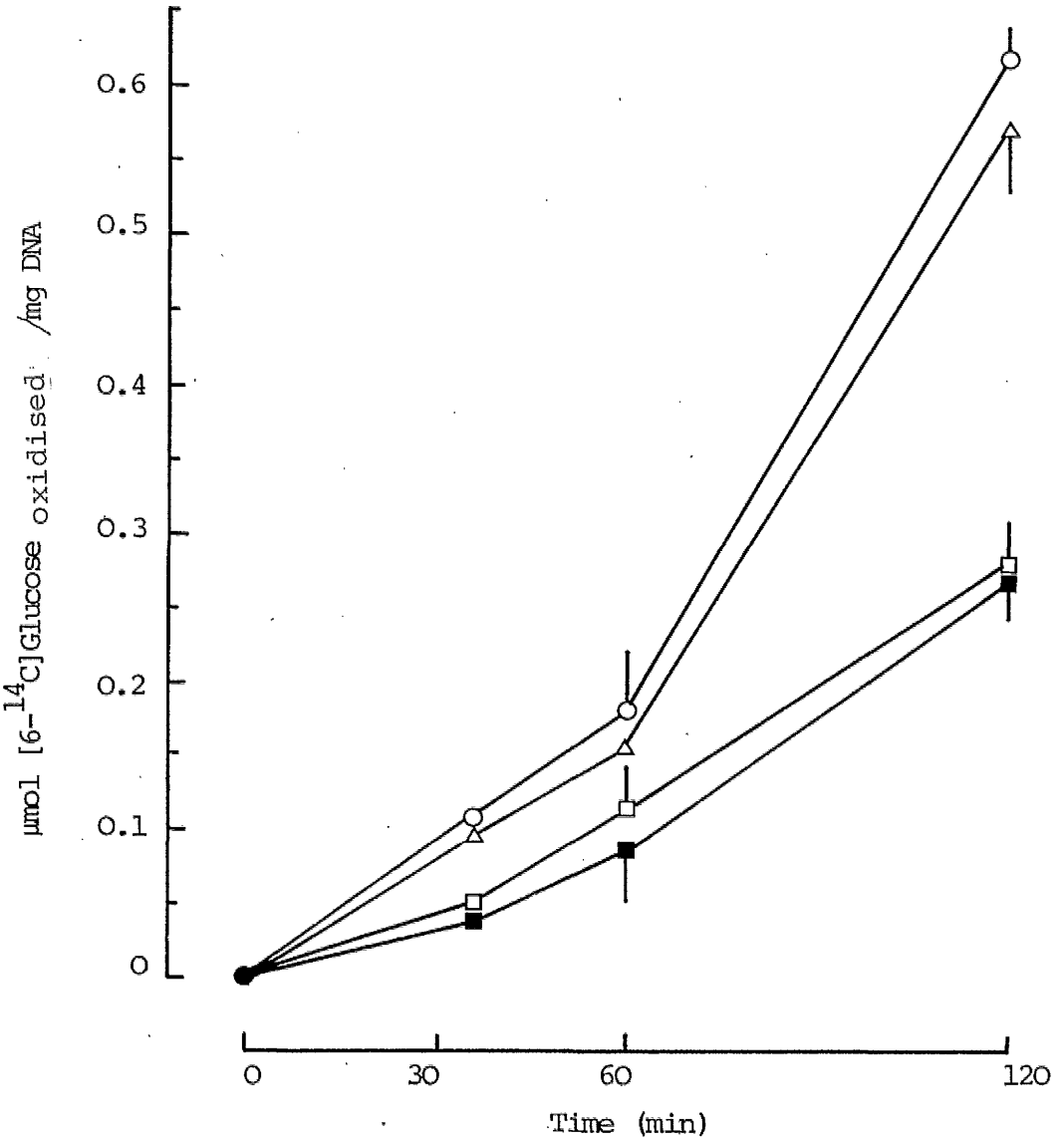


Fig. 5.4. Effect of Propionate and Dichloroacetate on [6-^{14}C] Glucose Metabolism

Rumen papillae were incubated with [6-^{14}C]glucose (O) (5mM). Incubations also contained glucose plus: dichloroacetate (△) (2mM); propionate (5mM) (■); and dichloroacetate and propionate together (□). All results are means ± S.D.

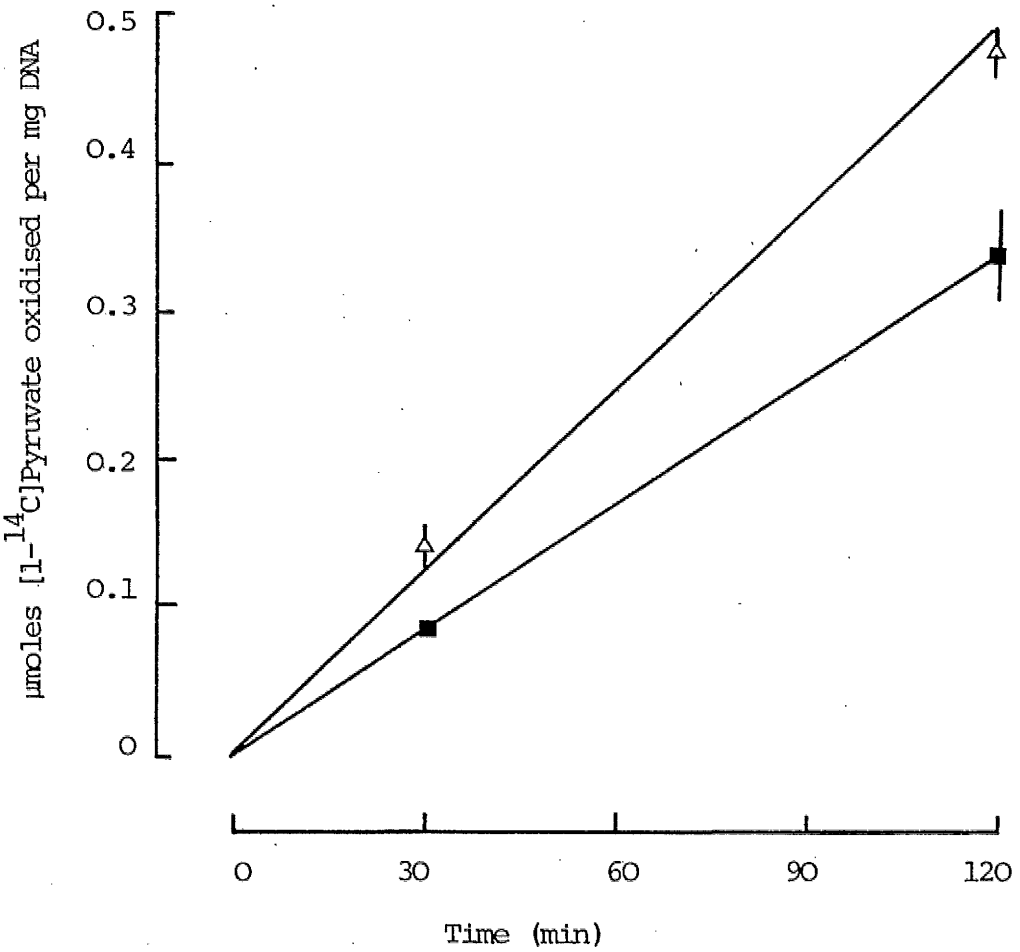


Fig. 5.5. Effect of Dichloroacetate on [1-^{14}C]Pyruvate Oxidation

Isolated rumen papillae were incubated with [1-^{14}C]pyruvate (1mM) (■) plus dichloroacetate (2mM) (Δ). $^{14}CO_2$ evolved from the tissue was collected and measured as described in section 2.13. Results are given as means ± S.D.

synergistic increase in lactate formation from glucose and propionate (Weekes, 1974), as some of the pyruvate, derived from glucose, would be diverted to lactate formation.

Lastly, it has been reported that NH_4Cl (10mM) significantly reduces net lactate formation from propionate, in comparison with incubations containing propionate alone. This inhibitory effect is caused, as Weekes (1974) proposes, by NH_4^+ draining 2-oxoglutarate away from the tricarboxylic cycle (and away from lactate synthesis) to glutamate formation. An alternative explanation might be that NH_4^+ can activate pyruvate dehydrogenase, even when the enzyme is subjected to inhibition by propionate (see Table 5.4). Decreased lactate production could then be accounted for in terms of pyruvate oxidation. Ammonium ions activate the pyruvate dehydrogenase complex in Ehrlich ascites tumour cells (Carrascosa *et al.*, 1982) and this is coupled with the observation that glycolytic lactate production decreases in the presence of NH_4^+ (Olarria *et al.*, 1981).

5.4. *Propionate Metabolism*

L-Lactate is the major end-product of the metabolism of propionate in rumen epithelium. However, the greater proportion of radioactivity from [1-^{14}C]propionate is accounted for as $^{14}CO_2$; the specific activity of the lactate formed is much less than that of propionate (Pennington and Sutherland, 1956b). A specific activity of lactate of one-half of that of propionate was predicted by Pennington and Sutherland (1956b). (This is because the hydroxyl group

added during the fumarase-catalysed reaction can be added to carbon 2 or 3 of fumarate (Lowenstein, 1967), giving L-malate radioactively labelled at carbon one or carbon four (see Fig. 3.)). Pennington and Sutherland (1956b) suggest that rapid interconversion of oxaloacetate and phosphoenolpyruvate which, together with rapid interconversion of oxaloacetate with fumarate, would lead to partial equilibration of the carboxyl-carbons of the dicarboxylic acids with the carbon of the incubation CO_2. In this present study the observation that PEPCK is located in the mitochondria would be evidence in support of this suggestion.

There was no inhibition of lactate production from propionate by n-butylmalonate, although malonate significantly decreased both CO_2 and lactate formation (see Fig. 5.6). n-Butylmalonate blocks malate transport across the mitochondrial membrane (see Table 4.7) and the absence of an effect by this inhibitor may mean that propionate is converted to oxaloacetate, which is subsequently decarboxylated to pyruvate (see Fig. 3.). However, the rate of lactate formation from propionate was not influenced by 3-mercaptopicolinate (MP) or 2-cyano-4-hydroxycinnamate (CHC), whether propionate was added as the only substrate (results not shown) or together with butyrate (see Table 5.5). Again, this would suggest that efflux of oxaloacetate, phosphoenolpyruvate or pyruvate (from oxaloacetate decarboxylation) from the mitochondria is not the major pathway through which propionate is metabolised. Therefore, because of the absence of an

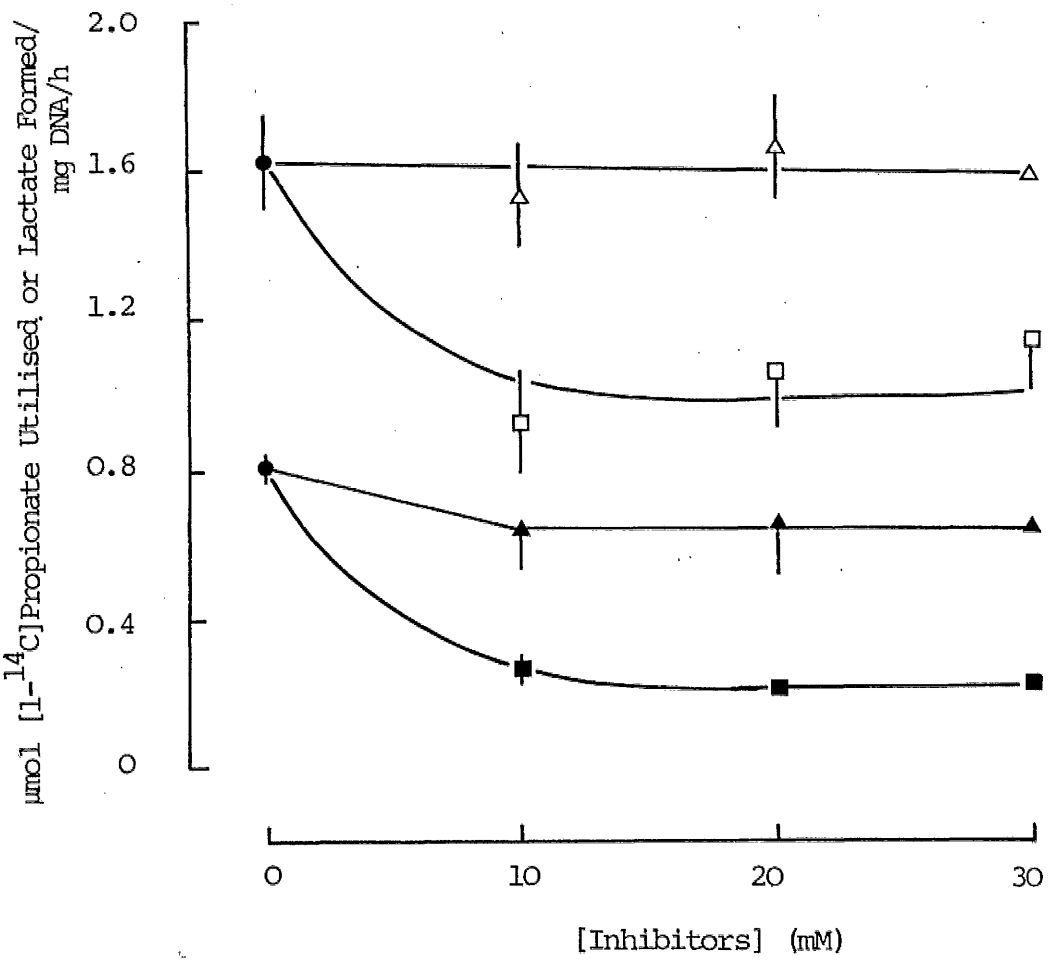


Fig. 5.6. Effect of Malonate and Butylmalonate on Propionate Metabolism

Isolated rumen papillae were incubated with [1-^{14}C]propionate (20mM). Malonate (□ and ■) and n-butylmalonate (△ and ▲) were added to the incubations and the lactate (□ , △) and $^{14}CO_2$ (■ , ▲) released from the tissue were measured as described in Chapter 2. All results are given as means ± S.D.

Substrate and Inhibitors	$^{14}CO_2$	Pyruvate	Lactate	[Lactate]/[Pyruvate]
	(μmol/mg DNA/2h)			Ratio
Propionate (15mM)	2.47 ± 0.16	0.11 ± 0.06	1.99 ± 0.16	18.1
Propionate, Butyrate (5mM)	2.80 ± 0.07	0.11 ± 0.06	3.75 ± 0.38	34.1
Propionate, Butyrate, 2-cyano-4-hydroxycinnamate (0.17mM)	2.80 ± 0.58	0.31 ± 0.02	4.22 ± 0.66	13.6
Propionate, Butyrate, 3-Mercaptopicolinate (0.5mM)	2.75 ± 0.19	0.16 ± 0.04	4.59 ± 0.21	28.9

Table 5.5. Effect of 3-Mercaptopicolinate and 2-Cyano-4-hydroxycinnimate on propionate metabolism in Rumen Epithelium

Rumen papillae were incubated with propionate for 2h. Additional flasks also contained butyrate, 2-cyano-4-hydroxycinnamate and 3-mercaptopicolinate (incubation concentrations are given in parentheses). Results are means ± S.D. for triplicate incubations.

effect by n-butylmalonate, MP and CHC the possibility that the penetration of inhibitor into the epithelium is limited cannot be ruled out. For instance, the inhibition of lactate formation by malonate was about 65% (after accounting for endogenous lactate production; see Fig. 5.6) but in other tissues, e.g. muscle, 10mM-malonate causes an inhibition of the order of 90% (Tepperman and Tepperman, 1958). In other studies the malonate inhibition of lactate formation, from propionate, in rumen epithelium is also only a partial inhibition (Pennington and Sutherland, 1965b; Weekes, 1974).

As the results from the metabolic inhibitors experiments were inconclusive mitochondria were isolated from rumen epithelium and propionate metabolism was studied with this system. It was expected that propionate conversion in the isolated mitochondria would result in a build up of the mitochondrial metabolite which is the precursor for lactate synthesis in the cytoplasm. Results given in Fig. 5.7a, show that it was possible to measure some propionate oxidation. This oxidation was completely inhibited by malonate (see Fig. 5.7a). Butyrate was added to incubations, which contained propionate, to supply acetyl-CoA to the mitochondria. Rates of ketone body production from butyrate ranged from 200-250nmol per mg mitochondrial protein.30min^{-1}.

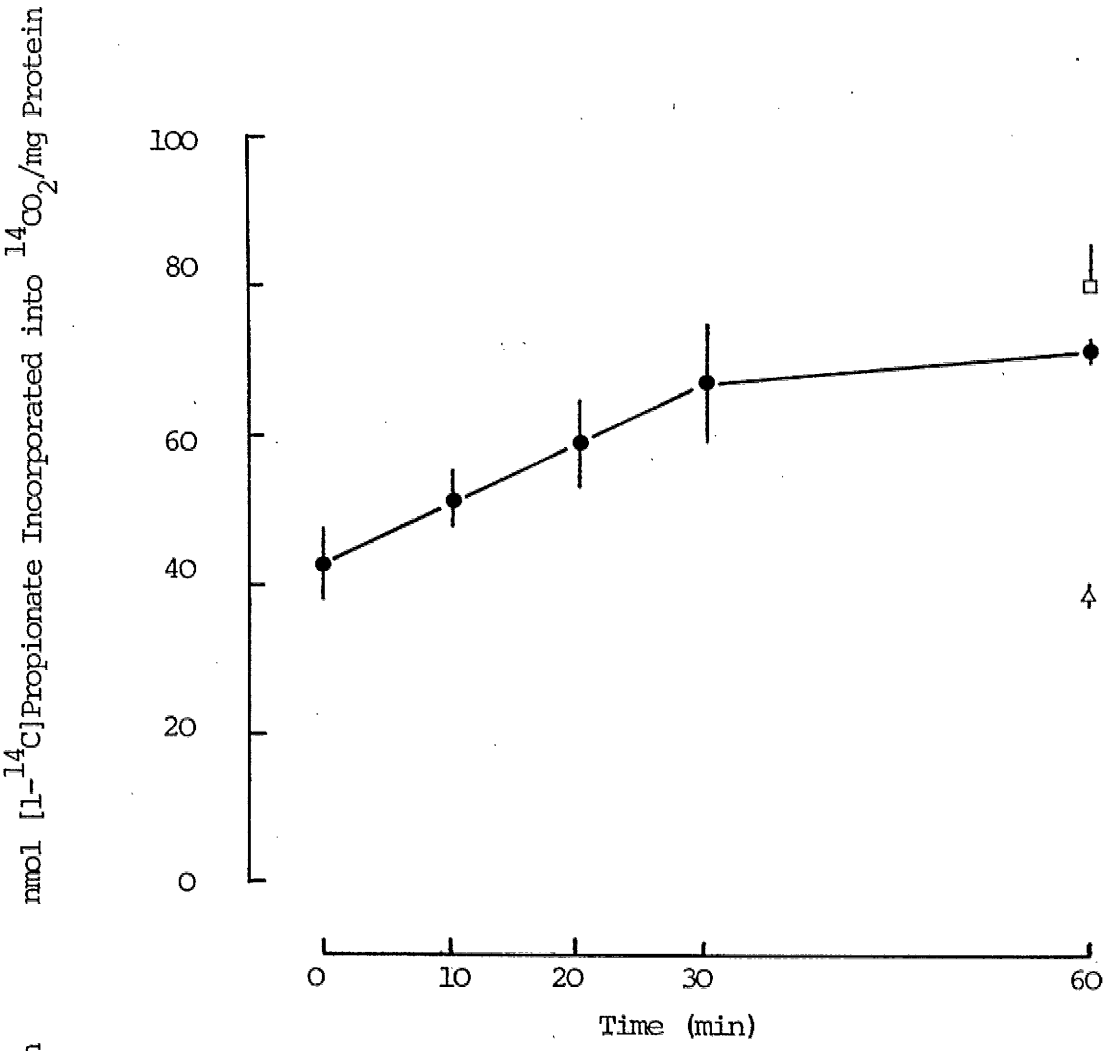
FIG. 5.7. [1-^{14}C]Propionate Oxidation in Isolated Rumen Epithelial Mitochondria

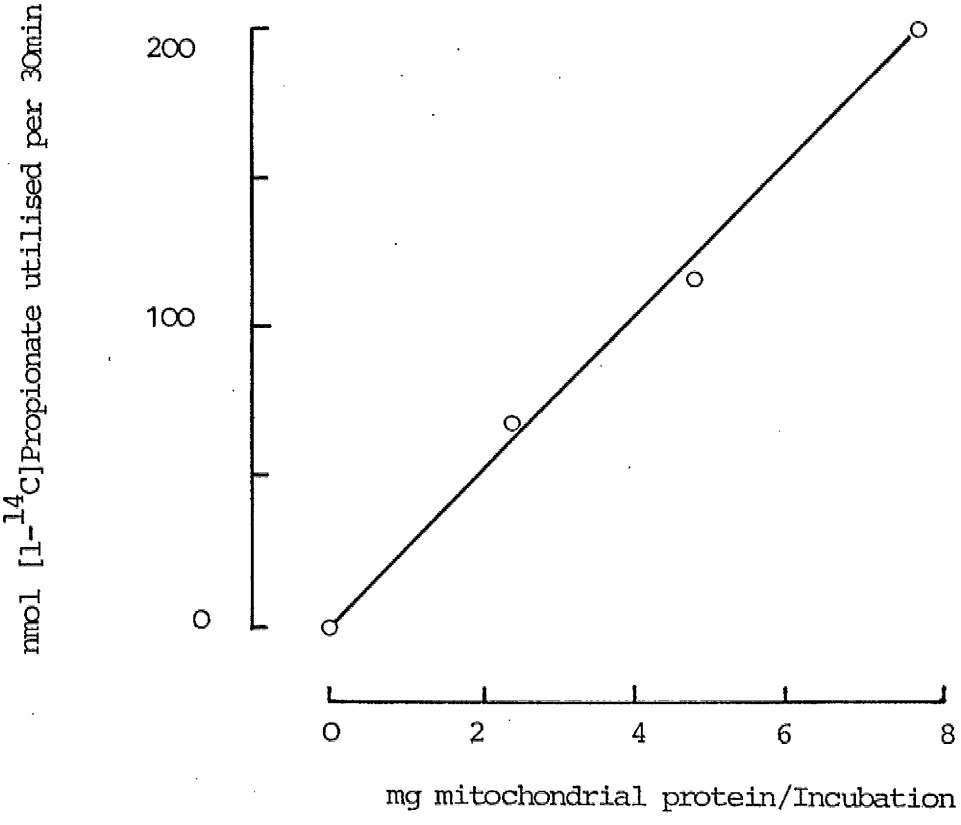
Top - Effect of Malonate (△) and Butyrate (□)

Mitochondria were isolated from sheep rumen epithelium as described in section 2.8. After the mitochondria were washed twice, they were incubated in 2ml of medium (given in section 2.8) with 10mM-[1-^{14}C]propionate and $^{14}CO_2$ released (●) was collected as described in section 2.13. Additional incubations contained 5mM-Malonate (△) or 2mM-butyrate (□). The mitochondrial protein content was determined with the Biuret protein assay described in section 2.12.2 and each flask contained 2.4mg of protein. Results are means ± S.D. from triplicate incubations.

Bottom - Oxidation as a Function of Mitochondrial Protein Concentration

Isolated epithelial mitochondria were incubated at 39°C with 10mM-[1-^{14}C]propionate as described above. $^{14}CO_2$ production (○) was measured for three different mitochondrial protein concentrations.


nmol [1-14C]Propionate Incorporated into 14CO2/mg Protein
100
80
60
40
20
0
0
10
20
30
60
Time (min)


nmol [1-14C]Propionate utilised per 30min
200
100
0
0
2
4
6
8
mg mitochondrial protein/Incubation

Rates of propionate oxidation were a linear function of mitochondrial protein concentration (see Fig. 5.7b). Measurement of metabolite accumulation in the incubation medium required that relatively high mitochondrial protein concentrations were used. However, 1-^{14}C propionate oxidation was similar whether high or low protein concentrations were used (see Fig. 5.8).

Accumulation of lactate, oxaloacetate, phosphoenolpyruvate, or pyruvate in the medium was not detectable. Although there was accretion of citrate this was not linear over the incubation period (citrate synthesis was about 2nmol/mg protein at 30 and 60 min). This was not true for malate (see Fig. 5.8) and because of accumulation of this metabolite it is proposed that the major route of propionate conversion to lactate is by efflux of malate from the mitochondria and subsequent oxidative decarboxylation of malate, by $NADP^+$ 'malic' enzyme, to pyruvate.

The marked stimulation of propionate metabolism by butyrate was not expected (see Table 5.5). Experiments with acetate did not result in similar effects being observed. It was found that butyrate was a most effective stimulator at relatively low concentrations (see Fig. 5.9). The results given in Fig. 5.9 are not representative, in terms of the magnitude of the stimulation, because in other experiments CO_2 and lactate production, although increased, were lower (see Fig. 5.10). However, although the effect of butyrate was variable there was always an observed stimulation of propionate metabolism.

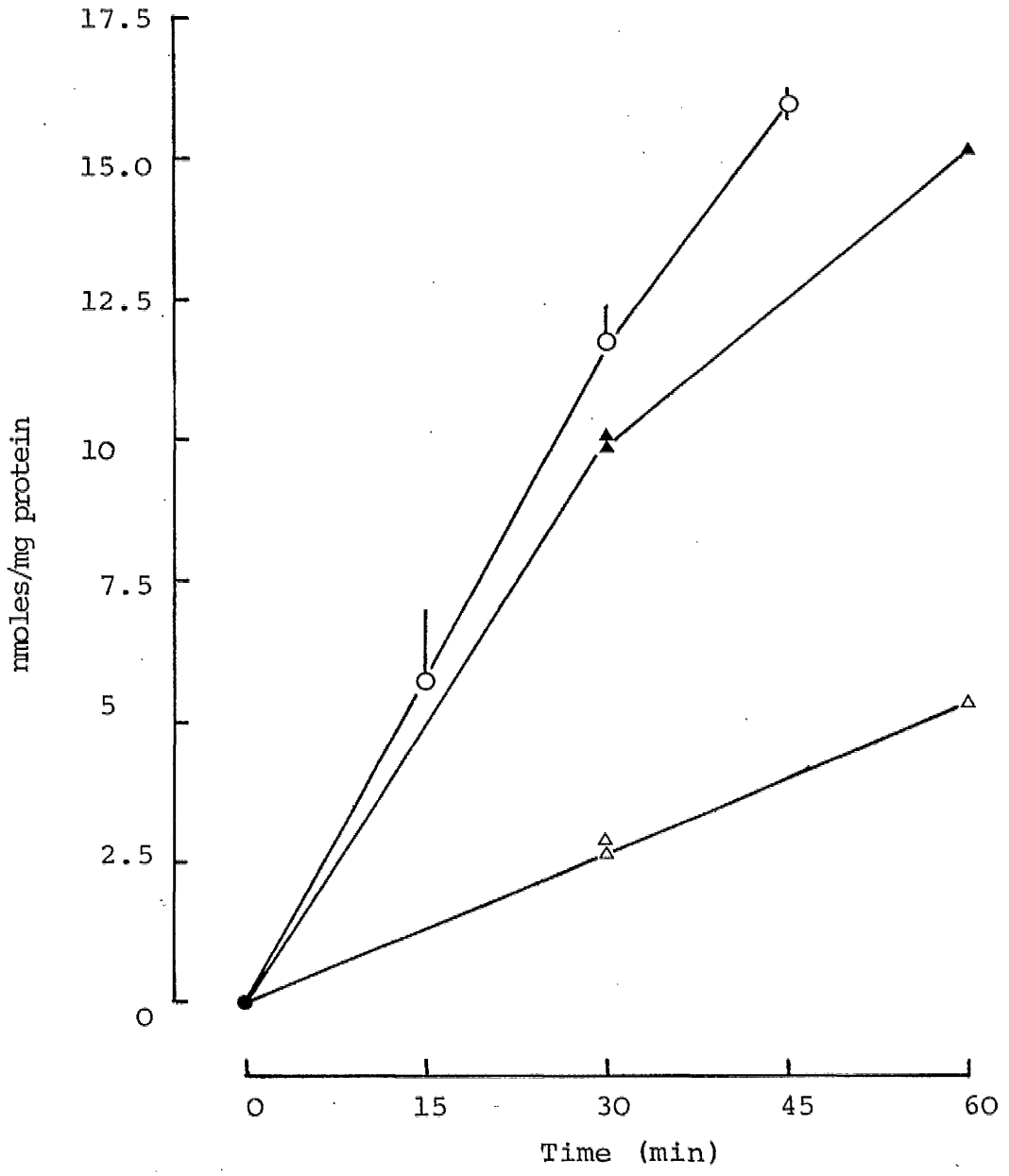


Fig. 5.8. Malate and CO_2 Formation in Isolated Mitochondria

Isolated rumen epithelial mitochondria were incubated with 15mM-[1-^{14}C]propionate and 5mM-butyrate as described in the legend to Fig. 5.7a. Malate (△) and $^{14}CO_2$ (▲) produced by the mitochondria were measured as described in Chapter 2. The final concentration of protein in the incubation flasks was 14.3 mg protein per ml. Also given is $^{14}CO_2$ formation (○) from 10mM-[1-^{14}C]propionate and 2mM-butyrate in incubations which contained about 10 times lower protein concentration (i.e. 1.6mg mitochondrial protein per ml). These results are given as means ± S.D. for triplicate incubations.

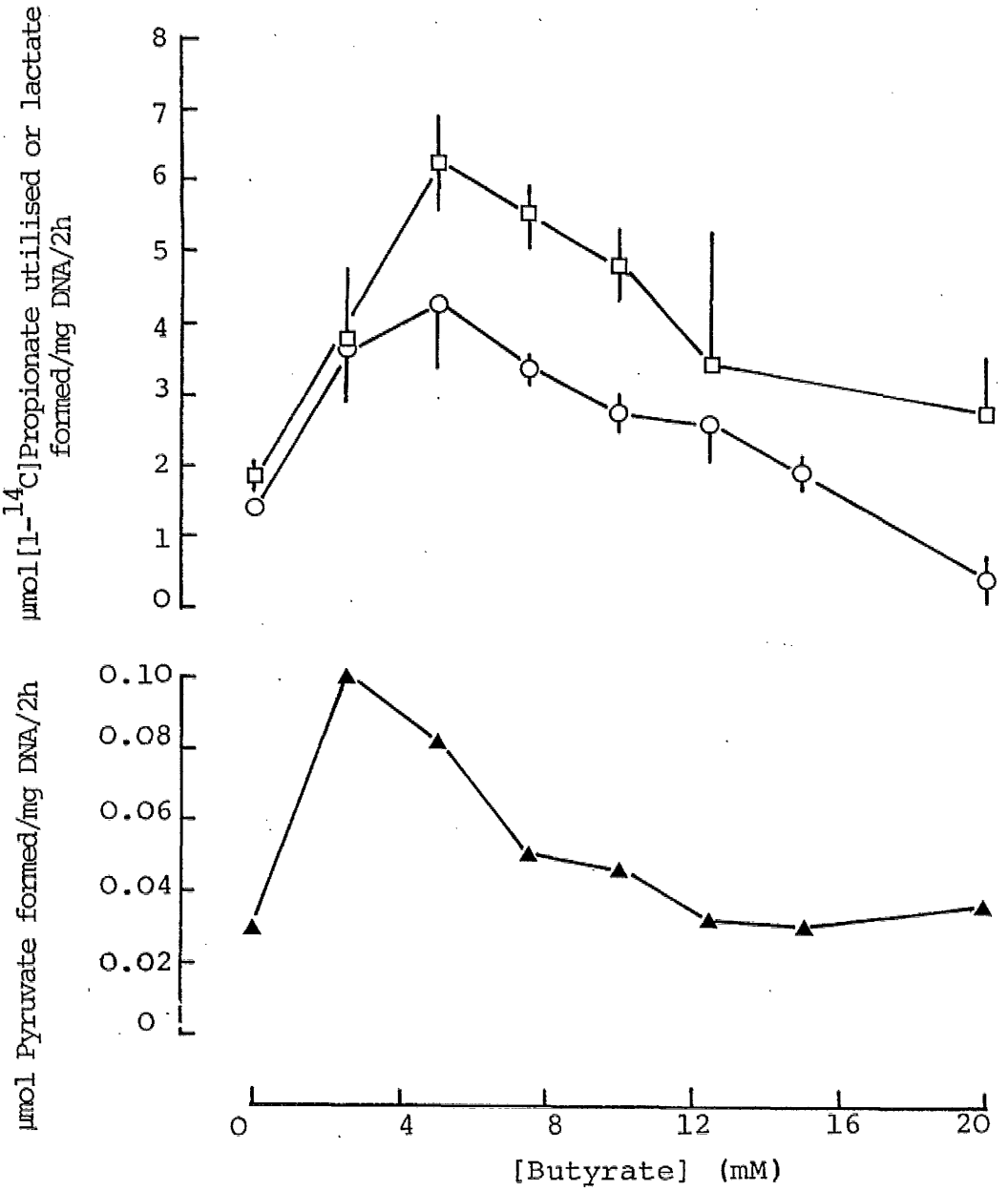


Fig. 5.9. Effect of butyrate on propionate Metabolism

[1-^{14}C]Propionate (15mM) was added to incubations containing rumen papillae. Pyruvate (▲), lactate (□) and $^{14}CO_2$ (○) released from the tissue were measured as described in Chapter 2. Other incubations contained various concentrations of butyrate (2.5 - 20mM). All results are means ± S.D.

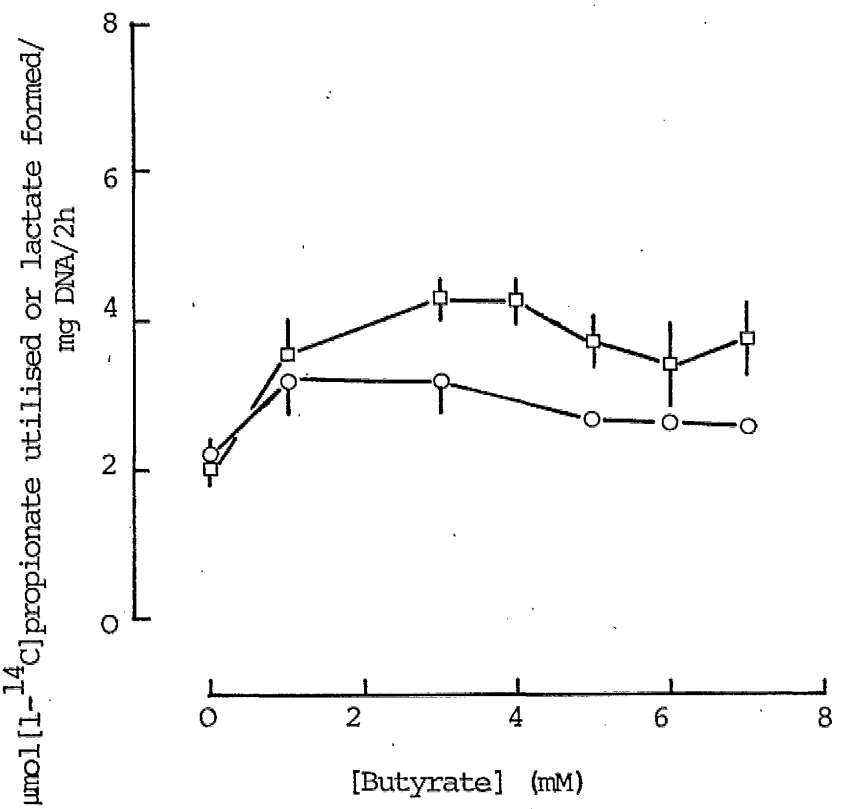


Fig. 5.10. Effect of Butyrate on $^{14}CO_2$ (○) and Lactate (□) Production from Propionate in Rumen Epithelium

Isolated rumen papillae were incubated with 15mM-[1-^{14}C] Propionate and butyrate as described in the legend to Fig. 5.9. Lactate (□) and $^{14}CO_2$ (○) produced by the tissue over a 2h incubation period are given in terms of means ± S.D. for triplicate incubations.

Butyrate is a strong inhibitor of propionyl-CoA synthetase activity in homogenates prepared from cattle and sheep rumen epithelium (Ash and Baird, 1973; Scaife and Tichivanganam, 1980). Therefore, stimulation of propionate utilisation by butyrate may not be caused by an increase in acetyl-CoA supply, from butyrate, to convert intramitochondrial oxaloacetate to citrate. Smith (1971) suggests that the lowering in oxaloacetate concentration would result in a de-inhibition of succinate dehydrogenase and thus cause an increase in propionate metabolism.

Another suggestion which would overcome the inhibition of the propionate-activating capacity, caused by butyrate, is that an enzyme exists in rumen epithelium which can catalyse the transfer of the coenzyme A moiety from butyryl-CoA to propionate. Pritchard and Tove (1960) were first to suggest this after they had found that butyrate stimulated the metabolism of propionate in liver from rats and non-fasted sheep, though to a lesser extent than fasted sheep. This hypothesis is attractive because butyrate:acetoacetyl-CoA transferase activity has recently been measured in cell-free extracts of bovine rumen epithelium (Emmanuel and Milligan, 1983). This enzyme was also able to transfer CoA to acetate and propionate. It may well be that the enzyme has a broad specificity and can utilise acetoacetyl-CoA but higher activities may be found with butyryl-CoA as the main substrate.

Pritchard and Tove (1960) postulate that if the activation of butyrate is more rapid than the activation of propionate, then a net reaction involving the activation of butyrate with CoASH and the transfer of CoA from butyryl-CoA to propionate would occur more rapidly than the direct activation of propionate. This would happen more readily at lower butyrate concentrations as the inhibition of propionyl-CoA synthetase activity caused by butyrate would not be as great. Of course this hypothesis requires much more evidence, such as the determination of the existence of a specific CoA-transferase in the epithelium and further work along these lines is needed.

5.5. *Fatty Acid and Cholesterol Synthesis in Rumen Mucosa*

The presence of lipid droplets in rumen epithelium has been recorded (Hable, 1959), though this is ascribed to triglycerides produced during keratinisation (Lavker, 1975). Garton (1965) suggests that the droplets may be the result of fatty acid uptake and synthesis. Water permeation in keratinised tissues is controlled by intercellular lipid lamella based on gangliosides, ceramides, cholesterol and free fatty acids (Birkby *et al.*, 1982). However, there are few published values for rates of lipid synthesis in rumen epithelium *in vitro*. Ingel *et al.* (1982) have measured incorporation of [1-^{14}C]-acetate into fatty acids in sheep liver, adipose

and intestinal tissues *in vivo*. They found that fatty acid synthesis in rumen epithelium contributed about 1% to the combined tissue rates of lipogenesis.

Rates of lipogenesis in rumen mucosa *in vitro* were also low (see Table 5.6), compared to fatty acid synthesis in isolated sheep hepatocytes (rates in the latter cells are about 0.8μg atoms ^{3}H incorporated into fatty acids per g wet wt. per h: N.P.J. Brindle, personal communication). Rumen epithelial fatty acid synthesis was not stimulated, to any extent, by potential lipogenic precursors (see Table 5.6).

However, of the substrates tested butyrate and propionate, when added alone, were the main precursors for lipid and cholesterol synthesis, respectively (see Table 5.7). Seto *et al.* (1971) reported that butyrate was quantitatively the best substrate for bovine rumen mucosal fatty acid synthesis *in vitro*.

The pathways of fatty acid and cholesterol synthesis draws upon the same cytoplasmic acetyl-CoA precursor pool. Therefore, measurement of these two synthetic rates is vindication of observations of appreciable acetoacetyl-CoA thiolase and HMG-CoA synthase activities in rumen mucosal cytoplasmic extracts (see Table 3.3).

Substrates Added	Lipogenesis	Cholesterogenesis
	(nmol acetyl units incorporated/g wet wt/3h)	
None	58 ± 15	20 ± 10
Glucose, Acetate, Propionate, L-Lactate	100 ± 22	36 ± 7
Glucose, Acetate, L-Lactate	108 ± 40	143 ± 44
Glucose, Acetate, Propionate, Butyrate	200 ± 57	174 ± 113

Table 5.6. Fatty Acid and Cholesterol Synthesis in Rumen Papillae

Rumen papillae were incubated with combinations of glucose (5mM), acetate (5mM), propionate (5mM), butyrate (2mM) or lactate (5mM). $^{3}H_2O$ (0.1 mCi/ml) was added to the incubations and after 3h ^{3}H-labelled fatty acids and cholesterol were extracted and measured as described in section 2.15. Zero-time control were also included to measure non-specific radioactive incorporation into the lipid fractions. Results are means ± S.D.

Substrates	Fatty Acids	Non-Saponifiable Fatty Acids	Cholesterol	Aqueous
None	2.1 ± 0.2	0.8 ± 0.6	0.1 ± 0.1	9.0 ± 2.5
D-[U-^{14}C]Glucose	15.1 ± 1.0	6.2 ± 0.3	2.0 ± 0.3	46.1 ± 5.2
L-[U-^{14}C]Lactate	17.6 ± 0.5	7.2 ± 3.3	2.6 ± 1.7	30.6 ± 2.4
[U-^{14}C]Acetate	3.6 ± 0.6	1.6 ± 0.3	0.8 ± 0.2	5.6 ± 1.9
[1-^{14}C]Propionate	16.2 ± 1.2	7.1 ± 1.5	4.0 ± 0.4	20.1 ± 2.1
[1-^{14}C]Butyrate	88.5 ± 7.2	4.2 ± 1.8	0.5 ± 0.1	22.1 ± 2.9

Table 5.7. Incorporation of [^{14}C]Substrates into Lipogenic Fractions Extracted from Sheep Rumen Epithelium

^{14}C-labelled substrates (10mM, 0.5 μCi/ml) were incubated with rumen papillae and various fractions isolated as described in section 2.15. Results reported in terms of nmol ^{14}C-labelled substrate incorporated per g wet wt per 2h and are in terms of means ± S.D.

Propionate was the best carbon source for cholesterol biosynthesis and a similar observation has been made for rat-liver extracts (Davis *et al.*, 1975). Incidentally the final step in cholesterol catabolism is the release of propionic acid from bile acid (Hagly and Krisans, 1982). In rat liver the two carbon unit from propionate which enters isoprenoids is derived from the methylene and methyl carbon atoms (Davis, 1978). The intermediary pathway does not appear to involve a common acetate pool nor can it be explained by involvement of citric acid intermediary metabolism. One suggestion is that β-oxidation of propionate to malonyl-CoA occurs (Davis, 1978), as malonyl-CoA may be involved in isoprenoid synthesis (Brodie *et al.*, 1963; Rudney *et al.*, 1966).

It is concluded that lipid synthesis in rumen epithelium, although low, is measurable and is probably necessary for primarily intracellular membrane or lamellae formation.

5.6. *Summary*

$^{14}CO_2$ production is greatest from ^{14}C-labelled propionate and butyrate. In comparison, rates of glucose oxidation are much lower. Short-chain and long-chain fatty acids decrease both lactate and CO_2 production from glucose in rumen epithelium, an effect which possibly involves regulation of pyruvate dehydrogenase activity. It is proposed that NH_4^+ decreases lactate production from propionate by stimulation of pyruvate dehydrogenase activity, thus increasing pyruvate oxidation.

Evidence is presented which indicates that the main pathway for propionate metabolism is through malate efflux from the mitochondria. Malate in the cytoplasm is then converted to pyruvate by $NADP^+$ 'malic' enzyme. Butyrate, at relatively low concentrations, stimulates propionate metabolism. Possible means to bring about this stimulation are discussed.

Rates of fatty acid and sterol synthesis are low but measurable; butyrate and propionate are, quantitatively, the most significant substrates for both pathways respectively.

C H A P T E R 6

INCORPORATION OF RADIOLABELLED LEUCINE INTO PROTEIN IN RUMEN EPITHELIUM

6.1. *Introduction*

Protein synthesis is important for the maintenance and function of cells and organs. Therefore, it seems surprising that regulation of this process in rumen epithelium has not been studied to any extent, considering that proliferation and differentiation of this tissue may be under hormonal control (Sakata *et al.*, 1980a)and may also be influenced by ruminal VFA levels (Sakata and Tamate, 1980b).

Rumen mucosa does not turn over as fast as other gastrointestinal tissues, probably as a result of the protection provided by the outermost layers of keratinised cells. However, the study of the influence of nutrients and metabolites on rumen mucosal protein synthesis may provide information towards an understanding of factors which regulate this process in the epithelial tissue.

Incorporation of radioactively-labelled amino acids into protein is a convenient method for measuring protein synthesis. Some aspects of rumen mucosal protein synthesis, measured by this method, are described in this chapter.

6.2.1. *Materials and Methods*

Stomach pouches were transported from a local abattoir and washed in the laboratory as described in Chapter 2. Papillae were isolated from the anterior ventral sac and, after washing, suspended in an amino acid incubation medium before addition to incubation flasks.

6.2.2. *Amino Acid Incubation Medium*

The medium used to investigate protein synthesis in isolated rat hepatocytes (Dr. S. McCune, personal communication) was employed in this study and consisted of:

	Final Concentrations
Minimum Essential Medium (MEM; Eagles, 1959) with Hanks' Salt	Diluted 10 times
Defatted Bovine Serum Albumin (BSA)	1% (w/v)
$NaHCO_3$	12.5mM
Glutamine	2mM
Penicillin G	0.5µg/ml
Streptomycin sulphate	1.0µg/ml
L-[4,5-^{3}H]leucine	1-3.3µCi/ml

After addition of all reagents, except L-[4,5-^{3}H]leucine, the solution was gassed with O_2:CO_2 (95:5%) and adjusted to pH 7.4, if necessary. The final concentration of leucine was 0.4mM.

6.2.3. *Modified Minimum Essential Medium (Eagles)*

A modified buffer was prepared free of glucose and leucine, normal constituents of the commercially supplied buffer.

Reagents (final concentration, mg/l):

L-Arginine HCl	126.4	NaCl	8000.0
L-Cystine (Na_2)	38.4	KCl	400.0
L-Glutamine	292.0	KH_2PO_4	60.0
L-Histidine HCl.H_2O	41.9	Ca D-Pantothenate	1.0
L-Isoleucine	52.9	Choline Chloride	1.0
L-Lysine HCl	73.1	Folic acid	1.0
L-Methionine	14.9	i-Inositol	2.0
L-Phenylalanine	33.0	Pyridoxal-HCl	1.0
L-Threonine	47.6	Riboflavin	0.1
L-Tryptophan	10.2	Thiamine HCl	1.0
L-Tyrosine	36.2	Penicillin G	0.5
L-Valine	46.9	Streptomycin Sulphate	1.0

Modified MEM (Eagles) was made up 10 times concentrated. $CaCl_2$ (186mg/l) and $MgSO_4$ (200mg/l) were added to the diluted buffer, after gassing with O_2:CO_2 (95:5%). The buffer was adjusted to pH 7.4, if necessary.

6.2.4. *Conditions for Incubations In Vitro*

Papillae were normally incubated in Erlenmeyer flasks containing 2ml gassed incubation medium; incubations were started by addition of [^{3}H]leucine. Conditions were essentially as described in section 2.8 except that, over long incubation periods, flasks were gassed with O_2:CO_2 (95:5%) every hour.

Method I: Initially, incubations were terminated by addition of 0.2ml 50% (w/v)-trichloroacetic acid (TCA) and kept on ice until both medium and papillae were homogenised with a ground glass homogeniser.

Method II: In later experiments, incubations were stopped by addition of 4ml incubation medium containing 5mM-'cold' leucine (4°C); medium was then removed by aspiration and 4ml 5% (w/v)-TCA (4°C) was added to the residual papillae before homogenisation with a ground glass homogeniser.

6.2.5. *Estimation of [^{3}H]leucine Incorporation into Protein*

After homogenisation, portions of extract were removed and stored at -80°C (for ATP and DNA assays, Chapter 2). Incorporation of [^{3}H]leucine was estimated by the following procedure: 0.2ml of vigorously mixed homogenates were transferred to Beckman tubes, the tubes were centrifuged at 12 000g for 2 min and excess liquid was removed by aspiration; pellets were washed with 0.3ml of ice-cold 5% (w/v)-TCA by vortex mixing and dispersion with a piece of fine metal wire before centrifugation and aspiration. The washing procedure was repeated twice more before the last 1mm of the tubes were cut off. 0.03ml Nuclear Chicago Solubiliser (NCS) was added to each tube; after 30 min the tubes and solubilised pellets were added to 1ml PCS; the whole was vigorously mixed before counting for ^{3}H in a Beckman LS 9800 Scintillation Counter.

High random coincidence counts were observed in samples. This may have been the result of the strongly alkaline nature of NCS producing two separate measurable events within the resolving time of the photomultiplier. Acidification of the scintillant mixture resolved this problem.

The amount of protein synthesised was calculated from the [^{3}H]leucine incorporated into TCA-insoluble pellets divided by the specific radioactivity of [^{3}H] leucine in the incubation medium. Samples were corrected, with appropriate zero-time controls, for non-specific radioactivity incorporated into the pellets.

6.2.6. Method for Measuring Protein Degradation

Isolated papillae (about 3g wet wt.) were incubated for 3h at 39^{o}C, in 20ml incubation medium containing [^{3}H]leucine (3μCi/ml). After the incubation period was completed the medium was removed by aspiration and 25ml of medium, with additional 'cold'-leucine (5mM), was added to the papillae before re-incubation for 20 min; this washing procedure was repeated twice more before collection of papillae and distribution into Erlenmeyer flasks containing gassed incubation medium (2ml).

Further incubations were carried out and, after the designated experimental period, 0.1ml of medium was removed and counted for ^{3}H in 1.2ml PCS, the remaining medium was aspirated; 4ml of 5% (w/v)-TCA was added to the residual

papillae before homogenisation; 0.1ml of the 'TCA-fraction' supernatant was also counted for ^{3}H in 1.2ml PCS.

6.2.7. *ADP and AMP assays*

The ATP concentration of test samples was always measured before ADP and AMP contents were determined. ADP was enzymatically converted to ATP by pyruvate kinase (EC 2.7.1.40), whilst ATP was formed from AMP through the coupled pyruvate kinase/adenylate kinase (EC 2.7.4.3) system.

ADP measurements were carried out by addition of 2µl 175mM-KCl/30mM-$MgCl_2$ and 3µl 30mM-phosphoenolpyruvate containing 80 Units/ml of pyruvate kinase. The mixture was incubated at 20°C for 30 min before ATP was assayed as described in section 2.18.3.. AMP was determined in a similar manner except that the phosphoenolpyruvate solution contained, in addition, 279 Units/ml adenylate kinase.

Adenylate concentrations in samples were determined from a standard curve (see Fig. 6.1).

6.2.8.1. *Polyacrylamide Gel Electrophoresis*

Polyacrylamide gel electrophoresis (PAGE) of denatured protein was based on the method reported by Laemmli and Favre (1973). Analysis by electrophoresis was carried out with 10% disc gels at 20°C.

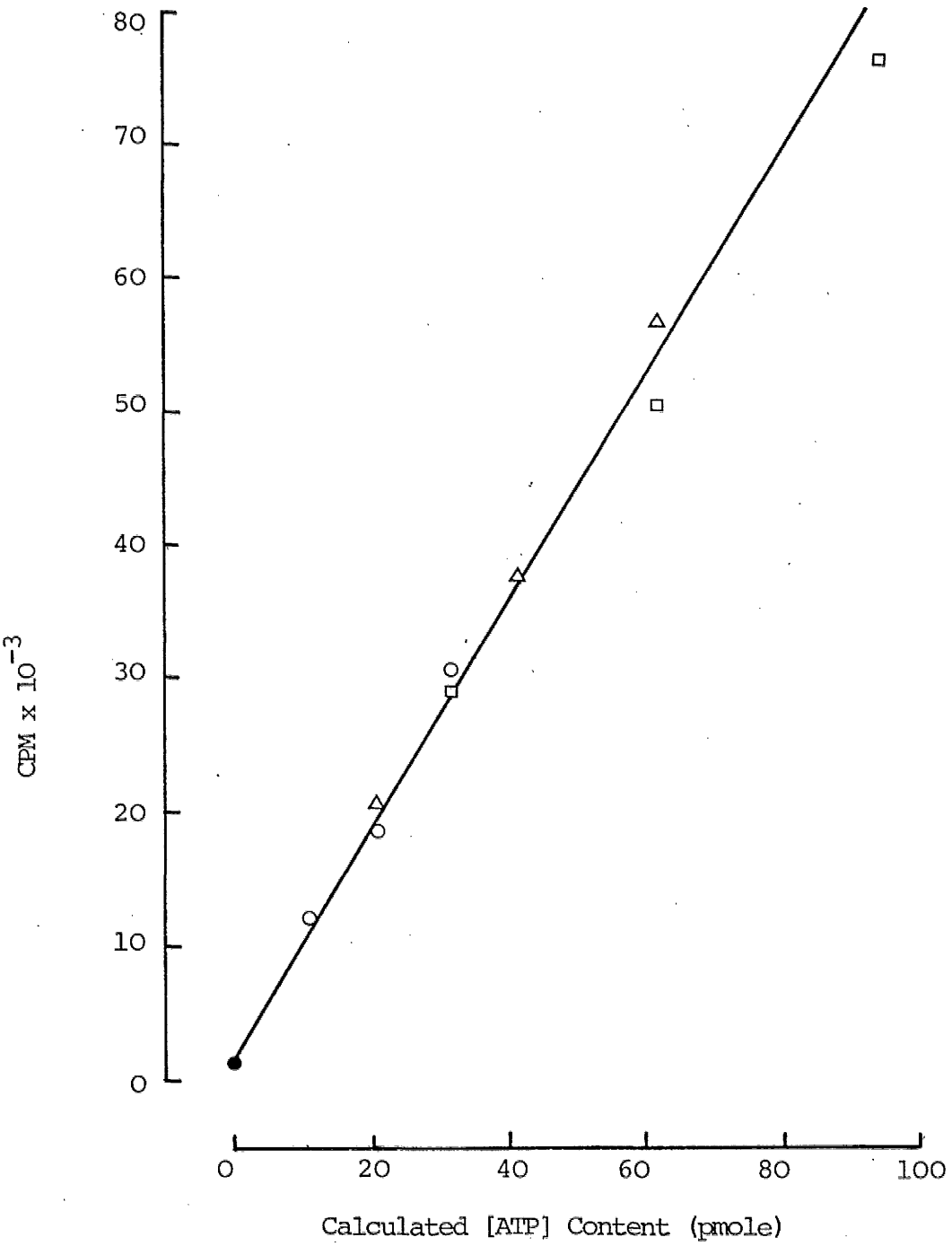


FIG. 6.1. Linearity of ADP and AMP Luciferase Assay

A standardised solution containing ADP, AMP and ATP (each at a final concentration of 1.05μM) was assayed for ATP content by the luciferase method. Samples (100μl) were either treated with pyruvate kinase (ATP + ADP) △ , pyruvate kinase/adenylate kinase (ATP + ADP + AMP) □ , or not treated at all (ATP) ○ before 10, 20 and 30μl samples were removed and assayed for ATP as described in section 2.18.3.

Stock Solutions:

A. Acrylamide; 30% (w/v)-acrylamide
0.8% (w/v)-N,N'-bismethylene acrylamide

B. Separating gel buffer, pH 8.8, 18.15% (w/v)-Tris base
0.4% (w/v)-Sodium dodecylsulphate (SDS)

C. Stacking gel buffer, pH 6.8; 6.0% (w/v)-Tris base
0.4% (w/v)-SDS

D. Electrode buffer, pH 8.8; 0.6% (w/v)-Tris base
2.89% (w/v)-glycine
0.2% (w/v)-SDS

E. Ammonium persulphate solution; 0.3% (w/v)-$(NH_4)_2S_2O_8$, freshly prepared.

Preparation of Separating and Stacking Gels: immediately before preparation of gels the following solutions were mixed together;

Solutions	Volumes (ml) required for Separating Gel	Stacking Gel
H_2O	8.31	5.64
Buffer solution (B or C)	5.00	2.50
Acrylamide (A)	6.67	1.75
N,N,N',N'-Tetramethyle-thylenediamine	0.02	0.01
Ammonium Persulphate solution (E)	0.20	0.10

The separating gel solution was dispensed into 8cm x 0.5cm siliconised glass tubes ($^3/4$ of the volume); after careful addition of H_2O, the separating gels were allowed to polymerise before 0.2ml of the stacking gel solution was added to the tubes.

Sample Buffer: contained in the sample buffer was;

0.76g	Tris base
2.0g	SDS
10ml	glycerol
37mg	Na_2 EDTA
40ml	H_2O

This solution was adjusted to pH 6.8 with HCl and made up to 50ml with H_2O.

3-Mercaptoethanol and bromophenol blue were added to the sample buffer, before protein samples were denatured, at 0.9ml and 5mg per 50ml, respectively, to give the 'complete sample' buffer.

Destain I: 900ml methanol and 184ml glacial acetic acid were mixed before being made up to 2 litres with H_2O.

Destain II: 100ml methanol was added to 150ml glacial acetic acid; this solution was brought to a final volume of 2 litre with H_2O.

Coomassie Solution: 1.2g Coomassie brilliant blue R was dissolved in 250ml destain I by stirring overnight and stored in a dark container after passing through Whatman No. 1 filter paper. Best results were obtained with freshly prepared solutions.

6.2.8.2. *Preparation of Samples and Analysis by Electrophoresis*

'Complete sample' buffer (50µl) was added to approximately 100µg of protein and heated at 100°C for 5 min before loading the denatured protein on to the stacking gel. Electrophoresis was carried out at 2mA/gel and continued until the bromophenol blue band was near the end of the separating gel. The dye bands were marked with pieces of fine wire before the gels were placed in Coomassie solution for at least 30 min. Several changes of destain I were used to remove unbound Coomassie stain. Gels were rehydrated in destain II (diluted 1:1 with water) before scanning or slicing.

6.2.8.3. *Scanning and Slicing of Gels*

Scanning was carried out at 635nm using an LKB 2202 Ultrascan Laser Densitometer (LKB Instruments, South Croydon, U.K.), connected to a chart recorder. This process was normally carried out before the slicing of gels with a Mickle Gel Slicer (Mickle Engineering Ltd., Gomshall, Surrey). Slicing involved freezing the gel with crushed solid-CO_2, before mounting on the platform of the

gel slicer. Usually 60-70 slices were obtained per gel; 0.2ml of '20 vol.'-H_2O_2 was added to each slice, contained in scintillation tubes, before heating at 80°C for 3 to 4h. PCS (1ml) was added to each tube and, after being mixed, they were counted for ^3H in a scintillation counter. H_2O_2 bleached the Coomassie stain in the gel and as a result no luminescence was observed.

6.2.8.4. *Calibration of Gels*

A solution containing denatured standard proteins was analysed, along with the samples, by electrophoresis. The standard protein solution contained (subunit molecular weights are in parentheses): β-D-galactosidase (EC 3.2.1.23, 130 000); catalase (EC 1.11.1.6; 60 000); pyruvate kinase (57 000); creatine kinase (EC 2.7.3.2; 40 000); myoglobin (17 200). The subunit molecular weights of rumen epithelial proteins were estimated from a semi-log plot of molecular weights of standard proteins versus their R_f values.

6.2.9. *Extraction of Prekeratin*

Prekeratin was extracted from rumen epithelium by the method described by Matoltsy (1964), as modified by Skerrow (1974). Papillae incubated in the presence of L-[4,5-^3H]leucine (3μCi/ml), for 5h at 39°C, were separated from the medium before washing in three changes of 'cold'-incubation medium; 10ml 0.1M-citric acid/sodium citrate (CASC) buffer, pH 2.6, was added to the papillae before homogenisation with a ground glass homogeniser. The

homogenate was centrifuged at 3000g for 10 min at 20°C and the supernatant was decanted.

The pH of the supernatant was raised to pH 3.5 by addition of 0.1M-NaOH dropwise and constant stirring. At this pH prekeratin is reported to be one of the first proteins to be precipitated (Skerrow, 1977; Matoltsy *et al.*, 1981). The precipitate was collected by brief centrifugation and dissolved in 3ml 0.1M-CASC buffer, pH 2.6. Further purification was achieved by reprecipitation (twice) before dissolution of the pellet in the minimal volume of 0.1M CASC buffer, pH 2.6. Protein concentration was measured using the dye-protein assay (section 2.12.1).

6.3. *Results*

6.3.1. *Time Course of Rumen Mucosal Protein Synthesis*

After an initial lag phase, L-[4,5-^{3}H]leucine was incorporated into protein in rumen epithelium as a linear function of time (see Fig. 6.2). However, average standard deviations were in the range of 14-17% when expressed as a percentage of the mean. Experimental errors may have been accentuated by [^{3}H]leucine binding tightly to defatted BSA (Rotermund *et al.*, 1970), present in the incubation medium. Papillae were separated from the medium, as little of the newly-synthesised protein was secreted by epithelial cells (see Fig. 6.3), resulting in experimental errors being reduced to 7% or less (see Fig. 6.4).

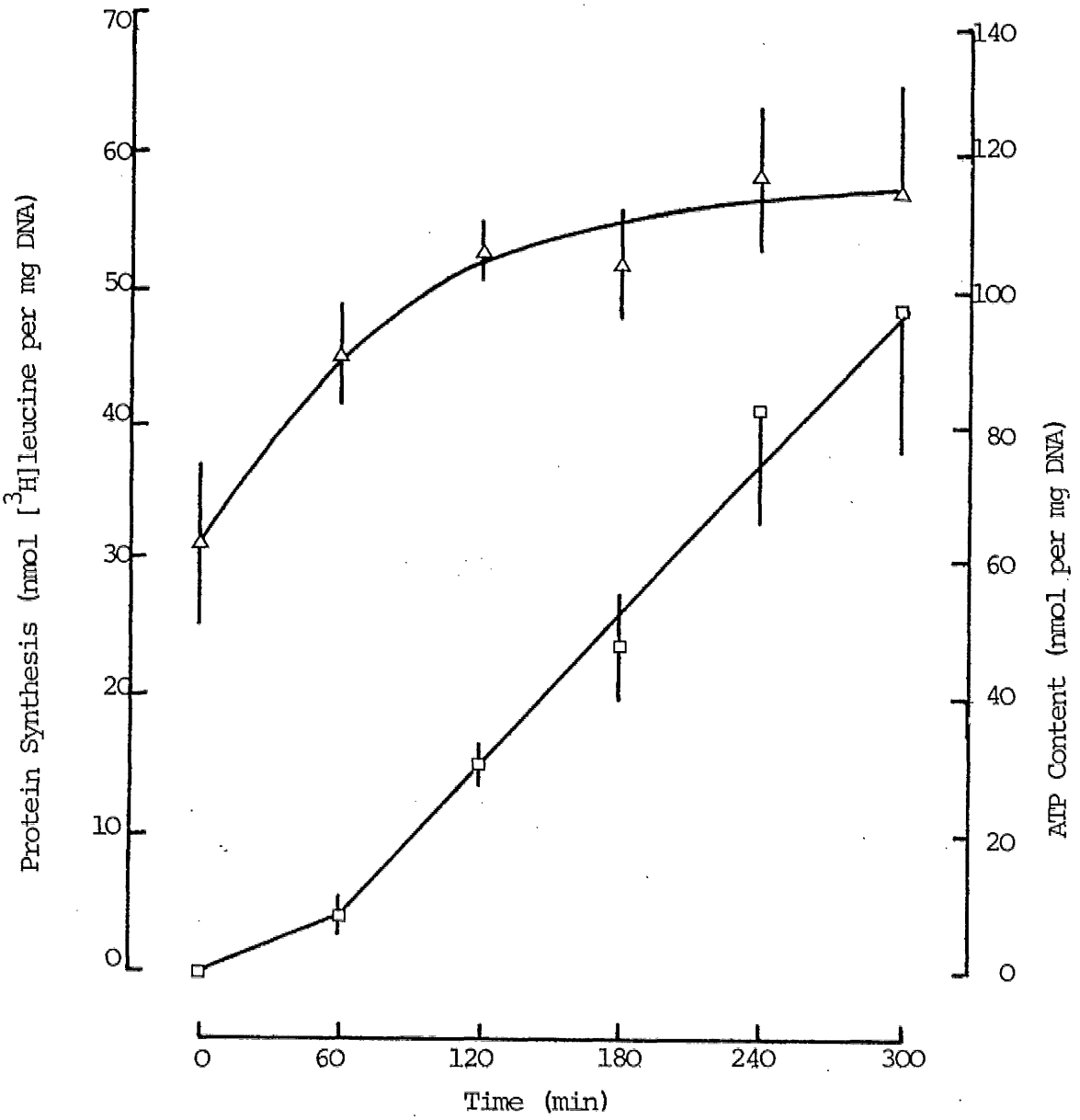


FIG. 6.2. Incorporation of L-[4,5-^{3}H]leucine in Protein as a function of Incubation Time

Rumen papillae were incubated in 2ml of medium (see section 6.2.2) containing L-[4,5-^{3}H]leucine (2μCi/ml). Incubations were carried out as described in section 6.2.4, by Method I. Incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble pellets (□), and ATP contents (△), are given. The results are reported as means ± S.D. for triplicate incubations.

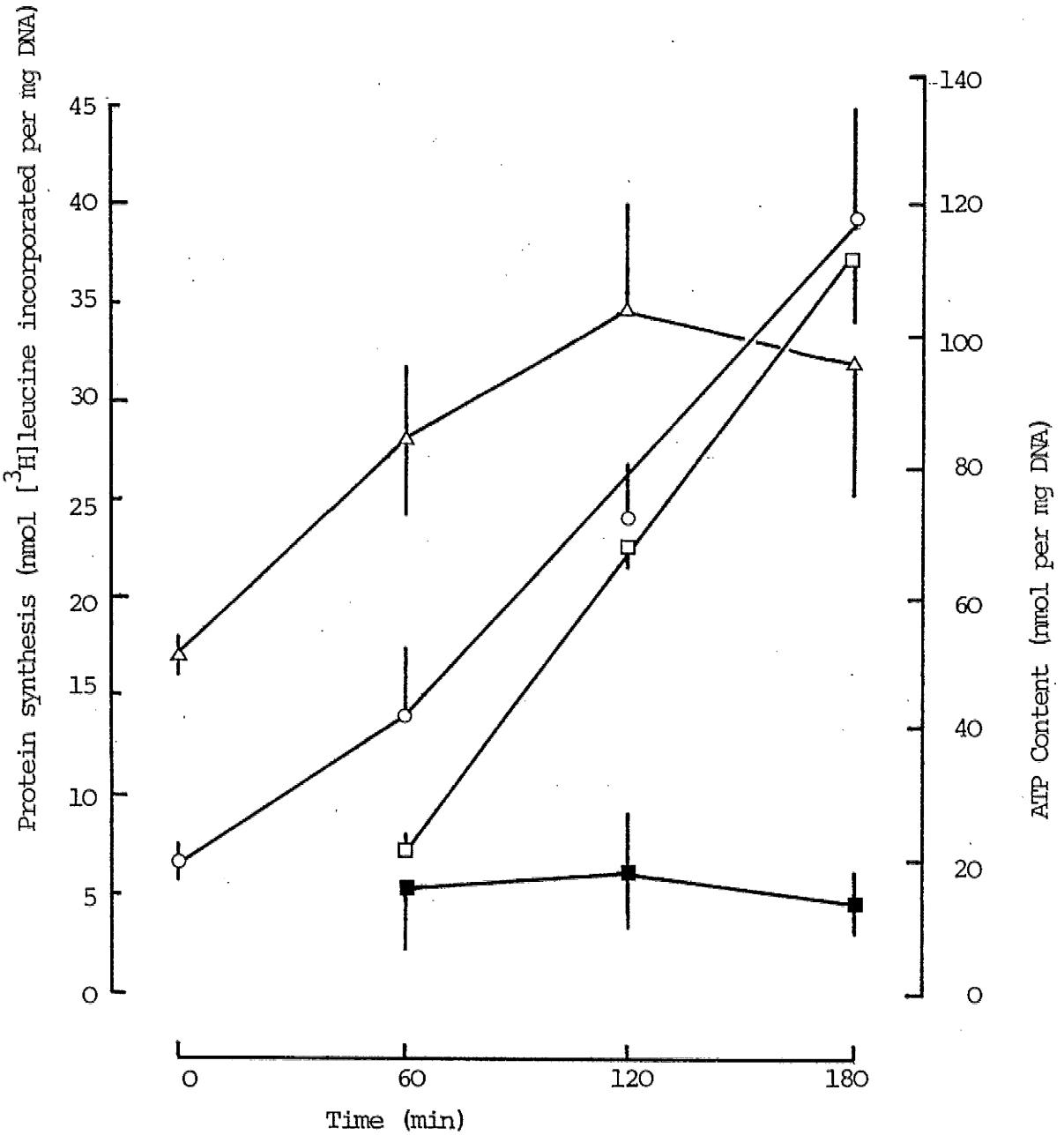


Fig. 6.3. Secretion of ^{3}H-labelled Protein into Incubation Medium by Sheep Rumen Papillae

The time courses for the appearance of protein, labelled with L-[4,5-^{3}H] leucine, in cells pellets (□) and extracellular medium (■) are given above. Conditions and procedures were as described in section 6.2.5 (Method II). Also given is the incorporation of ^{3}H-labelled leucine into trichloroacetic acid-insoluble medium plus cells (○) and ATP contents (△). Results are means ± S.D. for triplicate incubations.

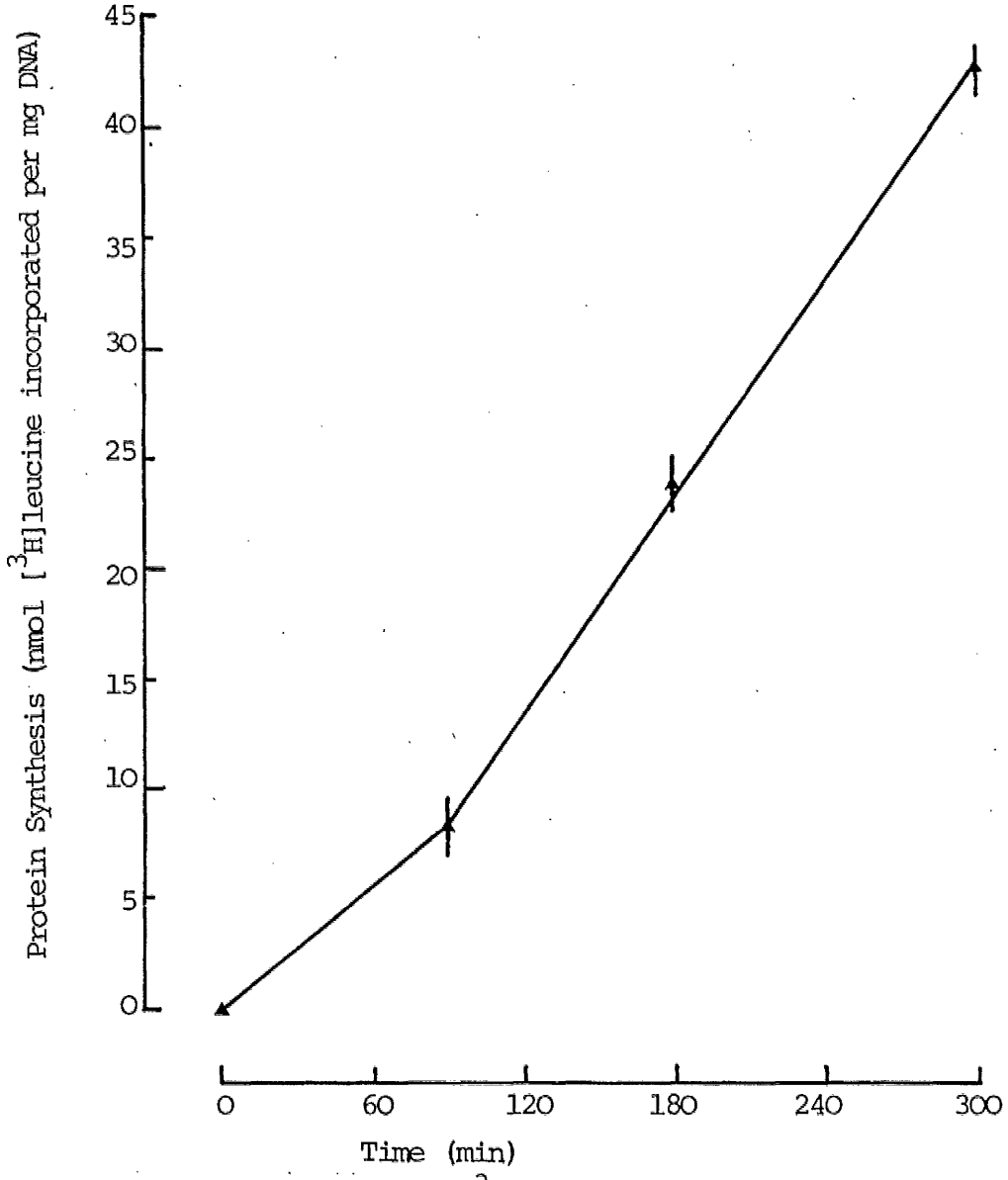


Fig. 6.4. Time Course of [^{3}H]leucine Incorporation into Rumen Epithelial Protein

Papillae were incubated as described in the legend to Fig. 6.3 and the amount of [^{3}H]leucine incorporated into protein was measured in papillae separated from the incubation medium (▲) (section 6.2.4, method II). The results are given as means ± S.D.

6.3.2. *Cell Viability*

During the incubation period the viability of rumen epithelial cells was assessed by measurement of ATP contents. Initially low ATP contents were observed but these steadily increased, levelling off 2h later (see Fig. 6.2 and 6.3). The ATP contents were in the range of values found for rumen papillae incubated in modified-Krebs buffer (section 4.3.2).

Also given in this section are the total adenylate contents of papillae incubated in modified-Krebs buffer (Table 6.1). Rumen epithelium incubated with either propionate or butyrate appeared to maintain ATP contents, if not increase them, over the experimental period (see Fig. 6.5). In addition to this the ATP/ADP ratio and adenylate charge (see legend to Table 6.2) also remained high relative to control values. The ATP/ADP ratios were decreased slightly at the 2h time point for propionate (3.87) and butyrate (4.19) but the energy charge was similar to the 1h values, i.e. 0.72 and 0.74, respectively.

Papillae metabolising acetate as the sole carbon source had much lower values for the energy charge and ATP/ADP ratio, compared to papillae utilising other substrates. Although the ATP/ADP ratio and energy charge steadily increased, over the incubation period, in rumen epithelium supplied with acetate (2h values of 1.93 and 0.59, respectively) these values were still below the observed control values (i.e. 2.52 and 0.65 after 2h). A significant decrease of rumen mucosal ATP contents, in the

	30 min				60 min			
	Control	Acetate	Propionate	Butyrate	Control	Acetate	Propionate	Butyrate
ATP	92.8 ± 3.4	68.5 ± 48	94.1 ± 11.1	104.4 ± 6.7	80.5 ± 6.1	68.1 ± 7.9	132.0 ± 15.8	119.6 ± 27.1
ADP	36.6 ± 0.6	42.6 ± 0.5	36.3 ± 3.8	19.7 ± 2.0	36.8 ± 1.1	39.9 ± 6.1	34.6 ± 8.2	22.1 ± 4.5
AMP	21.3 ± 10.4	61.5 ± 4.8	43.3 ± 2.9	20.2 ± 4.5	37.6 ± 13.4	46.3 ± 7.3	50.3 ± 20.3	28.0 ± 2.7
TOTAL NUCLEOTIDES	150.7 ± 16.0	172.6 ± 8.4	173.7 ± 4.3	144.3 ± 7.4	154.9 ± 18.2	154.3 ± 9.8	216.9 ± 36.5	169.7 ± 29.4
ATP/ADP	2.54 ± 0.2	1.61 ± 0.2	2.59 ± 0.5	5.30 ± 0.9	2.19 ± 0.2	1.71 ± 0.5	3.90 ± 0.7	5.40 ± 0.4
Charge	0.74	0.52	0.65	0.79	0.64	0.57	0.69	0.77

Table 6.1. Effect of Volatile Fatty Acids on the Adenosine Nucleotide Content of Isolated Rumen Papillae

Rumen papillae were incubated over a 2h period in modified-Krebs buffer. ADP and AMP (section 6.2.7) and ATP (section 2.18.3) were assayed as described in the text. The final concentrations of butyrate and propionate were 15 and 12mM, respectively. Acetate was added to the incubation medium at a final concentration of 60mM but an equivalent amount of NaCl was removed from the modified-Krebs buffer. Results are given in terms of nmol/mg DNA (means ± S.D.) and are representative values for one experiment. Similar observations were made in a separate experiment. The energy charge was calculated from the expression given by Swedes *et al.* (1975):

$$\text{Energy Charge} = \frac{[ATP] + \frac{1}{2}[ADP]}{\Sigma [ATP] + [ADP] + [AMP]}$$

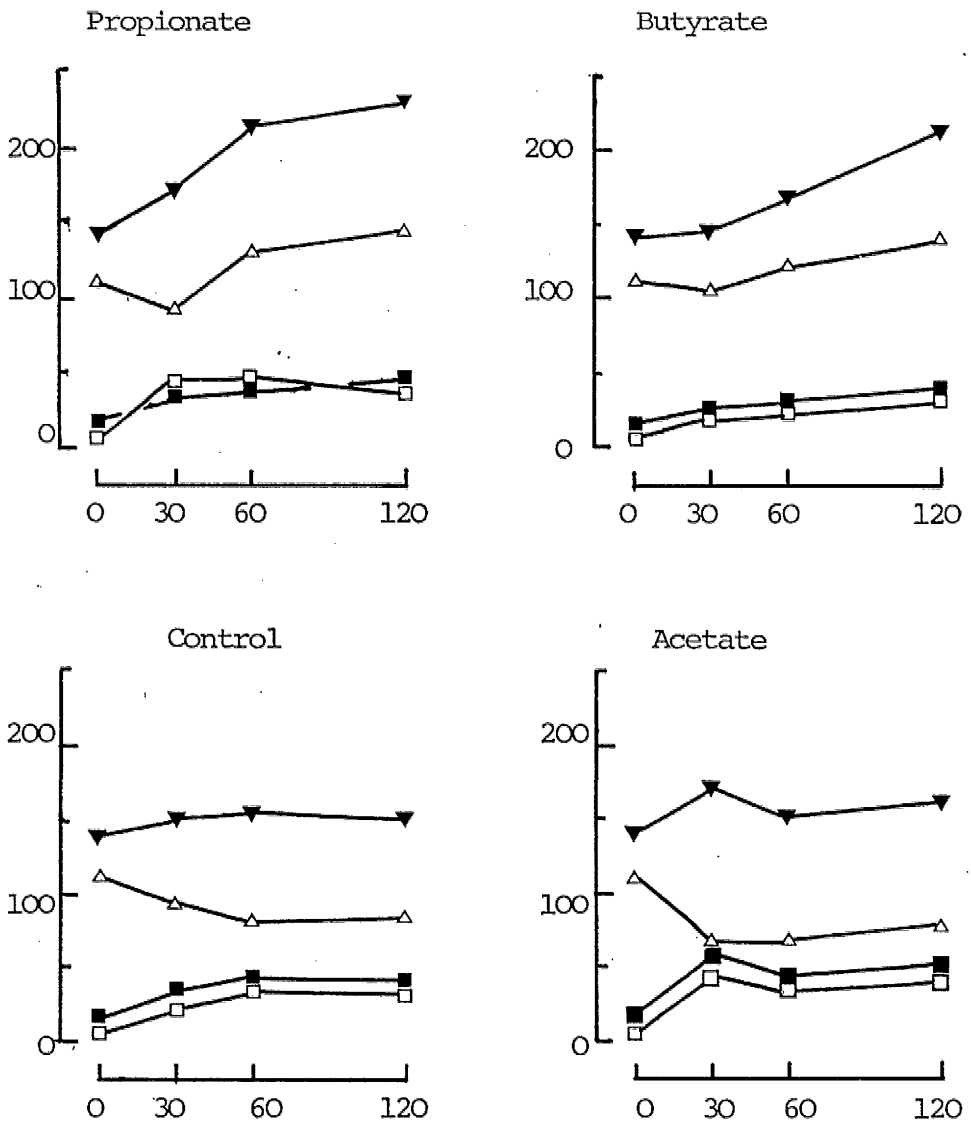


Fig. 6.5. Adenylate Content in Rumen Papillae Incubated with Volatile Fatty Acids

Papillae were incubated with volatile fatty acids as indicated in the legend to Table 6.1. Each point is the mean value of triplicate incubations. ATP (△), ADP (□), AMP (■) and total adenylate (▼) contents are given in terms of nmol per mg DNA. The time (min) is given along the x-axis.

presence of acetate, has already been reported in this thesis (section 4.3.2).

6.3.3. Influence of Insulin and Substrates on Rumen Mucosal Protein Synthesis

The rate of [^{3}H]leucine incorporation into protein synthesised by rumen epithelium was depressed by cycloheximide, an inhibitor of eukaryotic protein synthesis, by approximately 95% (see Table 6.2). NaF (1.3mM) inhibited protein synthesis by about 41%. This is similar in magnitude to NaF inhibition of rat hepatocyte protein synthesis (Schreiber and Schreiber, 1973).

Insulin or dibutyryl cAMP did not affect protein synthesis over a 2h incubation period (see Table 6.3). Because of the lag phase in the protein synthetic rate papillae were preincubated for 1h, in the presence of [^{3}H] leucine, before additions were made to the flasks. Also dexamethasone, a corticosteroid known to induce protein synthesis in other tissues (Hoshino *et al.*, 1981), was apparently ineffective in this system.

Propionate or butyrate added as sole substrates to rumen papillae did not significantly change the rate of protein synthesis (see Table 6.3). However, when these two substrates were added the rate of [^{3}H]leucine incorporation was increased significantly over the time course of the experiment. A similar observation was made in glucose-free incubation medium (see Table 6.4). In

Additions	Rate of Protein Synthesis (nmol [^{3}H]leucine incorporate/mg DNA per 3h)	
None	13.07 ± 1.92	
Cycloheximide (0.21mM)	0.69 ± 1.92	P < 0.001
NaF (1.3mM)	7.68 ± 1.13	P < 0.001
Dibutyryl cAMP (0.05mM)	13.52 ± 1.66	N/S

Table 6.2. Effect of Possible Inhibitors on Rumen Mucosal Protein Synthesis

NaF, cycloheximide (freshly prepared), and N^6, O^2 dibutyryladenosine-3',5'-cyclic phosphate (dibutyryl cAMP) were added to isolated rumen papillae incubated in the presence of L-[4,5-^{3}H]leucine. The final concentrations of additives are given in parentheses. After a 3h incubation period papillae plus medium were homogenised as described in section 6.2.4, by method I. Incorporation of [^{3}H]leucine into protein was measured as described in Section 6.2.5. Results are reported as means ± S.D. for triplicate incubations. The significance of differences between mean values were determined by Student's t test (N/S, not significant).

Additions	Rate of Protein synthesis (nmol per mg DNA)		
	30 min	120 min	240 min
Control	9.4 ± 0.3	23.9 ± 1.2	43.5 ± 1.0
Insulin (1μM)	9.2 ± 1.2	23.4 ± 2.7	-
Dibutyryl cAMP (50μM)	9.9 ± 0.9	24.0 ± 2.0	-
Dexamethasone phosphate (1μM)	-	26.3 ± 2.9	42.9 ± 6.0
Butyrate (10mM	11.3 ± 2.0	23.7 ± 0.2	-
Propionate (15mM)	10.7 ± 1.2	23.8 ± 1.7	-
Propionate (15mM) plus Butyrate (2mM)	10.4 ± 0.4*	29.9 ± 0.9*	-

Table 6.3. Effect of Hormones and Volatile Fatty Acids on Rumen Epithelial Protein Synthesis

Isolated rumen papillae were incubated with [^{3}H]leucine and protein synthesis measured as described in the legend to Table 6.2, except that method II (section 6.2.4) was used. Papillae were preincubated for 1h in medium containing L-[4,5-^{3}H]leucine (2μCi/ml) before additions were made to the flasks. Results are means ±S.D.. The significance of differences between mean values were determined by the Student's t test; *P < 0.05.

Additions	Rate of [^{3}H]leucine Incorporation (nmol per mg DNA per 3h)	%	
Control	4.3 ± 0.5	100.0	
Glucose (5.5mM)	10.6 ± 2.0	246.5	P < 0.05
Acetate (15mM)	3.6 ± 0.1	83.7	N/S
Propionate (15mM)	8.0 ± 2.3	186.0	N/S
Butyrate (15mM)	6.9 ± 1.0	160.5	P < 0.01
Propionate (15mM) plus Butyrate (2mM)	11.4 ± 1.4	265.1	P < 0.005
Acetate (10mM), Propionate (2.5mM) plus Butyrate (2.5mM)	8.3 ± 0.3	193.0	P < 0.001

Table 6.4. Effect of Potential Anabolic Agents on Rumen Epithelium Protein Synthesis

Papillae were incubated in modified minimum essential medium buffer (Eagles) with a final leucine concentration of 0.11mM (value reported for ovine plasma; Koenig and Boling, 1980). The final concentration of substrates are given in parentheses. P values were determined by Student's t test.

the absence of any exogenous substrates rumen epithelium synthesised protein at a relatively high rate (e.g. 40% of the glucose-stimulated rate).

The rate of protein synthesis in strips of rumen epithelium populated with either short-(<2mm) or long-(>10mm) papillae was also investigated. It was found that the rate of [^{3}H]leucine incorporation varied in both types of papillae, apparently depending on the nature of rumen mucosa (see table 6.5 and discussion).

6.3.4. Protein Degradation

Measurement of rumen epithelial protein degradation rates, in papillae preincubated with [^{3}H]leucine, was complicated by the apparent slow efflux of this radioactively labelled amino acid, even after extensive washing procedures. Rates of degradation could only be estimated from the sum of [^{3}H]leucine which appeared in both medium and TCA-supernatants (of papillae homogenates). Control and methylamine treated papillae gave rates of degradation, expressed as a percentage of the total protein radioactively measured in the tissue at the start of the incubation, of 2.9% hr^{-1} and 1.9% hr^{-1}, respectively, (see Table 6.6 and Fig. 6.6).

Identification of Rumen Epithelium	Protein Synthesis (nmol [^{3}H]leucine incorporated/mg DNA per 6h)		
	Short-Papillae	Long-Papillae	L/S
Yellow	86.2 ± 12.9	38.2 ± 5.7	0.44
	78.4 ± 34.3	27.9 ± 3.4	0.36
Black	43.2 ± 14.1	83.8 ± 2.6	1.94
	35.5 ± 13.9	54.5 ± 14.6	1.54
	60.9 ± 30.7	79.0 ± 6.6	1.30

Table 6.5. Protein Synthesis in Long and Short Papillae

Protein synthesis was measured in strips of long-and short-papillae, removed from the anterior ventral sac, as isolated short-papillae were difficult to obtain by shaving. Tissue was incubated as described in section 6.2.4 and homogenised with a ground glass homogeniser. Also given is the ratio of [^{3}H]leucine incorporation into long-and short-papillae. The results are means ± S.D. obtained from different rumen preparations.

	Control			+Methylamine	
	0	1h	3h	1h	3h
Release of [^{3}H]leucine into medium (DPM per mg DNA)	12936 ± 864	50697 ± 2636	81518 ± 273	44882 ± 2884	77308 ± 2792
Release of [^{3}H]leucine into TCA solution (DPM per mg DNA)	71904 ± 1627	49612 ± 1501	37212 ± 3913	45764 ± 3375	32537 ± 1080

Table 6.6. Sheep Rumen Epithelium Protein Degradation. Effect of Methylamine

Rumen papillae were preincubated with L-[4,5-^{3}H]leucine for 3h, as described in Section 6.2.6, before washing and transferring to 'cold'-incubation medium. Some flasks contained methylamine (10mM). The release of [^{3}H]leucine into the incubation medium and into the 5% (w/v)-trichloroacetic acid solution, (after homogenisation), was determined. Results are given as means ± S.D.. L-[4,5-^{3}H]leucine incorporated into rumen epithelial protein, at the start of the experiment, was 431821.9 ± 15209.3 (DPM per mg DNA).

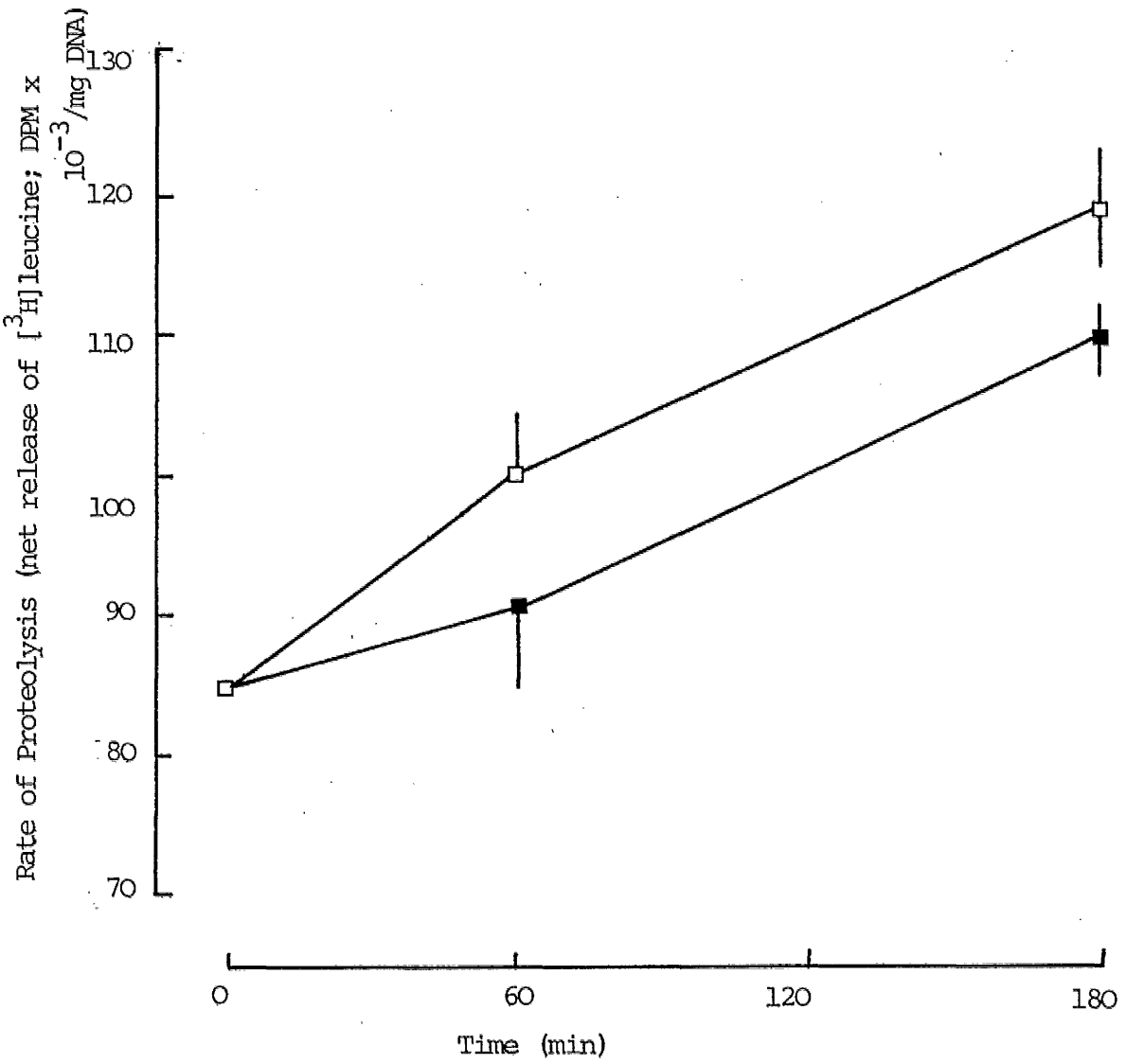


FIG. 6.6. Net Release of [^{3}H]leucine from Prelabelled Rumen Epithelial Protein

Papillae were incubated, as described in the legend to Table 6.2, with (■) and without (□) 10mM-Methylamine. Results are given as means ± S.D.

6.3.5. *Analysis of Denatured Protein from Rumen Mucosa*

Denatured protein from homogenates and cytoplasmic fractions of rumen epithelium, preincubated in the presence of [^{3}H]leucine, were analysed by electrophoresis. A representative profile from one analysis is shown in Fig. 6.7. Similar protein patterns were observed with homogenate samples but the radioactive peaks could not always be assigned to protein bands. In some experiments papillae were also incubated with dexamethasone phosphate (1μM) and propionate plus butyrate (15mM and 2mM, respectively) before analysis of rumen epithelial protein. In both cases no increase in the specific radioactivity of any of the protein band was found, compared to protein isolated from control papillae.

Extraction of a prekeratin fraction and analysis by electrophoresis revealed a profile consisting largely of three main peaks, with some minor protein bands (see Fig. 6.8). Matoltsy *et al.* (1981) have extracted and purified prekeratin from bovine snout epidermis and found that three major polypeptides comprised about 70% of the prekeratin sample and were present in nearly equal proportions. Similar observations have been made from human (Skerrow, 1977) and bovine prekeratin (Steinert, 1975). One proposal is that the major polypeptides form the backbone structure of *in situ* epidermal filaments and are thought to be deposited in three-stranded units (Skerrow *et al.*, 1973). Because of these observations it is assumed that the three major polypeptides identified in Fig. 6.8 formed the backbone structure of sheep rumen epithelium prekeratin.

FIG. 6.7. Analysis of Denatured Rumen Epithelium Cytoplasmic Protein by Electrophoresis

Shaved papillae were incubated in medium containing [^{3}H]leucine (section 6.2.2) for 5h before subcellular fractionation (section 2.7). Samples of cytoplasmic protein were denatured by heating at 100°C for 5 min before analysis by electrophoresis. After electrophoresis protein bands were stained with Coomassie brilliant blue R before scanning (-) at 635nm in an LKB 2202 Ultroscan Laser Densitometer; gels were sliced and counted for ^{3}H (O) as described in Section 6.2.8.3 (background radioactivity was not subtracted from any values). Arrows S and B are the stacking gel and bromophenol blue ends of the gel, respectively. Three peaks are also identified as possible prekeratin subunits (★, see Fig. 6.9).

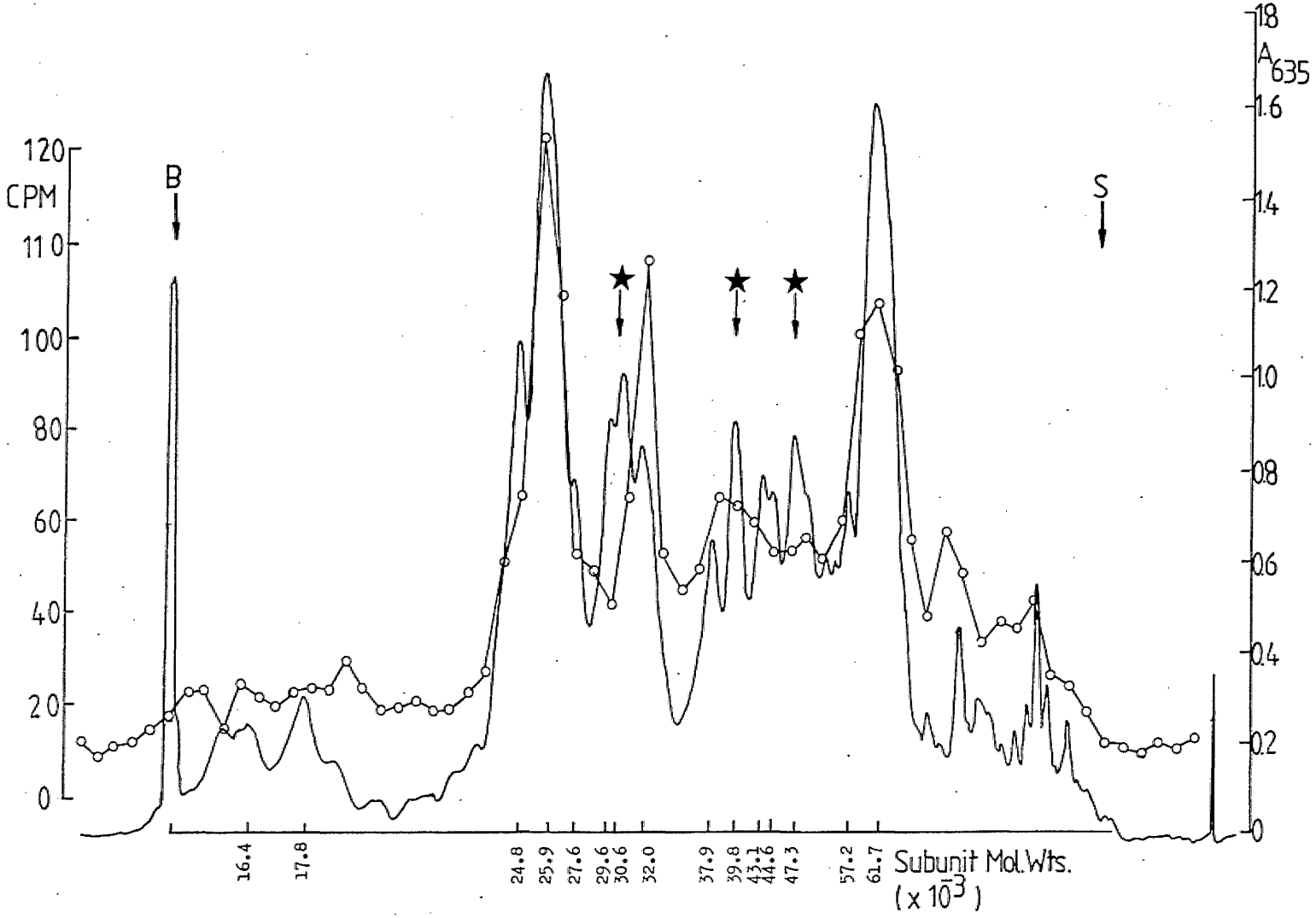
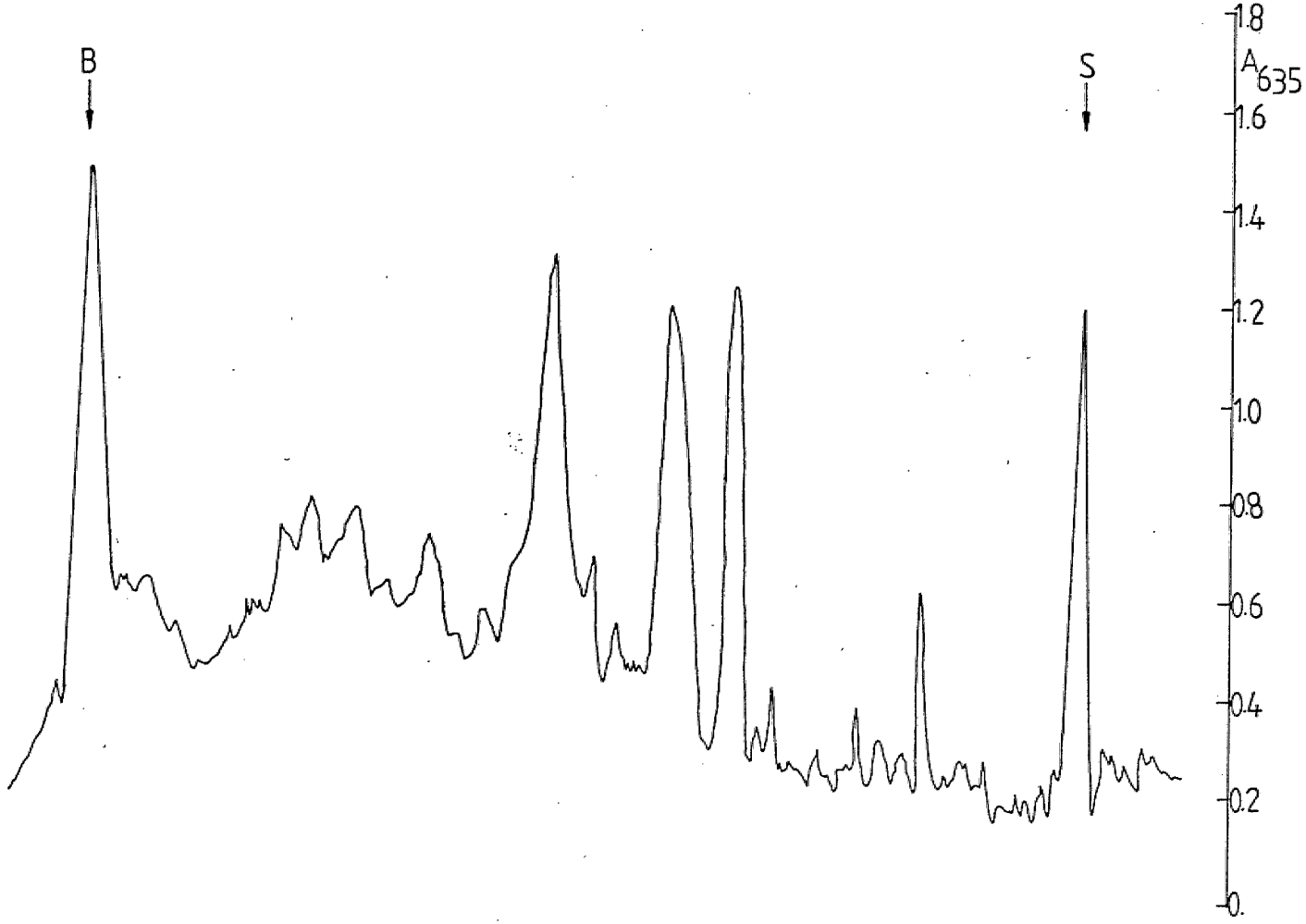

120
CPM
110
100
80
60
40
20
0
B
S
18
A635
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
16.4
17.8
24.8
25.9
27.6
29.6
30.6
32.0
37.9
39.8
43.1
44.6
47.3
57.2
61.7
Subunit Mol.Wts.
(x 10³)

FIG. 6.8. Identification of Possible Prekeratin Subunits

Papillae were incubated as described in the legend to Fig. 6.7. before extraction of prekeratin into 0.1M-citric acid/sodium citrate buffer, pH 2.6, as described in section 6.2.9. Gels were scanned and sliced as described above (Fig 6.7). It was observed, however, that no significant radioactivity was present in any of the gels (triplicate samples were analysed). The subunit molecular weights of the three major protein bands were estimated to be 47 000, 36 800 and 30 800 daltons (reading from stacking gel end, S, to bromophenol blue end, B).


B
S
1.8
A635
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.

6.4. *Discussion*

Rates of protein synthesis calculated by using the specific radioactivity of an extracellular amino acid are sometimes lower than rates obtained by using the specific radioactivity of charged tRNA (Ballard, 1982). The specific radioactivity of leucine-charged tRNA was not measured in this study. In other biological systems there is virtual equilibration of the intra- and extracellular amino acid pools at medium concentrations above 0.5mM (Ballard, 1982). In one experiment protein labelling was measured over 3h in extracellular leucine concentrations of 0.4mM and 5mM. The specific radioactivity of [^{3}H]leucine was constant. There was almost twice the increase in leucine incorporation into protein at the higher amino acid concentration. This may mean that the rates of protein synthesis measured in this study are underestimated because the extracellular leucine is not the appropriate precursor pool for rumen mucosal protein synthesis, as assumed in the calculations (see section 6.2.5).

Nevertheless, the almost complete inhibition of protein synthesis produced by cycloheximide rules out any significant bacterial contribution to total protein synthesis. This is in agreement with Bush's observation (Bush, 1982) that contamination of epithelial extracts, by bacterial infiltration into the rumen wall, is minimal. NaF suppressed rumen mucosal protein synthesis at a dose which inhibited chain initiation in other biological systems (Leader, 1972; Bagloni *et al.*, 1972).

In contrast to the effects produced by these inhibitors butyrate, glucose and propionate all stimulated protein synthesis in isolated rumen papillae (Table 6.4). Glucose stimulated protein synthesis to a greater rate than that produced by either of the VFAs. It has been suggested that the glycolytic pathway could exert some control over protein synthesis (Ayuso-Parrilla & Parrilla, 1975). In some tissues, under experimental conditions, glucose oxidation increases protein synthesis independently of any effect on the adenylate system (Ohler, 1969; David, 1975; Martinez-Izquierdo *et al.*, 1982). It is proposed that this glucose effect is related to an increased concentration of one or more of the metabolites of glucose-breakdown (Martinez-Izquiedo, 1982). Glucose 6-phosphate is implicated as being one of these metabolites (Ernst *et al.*, 1978; Giloh and Mager, 1975). However, no decrease in glucose-stimulated protein synthesis was produced by added propionate or butyrate (Table 6.3), even though these VFAs inhibited glucose oxidation *in vitro* (Chapter 5).

Alternatively there is evidence to indicate that decreased ATP concentrations and/or energy charge of the adenylate system results in a significant inhibition of protein synthesis (Tierney, 1971; Van Venrooig *et al.*, 1972; Ayuso-Parrilla and Parrilla, 1975).

In this study some of the results tend to support a relationship between protein synthesis and adenylate contents. For instance, both propionate and butyrate

maintained the ATP contents and cellular energy charge in rumen epithelium, as well as stimulating endogenous protein synthesis. Conversely, acetate produced a decrease in protein synthesis and depressed the cellular ATP content and energy charge, even though some acetate oxidation does occur in rumen epithelium.

Also the observed lag phase of [^{3}H]leucine incorporation (from 0-90 min) may be linked to the concomitant low ATP contents. However, a slow uptake of neutral amino acids, from the extracellular medium into the intracellular amino acid pool, could also explain the lag phase in protein synthesis. In sheep colonic mucosa, another fermentative organ, neutral amino acids appear to compete for the same transport mechanisms and may involve a Na^+-dependent active transport system (Scharrer, 1978). A slow efflux of [^{3}H]leucine from papillae prelabelled with this radioactive amino acid was observed. When papillae were prelabelled with [^{3}H]leucine and protein synthesis inhibited by saturating concentrations of cycloheximide this slow efflux still persisted. If the efflux of neutral amino acids in fact employs the same transport site as influx then this phenomemon is similar to the *trans*-inhibition of neutral amino acid transport System A, caused by the presence of intracellular substrates of this system (Shotwell and Oxender, 1983). *Trans*-inhibition may have occurred because papillae were routinely placed in an amino acid medium before addition to incubation flasks (section 6.2.1).

The slow efflux of [^{3}H]leucine from prelabelled papillae made estimation of proteolysis difficult because the blank value was high (i.e. 20% of the value of [^{3}H] leucine incorporated into protein). An inhibition of protein-breakdown by methylamine was observed. This inhibition may be caused by the lysosomotropic mode of action of the weak base amine, i.e. a rise in the internal pH of lysosomes, inhibiting the action of degradative enzymes (Seglen, 1976). Methylamine may influence rumen epithelial protein breakdown *in vivo* as it is a normal constituent of the rumen contents, at concentrations of about 1mM (Hill and Mangan, 1964). Other weak base amines, also present in rumen liquor (Fraser and Butler, 1964), could also affect rumen mucosal proteolysis.

Feeding sheep concentrate diets, low in fibre content, may result in parakeratosis of the rumen epithelium and often feed becomes impacted between papillae in a very dark, blackened colour (Jensen *et al.*, 1958; Warner, 1965; Weekes, 1974). The long-papillae, normally having greater contact with rumen contents than the short-papillae, achieved higher rates of protein synthesis when sampled from the blackened rumen. Perhaps a more systematic study would reveal whether a relationship does exist between abnormal rumen epithelial development and protein synthesis.

The results presented in this Chapter tend to confirm observations, originally made by Kalachyuk *et al.* (1975), that the rumen epithelium may possess a high capacity to synthesise protein.

6.5. *Summary*

A method is described for measuring rumen epithelial protein synthesis *in vitro*. Isolated rumen papillae incorporated ^{3}H-labelled leucine into protein, after an initial lag phase, as a linear function of time. Glucose, propionate and butyrate stimulate protein synthesis by 147%, 84% and 61% respectively. Acetate produced a decrease in protein synthesis, ATP content and adenylate energy charge.

Glucose, propionate and butyrate maintain the cellular ATP content, an effect possibly correlated with increases in protein synthesis. Glucose-stimulated protein synthesis is not influenced by either insulin or dibutyryl cyclic AMP.

Separation by PAGE of newly-synthesised proteins, labelled with ^{3}H-labelled leucine, shows that some of the protein subunits are highly labelled. However, extraction and analysis of rumen mucosal prekeratin indicates that this does not make up a major fraction of the newly-formed protein. Three major polypeptides were separated from the prekeratin fraction and their subunit molecular weights are given.

The rate of protein degradation is estimated to be 2.9 % hr^{-1} for rumen papillae prelabelled with ^{3}H-labelled leucine. Methylamine decreased the rate of proteolysis.

CHAPTER 7

AMINO ACIDS SYNTHESISED BY AND RELEASED FROM ISOLATED RUMEN EPITHELIUM

7.1. *Introduction*

Dietary protein fed to ruminants is degraded by rumen microbial proteases, releasing amino acids and ammonia; these substrates provide most of the nitrogen requirements for the bacterial biomass. In some feeding regimes non-protein nitrogen is added to reduce the protein contribution in the diet. For example, addition of urea to forage is common practice in the intensive beef producing industry (Preston & Willis, 1974). Urea is readily hydrolysed in the rumen, by bacterial urease, resulting in the formation of high concentrations of ammonia within the first hour of feeding. Urea also enters the rumen by both salivary secretions and direct diffusion (from the blood through the rumen wall). Entry of endogenous urea into the alimentary tract is positively related to plasma concentrations (Armstrong & Weekes, 1983).

Wallace *et al.* (1979) have identified an independent specialised bacterial flora attached to, and living on, the rumen epithelium. There has been speculation that the role of these bacteria in rumen nitrogen metabolism is related to the microorganisms developing increased ureolytic activity, this being inversely proportional to ammonia concentrations in rumen liquor. The hypothesis is that, at high rumen ammonia concentrations, when urease activity will be low, removal of plasma urea will be slower than when rumen ammonia concentrations are low and

the mucosal bacterial urease activity is high (Englehart *et al.*, 1978).

Comparable rates of urea hydrolysis and fixation of ammonia into microbial protein are not reached in the rumen, thereby restricting the efficiency of urea utilisation. At high concentrations of ammonia in the rumen, bacterial glutamate dehydrogenase catalyses the fixation of ammonia into glutamate, whereas the glutamine synthetase (EC 6.3.1.2) pathway predominates at lower concentrations (Allison, 1982). Alanine synthesis has been reported as another means of ammonia fixation in the rumen (Salter and Smith, 1981).

Rumen ammonia concentrations - normally ranging from 4 to 40 mM (Mahadevan *et al.*, 1976) - are also reduced by absorption. The main factor affecting absorption, besides concentration, is pH. Absorption is more rapid at high rumen liquor pH values. Of the total ammonia entering the rumen pool, about 40% is absorbed through the rumen wall, 46% is incorporated into microbial protein and 16% passes to the lower gut (Nolan, 1975). The rate of ammonia uptake into blood draining the portal viscera was estimated to be 29mmol per h in adult fed sheep (Wolff *et al.*, 1972).

Ammonia concentrations in the portal vein of adult dairy cows are about 0.3mM (Symonds *et al.*, 1981). This means that the concentration gradient between rumen and blood may be up to 100-fold in favour of the rumen

(Bartley *et al.*, 1976; Davidovich *et al.*, 1977a). Therefore, effective pathways of ammonia assimilation may be present in rumen epithelium to prevent ammonia toxicity in this tissue. Energy yielding processes, including the Krebs cycle and oxidative phosphorylation pathway, are inhibited by ammonia (Emmanuel *et al.*, 1980a).

Homogenates prepared from sheep rumen epithelium were able to produce ammonia when incubated with glutamine (Hoshino *et al.*, 1966). Boila *et al.* (1980a) reported, however, that glutamine was not readily converted to glutamate, although they could not account for the disappearance of some of the glutamine nitrogen after allowing for incorporation of glutamine nitrogen into amino acids synthesised by bovine rumen papillae.

In fed sheep glutamine and glutamate are removed from the blood by the portal drained viscera (Heitmann and Bergman, 1980a). Seven-times and thirty six-times more glutamine is removed than glutamate in fed and 3-day fasted sheep, respectively. These findings are consistent with the role of glutamine as a major respiratory substrate for enteric epithelial tissues (Windmueller and Spaeth, 1978; 1980; Hanson and Parsons, 1978).

The purpose of this study was to examine rumen epithelial nitrogen metabolism by using glutamine as a nitrogen source.

7.2. *Materials and Methods*

7.2.1. *Introduction*

Rumen papillae were obtained from a highly papillated area of the anterior ventral sac of sheep rumen as described in Section 2.5. Incubations were carried out in modified Krebs buffer (see section 2.3) as detailed in section 2.9 .

7.2.2. *Analysis of Amino Acids*

Amino acids were analysed from ice-cold incubation medium (pH 7.0) with an LKB 4400 Amino Acid Analyser (LKB Instruments, South Croydon, U.K.). Samples were loaded on to a column (270mm x 4mm) packed with Ultropac 8 resin.

The run conditions were as follows: buffer flow rate - 25ml/h; ninhydrin flow rate - 20ml/h. Column temperature settings: 45°C for 2 min, 64°C for 33 min, and 75°C for 19 min. Buffers (LKB prepared): 0.2M-Sodium citrate, pH 3.42 for 16 min; 0.2M-sodium citrate, pH 4.25 for 26 min; and finally 0.2M sodium citrate, pH 10.0 for 12 min. Analysis was normally carried out on the same day as the experiment.

After each analysis the column was re-equilibrated with 0.2M-sodium citrate buffer, pH 3.0 at 4.5°C for 24 min. Amino acids used for calibration were Pierce CH Standards containing 10nmol of each amino acid.

7.3. *Results and Discussion*

Initially metabolites formed, after incubation of rumen papillae with glutamine or glutamate, were measured enzymically (see section 2.17). The results from a representative experiment are given in Table 7.1.

Addition of glutamate alone did not change the rate of formation of any of the measured metabolites. Incubation of papillae with glutamine resulted in formation of glutamate, alanine, aspartate and ammonia. Glutamate accounted for most of the glutamine removed from the incubation medium, if it is assumed that endogenous glutamate production is unchanged by added substrates.

Ammonia, alanine and glutamate have already been shown by Matthews and Wiseman (1953), Aikawa *et al.* (1973) and Windmueller and Spaeth (1974) to be major end-products of intestinal epithelial glutamine metabolism *in vivo*. Some 27% of the nitrogen, from glutamine, could be accounted for in alanine or aspartate.

Net release of citrulline into the portal vein by gut tissues *in vivo* has been observed in fed (Heitmann and Bergman, 1980a; Wolff *et al.*, 1972) and fasted sheep (Koenig and Boling, 1980; Heitmann and Bergman, 1980a;

	Substrates Added		
Products Formed	Control	L-Glutamate	L-Glutamine
L-Lactate	+1.50	+1.52 ± 0.23	+1.11 ± 0.06
L-Glutamate	+1.12	-	+2.26 ± 0.38
L-Glutamine	N.D.	N.D.	-1.23 ± 0.26
L-Alanine	+0.17	+0.14 ± 0.04	+0.39 ± 0.04
L-Aspartate	+0.16	+0.16 ± 0.02	+0.25 ± 0.01
L-Citrulline	+0.05	+0.06 ± 0.01	+0.05 ± 0.01
NH_4^+	N.D.	N.D.	+0.10 ± 0.01

Table 7.1. Metabolic Changes Upon Addition of Glutamate and Glutamine to Isolated Papillae Suspensions

Isolated rumen papillae were incubated with either glutamate (5mM) or glutamine (5mM) for 1h at 39°C. The results are means ± S.D. for metabolite removal (-) or production (+) expressed as µmol/h per mg DNA. N.D. - not detected.

Similar observations have been reported for man (Felig and Wahren, 1971) and dog (Weber *et al.*, 1976). The most convincing evidence for citrulline synthesis in gut tissues is its formation from ^{14}C-labelled glutamine (Windmueller and Spaeth, 1974). Similar experiments were not carried out in this study as citrulline was not detectable in the incubation medium as a product of either glutamate or glutamine metabolism (see Table 7.1). This agrees with Emmanuel's observation (Emmanuel,1980d) that operation of the urea cycle in rumen epithelium is almost insignificant compared to ruminant liver.

[U-^{14}C]glutamine was oxidised to $^{14}CO_2$ by rumen papillae, the time course being linear over 1h (see Fig. 7.1). This indicates that glutamine catabolism is initiated by glutaminase (EC 3.5.1.2), which has been identified as phosphate-dependent and located in the mitochondria (Pinkus and Windmueller, 1972). The activity of phosphate-independent glutaminase is very low in sheep tissues (Pell *et al.*, 1983).

[U-^{14}C]glucose was oxidised at a faster rate, compared to [U-^{14}C]glutamine, in rumen epithelium (see fig. 7.1). In colonic mucosa glutamine, as opposed to glucose, is the preferred substrate for respiration (Roediger, 1982). Furthermore, glutamine is metabolised to CO_2 by intestinal slices obtained from several animal species (Neptune, 1965) and even stimulates respiration in mucosal preparations from the dog (Frizzell *et al.*, 1974).

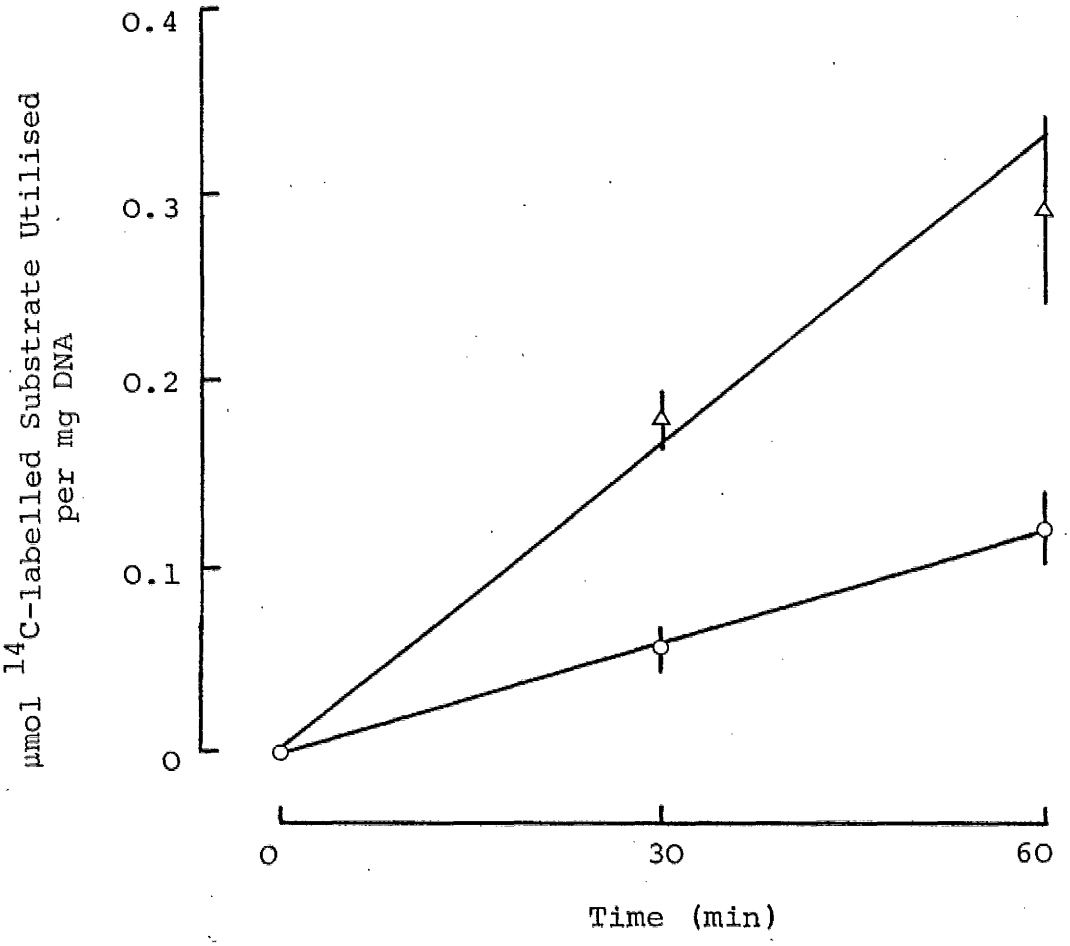


FIG. 7.1. Time Course of ^{14}C-labelled Glutamine Oxidation in Rumen Epithelium

[U-^{14}C]glutamine (O) and [U-^{14}C]glucose (Δ) were incubated with rumen papillae over a period of 1h. At the indicated time points the incubations were stopped by addition of 0.37M-$HClO_4$ and $^{14}CO_2$ collected as described in section 2.13. Results are means ± S.D.

In further experiments amino acid contents were determined with an LKB 4400 Amino Acid Analyser. Glutamine removal from the incubation medium by rumen papillae was not measured.

The results given in Figure 7.2 show that papillae incubated in the presence of glutamine, with or without butyrate, resulted in increases in the alanine, glutamate and ammmonia contents, as previously observed (see Table 7.1). An increase in alanine content was significant with propionate as the secondary carbon source; this increase was probably the result of elevated pyruvate content from metabolism of propionate in rumen epithelium. Propionate did not stimulate aspartate production (see Figure 7.2), an indication that there was no increase in the free oxaloacetate content.

In these experiments there was no significant increase in L-lactate production from any of the added substrates except in those incubations containing glutamine plus propionate. Also, no urea synthesis was observed in any of the incubations but the rate of [U-^{14}C] glutamine utilisation for $^{14}CO_2$ production was found to be 172nmol/mg DNA per h.

If it is assumed that the glutaminase pathway predominates in rumen epithelium then deamination should result in an equivalent amount of glutamate and ammonia being released (Watford *et al.*, 1979). Therefore,the proportion of glutamine not accounted for as glutamate

FIGS. 7.2, 7.3 and 7.4. Release of Amino Acids from Rumen Papillae Incubated with Glutamine and Volatile Fatty Acids

Each figure represents the release of the indicated amino acids from isolated papillae, incubated under different conditions. For each amino acid the conditions were as follows (reading from left to right): Control (0 min) plus glutamine (5mM), control (60 min), glutamine (5mM), glutamine plus butyrate (10mM) and glutamine plus propionate (10mM).

The glutamine peak appeared in the vicinity of threonine and serine peaks and as a result the data for these amino acids were lost. Serine and threonine levels for the control (60 min) were 185.5 ± 21.5 and 193.3 ± 33.0 nmoles/mg DNA per h, respectively.

Glutamine was freshly prepared before the start of experimental procedures and was added to the zero-time control s.

Results are means ± S.E.M. from three separate experiments.

Blocks with no error bars indicate only trace amounts of amino acids detected. Significance of difference between means (corrected for zero-time control *P < 0.05, **P < 0.01, ***P < 0.001.

FIG. 7.2

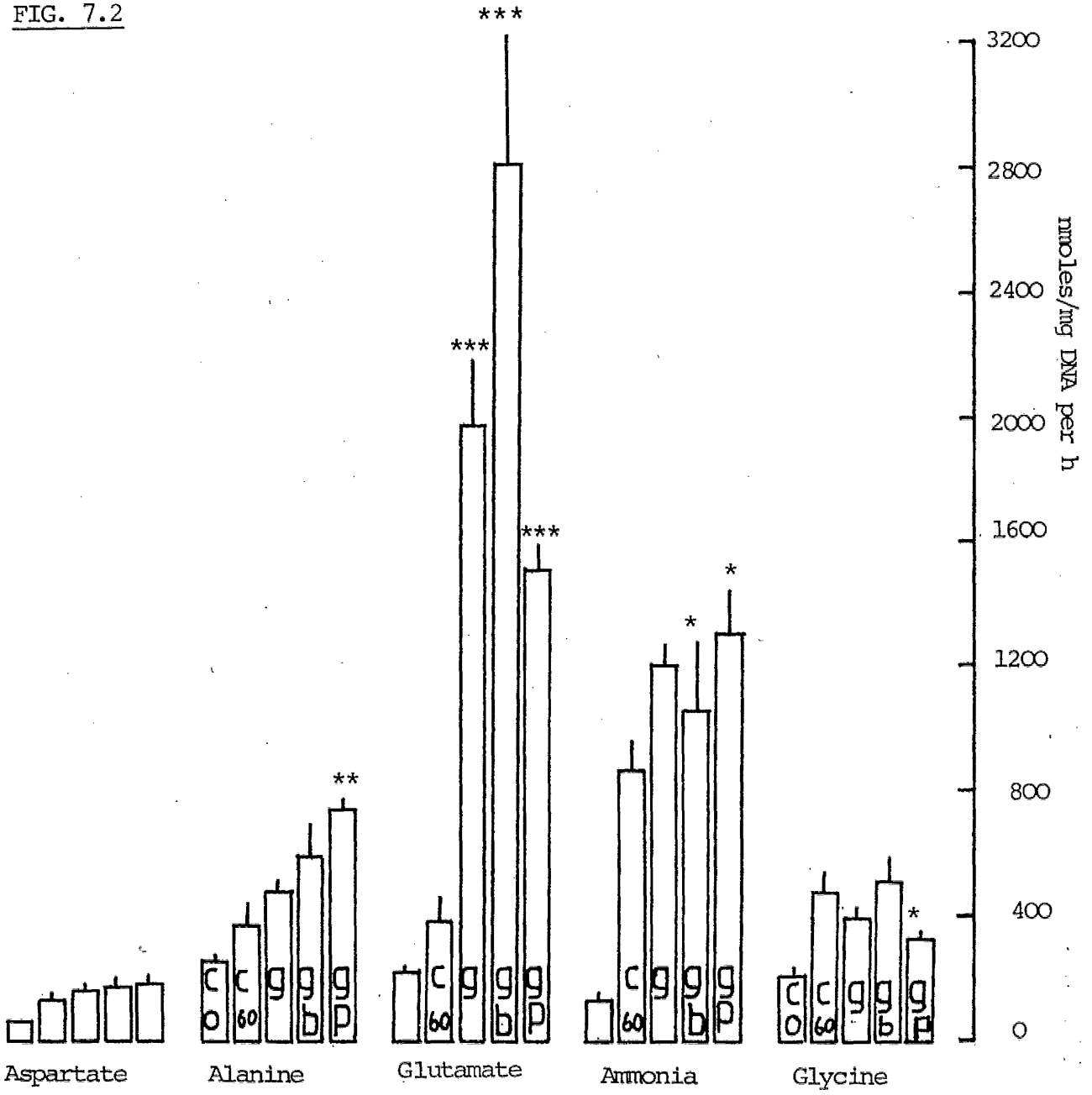

***
***
***
**
*
*
*
3200
2800
2400
2000
1600
1200
800
400
0
nmoles/mg DNA per h
c
o
c
60
g
g
b
g
p
Aspartate
Alanine
Glutamate
Ammonia
Glycine

FIG. 7.3

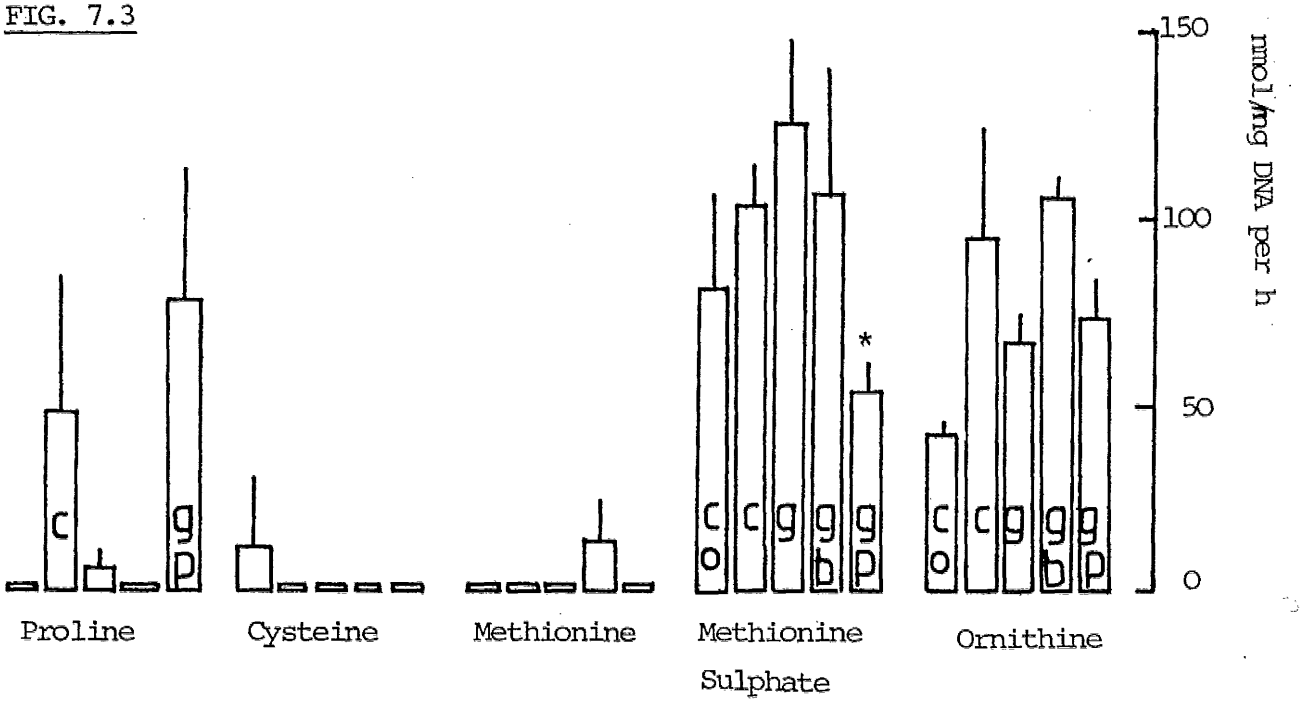
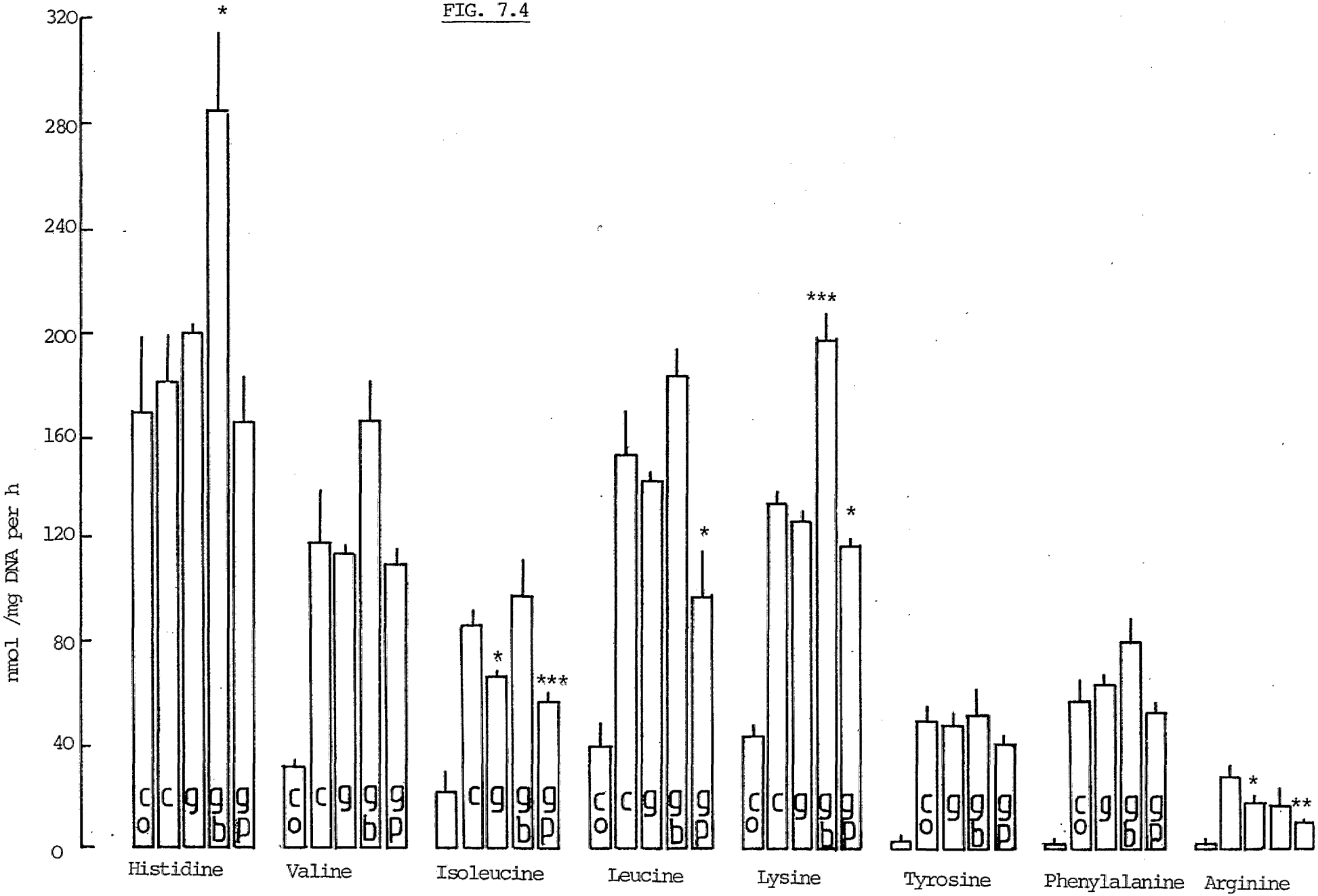

150
100
50
0
nmol/mg DNA per h
*
c
g
p
c
o
c
g
g
b
g
p
Proline
Cysteine
Methionine
Methionine
Sulphate
Ornithine

FIG. 7.4


nmol /mg DNA per h
320
280
240
200
160
120
80
40
0
c
o
g
b
p
Histidine
Valine
Isoleucine
Leucine
Lysine
Tyrosine
Phenylalanine
Arginine

alanine and aspartate can be calculated from the accumulation of ammonia.

It was found that recovery of released ammonia was less than expected (see Table 7.2). When ammonia release is presented as a percentage of the total of glutamate, alanine and aspartate contents the values for the different incubations are as follows; 46% (glutamine), 30% (glutamine + butyrate) and 53% (glutamine + propionate). This may indicate that ammonia, released from glutamine breakdown, is incorporated into other metabolites not measured in this study or that the original assumption is wrong.

One metabolite not measured was Δ'-pyrroline-5-carboxylate. Glutamine is reported to decay slowly at 20°C resulting in cyclisation to this metabolite (Oreskes & Kupfer, 1967; see Fig. 7.5). Cyclisation of glutamine to the imino acid is accelerated in the presence of bicarbonate or phosphate (Gilbert *et al.*, 1949) or at higher temperatures (Datta and Ottaway, 1975), such as those used in the amino acid analyser. For instance, Heitmann and Bergman (1980b) observed that 75% of [U-^{14}C] glutamine was consistently recovered after separation in the amino acid analyser. In this study glutamine added to the zero-time controls did not give rise to large amounts of ammonia, a by-product of cyclisation (see Fig. 7.5).

Products formed	Control + Glutamine	Control (60 min)	Glutamine (60 min)	Glutamine + Butyrate	Glutamine + Propionate
Ammonia	130 ± 22	858 ± 92	1201 ± 77	1056 ± 234	1299 ± 151
Glutamate (a)	218 ± 14	381 ± 80	1968 ± 222	2802 ± 439	1516 ± 96
Alanine (b)	148 ± 11	364 ± 83	467 ± 35	590 ± 103	729 ± 38
Aspartate (c)	66 ± 9	134 ± 10	159 ± 20	170 ± 31	190 ± 20
Σ (a) + (b) + (c)	432	880	2595	3562	2435

TABLE 7.2. Distribution of Glutamine-Nitrogen in Metabolites Released from Rumen Papillae

Results are mean values (nmol/mg DNA per h) ± S.E.M. Some of the glutamine nitrogen may have been incorporated into serine, as found by Boila and Milligan (1980), but this amino acid could not be determined (see legend to Fig. 7.3).

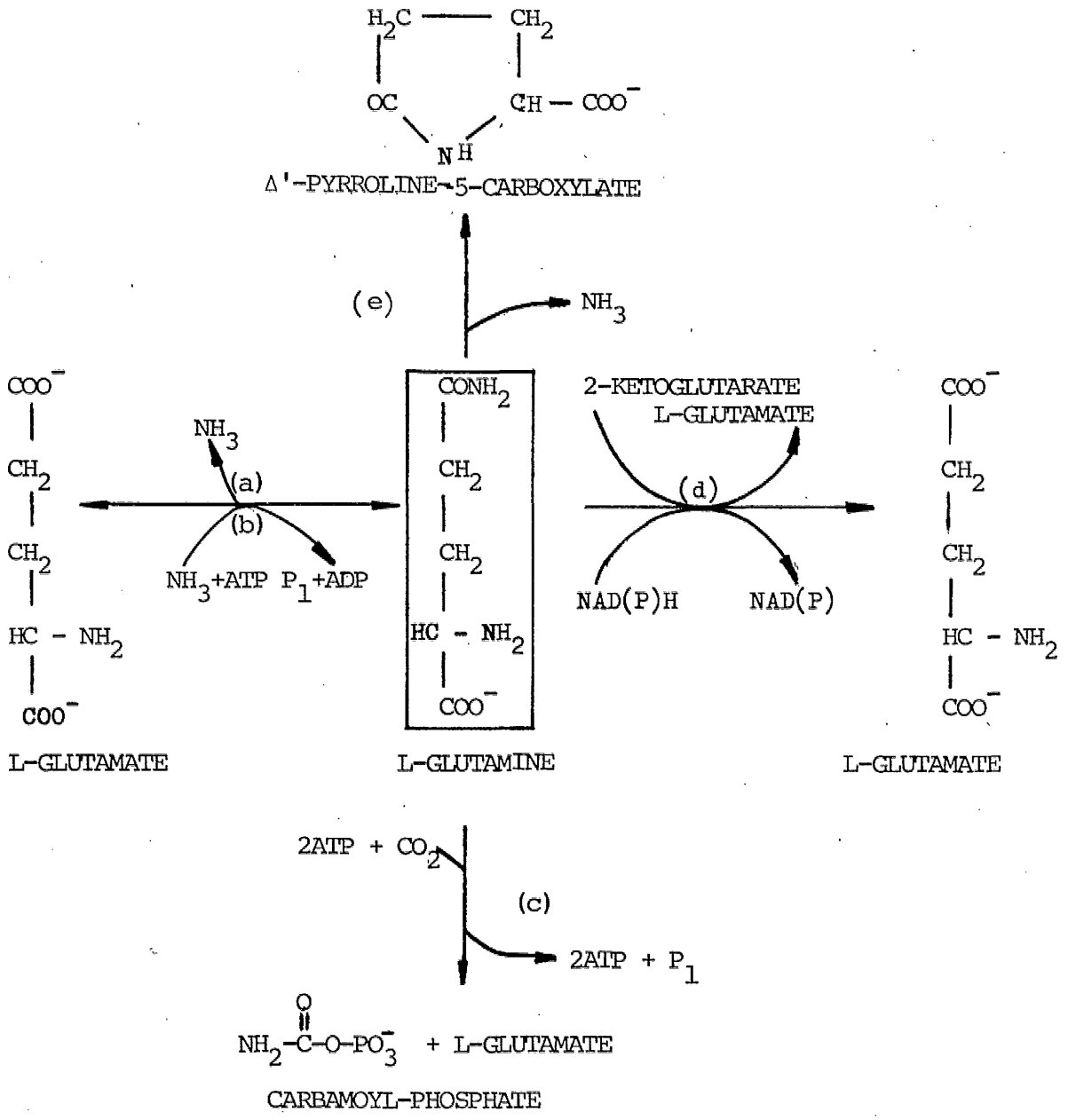


FIG. 7.5. Possible Pathways of Glutamine Metabolism

Reactions catalysed by; a, glutaminase (EC 3.5.1.2); b, glutamine synthetase (EC 6.3.1.2); c, carbamoyl-phosphate synthetase (EC 6.3.5.5); d, glutamate synthetase (EC 1.4.1.13 and EC 1.4.1.14); and e takes place as a result of spontaneous hydrolysis.

If turnover of nucleic acids occurs at a relatively high rate in rumen mucosa, as suggested by Kalachyuk's group (1974b; 1975), then a substantial portion of glutamine nitrogen maybe incorporated into nucleic acids. The first step is transfer of nitrogen from the donor molecule to carbamoyl-phosphate. Carbamoyl-phosphate supply may arise not only from the ammonia-dependent carbamoyl-phosphate synthetase, which has been detected in rumen epithelium (Emmanuel,1980a ; Salem *et al.*, 1973), but a glutamine-dependent enzyme. Glutamine-dependent carbamoyl-phosphate synthetase (EC 6.3.5.5) activity has been measured in *E. coli* (Anderson and Meister, 1965) and a variety of rat tissues (Yip and Know, 1970). Therefore, incorporation of glutamine nitrogen into nucleic acids may have accounted for some of the 'lost' nitrogen. The relatively high production rate of endogenous ammonia, after 1h incubation (see Fig. 7.2), may indicate a high turnover rate of nucleic acids in rumen mucosa as ammonia could arise from deamination of adenine nucleotides.

An alternative pathway which may exist in rumen epithelium is formation of glutamate from glutamine and 2-oxoglutarate (see Fig. 7.5). This reaction is catalysed by glutamate synthetase which uses NADPH (EC 1.4.1.13) (Tempest *et al.*, 1970; Miller and Stradtman, 1972) or NADH (EC 1.4.1.14) (Cohn, 1958) as coenzyme, although as yet this enzyme has only been studied in extracts from bacterial sources. If this route were to exist in the epithelial tissue it would account for the low observed net release of ammonia but high glutamate content.

It is possible that more than one of the described pathways for glutamine breakdown exist in rumen mucosa. If this were so then the flux through the pathways may depend on various conditions, such as α-oxoacids content. This tentative suggestion is put forward to explain the different results obtained with added propionate or butyrate to papillae supplied with glutamine. Obviously this problem warrants further investigation to resolve the pathway(s) of glutamine catabolism.

It was observed that propionate plus glutamine significantly decreased the release of glycine from sheep rumen papillae (Fig. 7.2). Similar results have been reported for bovine papillae (Boila and Milligan, 1980a). It was also found that the release of eleven other amino acids, in the presence of these two substrates, was lower than the 1h control and this being significant for the release of isoleucine, leucine, lysine and arginine (see Fig. 7.3 and Fig. 7.4).

The effect of propionate or butyrate on endogenous protein degradation is revealed in results given on Table 7.3. The essential amino acids shown on this table cannot be synthesised by mammalian tissues and must therefore originate from rumen epithelial protein. VFAs affect rumen epithelial protein degradation differently as shown by the total contents of the six essential amino acids.

Amino Acids Released	CONTROL + Glutamine	CONTROL (60 min)	GLUTAMINE (5mM)	GLUTAMINE + Butyrate (10 mM)	GLUTAMINE + Propionate (10 mM)
Isoleucine	22.2 ± 7.9	84.6 ± 5.9	65.6 ± 1.8	97.9 ± 13.3	57.5 ± 3.1
Leucine	39.5 ± 8.3	151.7 ± 16.5	141.9 ± 1.9	180.5 ± 7.8	97.6 ± 18.6
Lysine	43.7 ± 4.4	133.1 ± 4.5	126.2 ± 3.9	195.4 ± 8.8	117.2 ± 3.8
Phenylalanine	T	58.4 ± 7.1	63.7 ± 3.9	76.9 ± 8.6	53.3 ± 4.0
Valine	32.5 ± 2.3	116.3 ± 19.1	113.3 ± 4.4	164.9 ± 15.1	98.9 ± 4.4
Methionine	81.6 ± 24.5	102.8 ± 10.8	125.5 ± 22.1	119.7 ± 32.8	52.9 ± 8.7
TOTALS	219.5	638.5	636.2	835.3	477.4
%	34.4	100.0	99.6	130.8	74.8

TABLE 7.3. Profile of Essential Amino Acids Released from Sheep Rumen Papillae

See Legend to Fig. 7.3 for full experimental details. Results are means ± S.E.M.; T indicates trace values. Methionine is the sum of methionine plus methionine sulphate.

Essential amino acids were released from control papillae over the incubation period (see Table 7.4). Addition of glutamine produced no change in essential amino acid release. However, with butyrate as a secondary substrate the total was increased by about 30%, possibly indicating acceleration of proteolysis. Addition of propionate caused the opposite effect with the % total reduced by about 25%. Venous infusions of acetate and butyrate were shown to be less effective than propionate or glucose in depressing plasma essential amino acid concentrations in sheep (Eskland *et al.*, 1974). If essential amino acids are also broken-down by rumen epithelium then the catabolic rate may be affected differently by propionate or butyrate. If true, this could make some contribution to the different effects produced by VFAs on essential amino acid release from rumen papillae.

Lastly, the rate of endogenous protein degradation was estimated to be 2.3% hr^{-1}. This value was determined from the net release of 17 amino acids from rumen papillae into control medium (i.e. 1386 nmol/mg DNA). The average molecular wt. for amino acids was taken as 136.8 and the amount of protein degraded was calculated to be 1.05mg/g wet wt. per h (assuming, DNA/g dry wt. ratio of 27.6, section 4.2; wet wt./dry wt. ratio of 5, (Weekes, 1974); 44.9mg protein per g wet wt. for sheep rumen epithelium (Chandrasena *et al.*, 1979)). Of necessity this must only

be an approximation but it is of the same magnitude of the value determined for release of [^{3}H]leucine from prelabelled rumen epithelial protein (i.e. 2.9% hr^{-1}, see Chapter 6).

7.4. *Summary*

Glutamine metabolism in rumen epithelium results in some synthesis of aspartate and alanine; glutamate and ammonia are formed, compared to appropriate controls, in quantitatively significant amounts. Addition of propionate, but not butyrate, caused a significant increase in alanine release. There is no net formation of L-lactate, L-citrulline or urea from glutamine. The rate of glucose oxidation is nearly three times that of glutamine. Possible pathways for glutamine metabolism, which might exist in rumen epithelium, are discussed.

Substantial release of essential amino acids from control papillae occurs over the incubation period. From the rates of amino acids released the rate of proteolysis is estimated to be 2.3 % hr^{-1}, an approximation which is of the same magnitude as the value for protein degradation calculated from ^{3}H-labelled protein experiments. Glutamine did not influence the rate of essential amino acid release. However, butyrate stimulates this release by about an average of 30%, whereas propionate depresses it by approximately 25%.

A P P E N D I X

APPENDIX 1 - Sources of Chemicals, Enzymes, Radioisotopes and Hormones

1. *Chemicals*

Organic solvents, acid and alkali reagents and inorganic chemicals used were of the purest grade available from standard suppliers (i.e. B.D.H. Chemicals Ltd., Speke, Liverpool, U.K. or Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.). Other highly pure chemicals were obtained from the following suppliers:

Aldrich, Dorset, U.K.	n-Butylmalonic acid.
Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.	ADP
	AMP
	ATP
	NAD^+
	NADH
	$NADP^+$
	Oxaloacetic acid
	Triethanolamine.HCl
	N^6,2'-O-Dibutyryl-adenosine-3':5'-monophosphate
B.D.H. Chemicals Ltd., Speke, Liverpool, U.K.	Acrylamide
	N,N'-bis-methylene acrylamide
	Bromophenol blue
	Cocktail T scintillation fluid
	D-glucose

	NCS (solubiliser)
	PCS scintillation cocktail
	Sodium dodecyl sulphate
	Sucrose
	N,N,N',N'-Tetramethylenediamine
	Triton-X100 (scintillation grade)
British Oxygen Corp., Worsley, Manchester, U.K.	O_2:CO_2 (95%:5%; v/v)
Fisons Scientific Apparatus, Loughborough, Leics, U.K.	EDTA (disodium salt)
	Malonic acid
	Urea
Flow Laboratories, Irvine, Ayrshire, Scotland.	Eagles MEM (with Hank's Salts)
Hoffman La Roche Inc., Nutley, N.J., U.S.A.	(-)-Hydroxy citrate was a gift from Dr. A.C. Sullivan.
International Enzymes Ltd., Windsor, Berks, U.K.	Bovine Serum Albumin (fraction V)
P.L. Biochemicals Inc., Wisconsin, U.S.A.	Acetoacetyl-CoA, Lithium Salt
Ralph Emmanuel Ltd., Wembley, Middlesex, U.K.	aminooxyacetate (carboxy-methoxylamine hemi hydro-chloride)
	2-cyano-4-hydroxycinnamate.

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Aniline

Benzyl pennicillin (Na Salt)

Borohydride

DL-carnitine

Coomassie brilliant blue G250 and R250

Creatine Phosphate (Na Salt)

O-Dianisidine

Digitonin

Dithiothreitol

DNA (calf thymus)

L-Glutamine

HEPES

Hydrazine hydrate

DL-3-Hydroxybutyrate

Iodoacetamide

DL-Lactic acid

L-Lactic acid

2-Mercaptoethanol

Methylene blue

MOPS

Nicotinamide

Octanoic acid

2-Octanol

Oleic acid

Oxoglutarate

Palmitate (Na Salt)

Pyruvate (Na Salt)

Streptomycin sulphate.

Most amino acids and vitamins necessary for Eagle's Medium (1959).

Smith, Kline & French Lab., Philadelphia, U.S.A.

3-Mercaptopicolinic Acid

Miscellaneous

5-(Tetradecyloxy)-2-Furoic acid (dissolved in acetone) was a gift from Dr. S.A. McCune.

Phosphoenolpyruvate (monocyclohexylammonium salt) was prepared by the method of Clark & Kirby (1966)

ITP was made by treatment of ATP with NHO_2 according to the method of Kaplan (1957)

RS-Hydroxymethylglutaryl-CoA was synthesised by the method of Golfarb & Pitot (1971)

Acetyl-CoA was prepared by causing CoASH to react with diketene in a manner similar to that described by Simon & Shemin (1953)

2. *Enzymes*

Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.

Adenylate kinase (Myokinase; from hog muscle)
Citrate synthase (from pig heart)
Creatine kinase (from rabbit muscle)
D(-)-3-Hydroxybutyrate dehydrogenase (from *Rhodopseudomonas spheroides*)
Lactate dehydrogenase (from beef heart)
Malate dehydrogenase (from pig heart)
Phosphotransacetylase (from *Clostridium kluyveri*)
Pyruvate kinase (from rabbit muscle)
Urease (from Jack bean; *Canavalia ensiformis*)

International Enzymes Ltd., Windsor, Berks, U.K.

Peroxidase (from horseradish)

Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Glucose oxidase (Type V; from *Aspergillus niger*)
Glutamic-oxaloacetic transaminase (from porcine heart)
Luciferin : Luciferase (Type FLE-50; firefly lantern extract.

3. *Radioactive Chemicals*

Amersham International plc., Amersham, Bucks, U.K.

[U-^{14}C]Acetic acid, sodium salt (58mCi/mmol)

L-[U-^{14}C]Alanine (164 mCi/mmol)

n-[1-^{14}C]Butyrate, sodium salt (24 mCi/mmol)

D-[U-^{14}C]Glucose (281 mCi/mmol)

D-[6-^{14}C]Glucose (52.7 mCi/mmol)

L-[U-^{14}C]Glutamine (57.3 mCi/mmol)

L-[U-^{14}C]Lactic acid, sodium salt (160 mCi/mmol)

L-[4,5-^{3}H]leucine (46 Ci/mmol)

[1-^{14}C]Palmitic acid (57 mCi/mmol)

[1-^{14}C]Propionic acid, sodium salt (53 mCi/mmol)

[1-^{14}C]Pyruvic acid, sodium salt (25 mCi/mmol)

[2-^{14}C]Pyruvic acid, sodium salt (19 mCi/mmol)

Tritiated Water

4. *Hormones and Pharmacological Agents*

Glucagon (porcine monocomponents) was a gift from Eli Lilly & Co., Indianapolis, Indiana, U.S.A.

Beef monocomponent insulin was a gift from the Wellcome Foundation, Dartford, Kent, U.K.

Arg-Vasopressin (grade VI); (-)-Arterenol Bitartate (noradrenaline); L-Epinephrine (adrenaline); L-Isoproterenol (HCl salt); L-Phenylephrine (HCl salt); DL-Propanolol (HCl salt) were all purchased from Sigma Ltd., U.K.

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