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EFFECT OF NITRATE UPON THE DIGESTIBILITY
OF KIKUYU GRASS (✓PENNISETUM CLANDESTINUM)

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ABSTRACT

The factors affecting the accumulation of nitrate in kikuyu grass pastures and the effect of elevated nitrate levels upon digestion in the ruminant were investigated. A high potassium level in the soil seems to be the major factor stimulating the accumulation of excessive amounts of nitrate in kikuyu grass, when the nitrate content of the soil is also high. The continuous elongation of kikuyu grass tillers allows constant exposure of high nitrate containing stem tissue to the grazing ruminant.

Digestibility studies in vitro showed that nitrite, formed during the assimilatory reduction of nitrate to ammonia, reduces cellulose digestion, but the degree of reduction also depends upon the presence of readily available carbohydrates and protein in the digest.

Studies in vivo showed that the microbial population can adapt to metabolise high concentrations of nitrate (500 mg% N, m/m) in fresh kikuyu grass, without the accumulation of nitrite in the rumen. However, introduction into the rumen of nitrite in excess of the capacity of the nitrite reducing microbes, causes nitrite accumulation. Nitrite has no direct effect upon rumen cellulase activity. Due to the affinity of rumen carbohydrases for the substrate, attempts to isolate these enzymes by means of isoelectric focusing and other

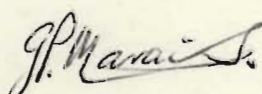
separation techniques met with limited success.

Nitrite strongly reduces the xylanolytic, total and cellulolytic microbial numbers with a concomitant decrease in xylanase and cellulase activity of the digest. Decreased microbial numbers could not be attributed to a less negative redox potential of the digest in the presence of nitrite, nor could the effect upon the cellulolytic microbes be attributed to an effect of nitrite on branched chain fatty acid synthesis required for cellulolytic microbial growth. A study of the effect of nitrite upon the specific growth rate of pure cultures of the major cellulolytic bacteria, Ruminococcus flavefaciens strain FDI, Butyrivibrio fibrisolvens strain Ce 51, Bacteroides succinogenes strain S 85 and Ruminococcus albus strain 22.08.6A and the non-cellulolytic bacterium Selenomonas ruminantium strain ATCC 19205 revealed the extreme sensitivity to nitrite of some of these bacteria and the relative insensitivity of others. Growth inhibition seems to depend primarily upon the extent to which these microbes derive their energy from electron transport-mediated processes.

PREFACE

Most of the experimental work described in this thesis was carried out in the Biochemistry Section, Cedara Agricultural Research Institute, Pietermaritzburg, from October 1983 to November 1985, under the supervision of Dr. Clive Dennison, Department of Biochemistry, University of Natal, Pietermaritzburg.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

A handwritten signature in dark ink, appearing to read 'J.P. Marais' with a stylized flourish at the end.

J.P. Marais.

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CHAPTER I

INTRODUCTION

Kikuyu grass is a forest margin species which occurs naturally on the highland plateaux of East and Central Africa where it thrives on fertile soil. From this natural habitat, it has been introduced to many countries and has spread over a wide geographical range. It was introduced into South Africa in 1910 when plants were established at the Botanic Gardens in Pretoria. A few years later kikuyu grass was transferred to the Cedara College of Agriculture (Taylor, 1941). Since then highly productive pastures have been developed and at present almost 30,000 ha is under kikuyu pastures in Natal (Dept. of Agriculture and Fisheries, 1981).

Kikuyu grass often comprises the bulk of the summer pasturage for milk production in the high rainfall areas of Natal. It responds well to high levels of nitrogen fertilizer but the performance of lactating cows on these pastures is much poorer than expected, based on proximate analyses, and tends to deteriorate towards the end of the growing season. Instances have also been reported of lush kikuyu pastures becoming totally unpalatable. Poor animal production seems to be associated with old, well established pastures which are heavily fertilised, often with slurry obtained from piggeries and dairies.

Although plants can effectively utilise both nitrate and ammonia, the ammonia, derived largely from ammoniacal fertilizers and the excreta of grazing animals, is readily oxidised to nitrate by soil organisms. This process is extremely rapid in moist, well aerated soils. Nitrate is therefore the predominant form of nitrogen available to pasture plants and is of primary importance for normal plant metabolism. In most instances, nitrate absorbed by the plant, is assimilated without the accumulation of large quantities of nitrate within the plant tissue, but many internal and environmental factors have been shown to affect the nitrate level (Wright & Davison, 1964; Huffaker & Rains, 1978). At present the regulation of the uptake and assimilation of nitrate by higher plants is not fully understood, but its accumulation would be favoured by any factor which leads to an absorption rate in excess of the assimilation rate.

Elevated levels of nitrate could pose a serious threat to animal health. Numerous instances of poisoning and death of ruminants on cultivated pastures containing high levels of nitrate have been described in the literature (Turner & Kienholz, 1972; Kemp, Geurink, Haalstra & Malestein, 1976; Kromann, Weikel & Falen, 1976; Jones & Jones, 1977). Although no animal deaths associated with elevated nitrate levels in kikuyu pastures in Natal have been reported, extremely high levels of nitrate are common in these pastures.

In the rumen, fodder nitrate is reduced to ammonia by some

rumen microbes, with nitrite as an intermediate product. Under certain conditions the rate of nitrite formation may exceed the rate of nitrite reduction to ammonia, resulting in elevated nitrite levels. Although nitrate as such seems to be relatively non-toxic to animals, nitrite may cause the following harmful effects:

a) Formation of methaemoglobin. Haemoglobin in the blood acts as an oxygen carrier by binding oxygen to the ferrous iron (Fe^{++}) in the haem at high oxygen partial pressures and releasing it again when the partial pressure is decreased. Nitrite is absorbed through the rumen wall into the blood stream. Being an oxidising agent, it converts the ferrous iron to ferric iron (Fe^{+++}) thus forming methaemoglobin. Due to this valence change, the reversible affinity of the iron for oxygen is lost and the haem, therefore, loses its function as an oxygen carrier, resulting in anoxia of the animal.

Vertregt (1977) investigated the nature of the reactions leading to the formation of methaemoglobin in bovine blood in the presence of nitrite, while Kemp, Geurink, Haalstra & Malestein (1977), Geurink, Malestein, Kemp & Van't Klooster (1979) made a comprehensive study of the factors leading to elevated methaemoglobin levels in the blood.

b) Lowering of blood pressure. Nitrite acts as a vasodilator, causing circulatory disturbances such as a reduction in blood pressure (Ashbury & Rhode, 1964). Holtenius (1957), in an

extensive study of nitrate toxicity in ruminants, was unable to demonstrate a reduction in oxygen transport in sheep suffering from severe methaemoglobinaemia and concluded that the drop in blood pressure, induced by the nitrite ion, is the main cause of death of the affected animals.

c) Formation of carcinogens. In recent years, a number of observations have led to concern about the potential risk to animal and human health, due to possible carcinogenic effects as a result of interactions between nitrite and substances such as amines and amides, to produce N-nitroso compounds (Van Broekhoven & Davies, 1980, 1981). A large number of these N-nitroso compounds are known carcinogens, causing cancer in many animal species (Magee & Barnes, 1956; Fishbein, 1979). The transfer of N-nitroso compounds from the rumen into the milk and consequent exposure to man, has been suggested (Juskiewicz & Kowalski, 1974).

Concern about the possible formation of carcinogens due to the widespread use of nitrite in cured meats, led to a thorough investigation by the United States National Academy of Sciences into the effect of nitrate and nitrite on human health (Committee on nitrite and alternative curing agents in food, 1981).

Apart from the above direct effects on animal and human health, nitrite and/or nitrate may, more subtly, also influence production in ruminants by adversely affecting the

rumen microbial population. Although earlier workers found no harmful effects other than a transient methaemoglobinaemia in animals consuming sub-lethal amounts of nitrate (Jamieson, 1959), a few workers subsequently reported a reduction in the digestibility of forages in vitro in the presence of nitrate (Hall, Gaddy & Hobbs, 1960; Marais, 1980).

At present, relatively little is known of the chemical changes within the rumen leading to a decline in digestibility and alterations in microbial population associated with the intake of high nitrate feeds. This lack of information prompted the investigation reported here.

In this study, attention was focussed on three important aspects. Firstly, an attempt was made to obtain a better understanding of the factors regulating nitrate metabolism in kikuyu grass and to establish the reasons for the accumulation of nitrate in certain kikuyu pastures. Secondly, by means of digestibility studies in vitro experiments were conducted to determine which substances reduce the digestibility of kikuyu. Attempts were also made to determine the mode of action of these substances. Finally, the effect of nitrite upon the specific growth rate of pure cultures of some of the more important rumen bacteria and in particular of the key cellulolytic microbes, was studied.

CHAPTER 2

FACTORS INFLUENCING THE LEVEL OF NITRATE IN KIKUYU GRASS PASTURES

Plants maintain a negative electrical potential between the cytoplasm of root cells and the ambient medium (Higinbotham, 1973). Nitrate ions are readily absorbed against this electro-chemical potential gradient by a process requiring metabolic energy (Raven & Smith, 1976; Huffaker & Rains, 1978). Although the mechanism involved is not known, several authors have postulated the involvement of a specific nitrate transport protein (Jackson, Flesher & Hageman, 1973; Neyra & Hageman, 1975). In order to maintain a constant potential within the plant tissue, absorption of nitrate is accompanied by the outward movement of internally generated bicarbonate and hydroxyl ions. Furthermore, some of the absorbed nitrate is continuously lost from the root, even at high ambient nitrate concentrations. Absorption has, therefore, been described in terms of a pump and leak model (Jackson, Flesher & Hageman, 1973; Jackson, Kwik, Volk & Butz, 1976).

According to Haynes (1980) the absorption of nitrate and other anions by plant roots, is influenced by the presence of cations in the ambient medium, but these ionic interactions within the apoplasm, which is the continuum of the external

ionic medium within the root, are poorly understood. Franklin (1970) showed that cations, especially divalent and trivalent cations such as calcium and aluminium, greatly stimulate the uptake of phosphate by the plant. He postulated that these cations neutralise the fixed negative charges (consisting of immobile carboxyl groups associated largely with pectins) on the cell walls surrounding the apoplasm. Anions such as nitrate and phosphate will therefore encounter less electrical interference when passing through the cell wall and their absorption is thus enhanced.

Once absorbed by the roots, nitrate, or its reduction products, are translocated throughout the plant. A scheme describing this process has been proposed by Ben-Zioni, Vaadia & Lips (1970, 1971). According to this model, nitrate is absorbed across the plasmalemma of the root cells in exchange for bicarbonate ions. The absorbed nitrate ions, in the presence of a cation (usually potassium) are transported via the intercellular plasmodesmata to the xylem. In the xylem it is translocated to the shoots where nitrate is transformed into organic nitrogen with the simultaneous synthesis of carboxylates. The carboxylate salts (mainly potassium malate) are translocated to the roots by means of the phloem stream, where they are decarboxylated to form bicarbonate ions which are again exchanged for nitrate ions (Fig. 2.1).

According to Van Egmond (1978), this model in which anions are

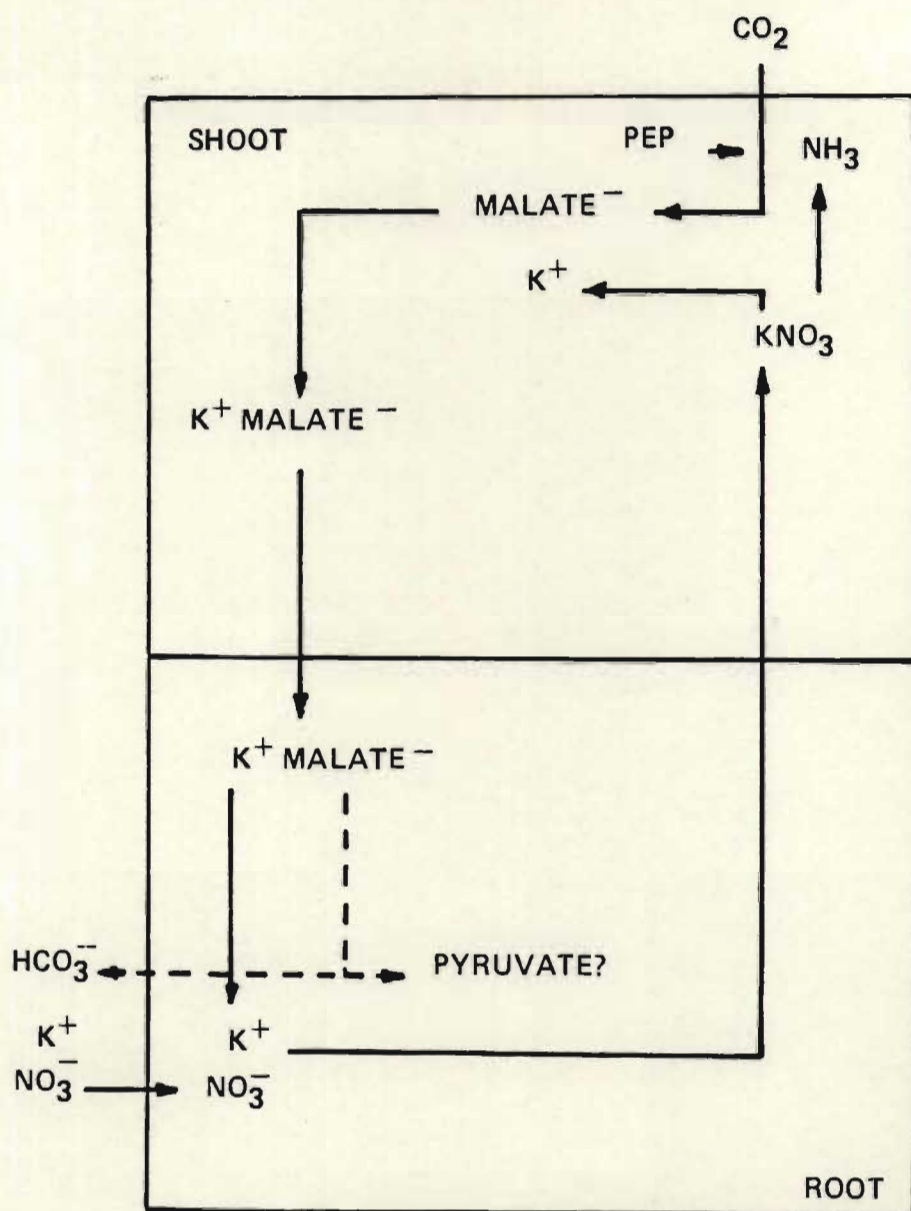


FIG. 2.1 Ben Zioni-Lips Model, describing the movement of malate, nitrate and potassium ions between root and shoot. PEP = phospho-enol pyruvate (from Van Egmond, 1978)

absorbed in excess of cations, applies particularly to members of the Gramineae. In contrast, most dicotyledonous herbaceous plants have a cation/anion uptake ratio close to unity. In these plants the translocation of carboxylates to the roots and their decarboxylation, leading to the exchange of nitrate for bicarbonate, seems to play a minor role.

In most plants the reduction of nitrate and its conversion into organic form, take place in both roots and leaves but considerable differences exist between species (Jackson, 1978). In cereals most of the nitrate is first translocated to the leaf mesophyll cells, where reduction takes place (Chantarotwong, Huffaker, Miller & Granstedt, 1976).

It is generally accepted that nitrate and its reduction products, such as amino acids, are compartmentalised in the plant into a relatively small metabolic pool (presumably in the cytoplasm) and a much larger storage pool (presumably in vacuoles)(Jackson, 1978). The movement of nitrate into and out of the metabolic pool is regulated by the plasmalemma and tonoplast. The studies of Shaner & Boyer (1976a and b) and Huffaker & Rains (1978) suggest that nitrate enters the cytoplasm much less readily from the vacuoles than from the xylem elements in the case of maize and sudan grass. Very little nitrate is retained in the cytoplasm and its presence is ephemeral, but this nitrate seems to be responsible for the induction of nitrate reductase, which is the key enzyme regulating nitrate assimilation in the plant.

Nitrate assimilation is a photosynthetic process driven by sunlight energy with water acting as the ultimate reductant (Guerrero, Vega & Losada, 1981). In most higher plants reduction of nitrate to nitrite is catalysed by a nicotinamide adenine dinucleotide (NADH)-dependent nitrate reductase having flavin adenine dinucleotide (FAD), cytochrome b-557 and molybdenum as co-factors. Although it has been suggested that the enzyme is associated with the outer surface of the chloroplast (Beevers & Hageman, 1969), it is generally considered a cytoplasmic enzyme (Hewitt, Hucklesby & Notton, 1976). In contrast, nitrite reductase in the leaves of higher plants is localised in the chloroplasts (Rathnam & Edwards, 1976; Harel, Lea & Miflin, 1977). Ferredoxin-nitrite reductase catalyses the reduction of nitrite to ammonia in a single step. In turn, the ammonia is incorporated into organic compounds, mainly free amino acids and proteins.

The major route of conversion of ammonia into the organic form in higher plants is through the keto-acid, l-oxoglutarate by means of the glutamate synthase cycle localised in the chloroplasts (Lea & Miflin, 1974). Ammonia can also be released from the organic form during photorespiration in plant tissue, particularly from glycine, asparagine and arginine (Keys, Bird, Cornelius, Lea, Wallsgrove & Miflin, 1978). This ammonia generated in the plant is immediately assimilated and does not accumulate.

However, the flow of nitrogen into an organic form and the nature of the products of assimilation are determined largely by the carbon flux into keto acids.

Many internal and environmental factors may therefore regulate or affect the process of nitrate absorption and assimilation leading to elevated levels of nitrate in the plant. Several authors have documented the accumulation of such high levels in pasture grasses (Reid & Strachan, 1974; White & Halvorson, 1980).

In the investigation reported in this chapter, four different experiments were conducted in an attempt to obtain a better understanding of the factors controlling the level of nitrogenous compounds in kikuyu pastures. In the first experiment, conducted on a newly established pasture, the effect of nitrogen fertilizer level on the nitrate content of the grass was followed over the growing season. In the second experiment, done on an old, established pasture, a comparison was made between palatable areas and areas which have become unpalatable to grazing sheep. In the third experiment, also on an old kikuyu pasture, grass known to contain high levels of nitrate was compared with grass known to be low in nitrate. In the fourth experiment the effect of potassium upon nitrate uptake was investigated by growing kikuyu grass under controlled conditions in defined aqueous nutrient media in a glasshouse.

2.1 Experimental plots and fertilizer treatment

Experiment 1 Thirty six plots (10m^2) were measured out in a newly established kikuyu pasture not used for grazing. Six randomly distributed replicate plots each received limestone ammonium nitrate (LAN) at a rate of 0, 100, 200, 300, 400 or 500 kg N.ha^{-1} per annum, applied during the growing season in four equal dressings. At the beginning of the growing season all plots were defoliated and representative soil samples were taken before the first LAN dressings were applied. Three of the replicate plots at each rate of LAN application were cut (cutting height, 5 cm) at 3-week growth stages throughout the growing season, while the remaining plots were cut at intervals of 6 weeks for chemical analysis.

Experiment 2. Two experimental plots (470m^2) were measured out, five meters apart within an old established kikuyu pasture. Plot 1 was situated in an area which had been readily grazed by sheep during previous seasons, while plot 2 was in an unpalatable area. At the beginning of the growing season representative soil samples were collected from each plot and both plots were fertilized with LAN at a rate of 120 kg N.ha^{-1} . A further LAN application (120 kg N.ha^{-1}) was applied towards the end of the growing season. The kikuyu grass in plot 1 grew faster than that in plot 2 and in order to take into account differences in composition due to variation in plant age and height, the two plots were sub-divided into A and B sections. The A sections were cut (cutting height, 5 cm) when the grass in each attained a

height of 20 cm, while both B sections were cut at the same time when the grass in plot 2B attained a height of 20 cm. During the growing season, three cuts were made from each section.

Experiment 3. Three experimental plots (1500m^2) were measured out in old established kikuyu pastures. All plots were fertilised with LAN ($250\text{ kg N}\cdot\text{ha}^{-1}$) at the beginning of the growing season. The kikuyu in plots 1 and 2 was known to accumulate high levels of nitrate while the nitrate content of the grass in plot 3 was low. Towards the end of the growing season (early February) representative soil samples were taken and each plot was sub-divided into 10 strips of 150m^2 each. One strip of each plot was defoliated (cutting height, 8 cm) every second day and the defoliated strip in plot 1 fertilized with a heavy dressing of LAN ($1200\text{ kg N}\cdot\text{ha}^{-1}$). Representative samples of regrowth were taken for chemical analyses from all strips 15 days after the last defoliation. Individual tillers from the oldest regrowth ($\pm 25\text{cm}$) were also collected and divided into leaf and stem fractions for analysis.

Experiment 4. Kikuyu tillers were grown in nutrient solutions based upon medium II of Shive & Robbins (Hewitt, 1952) with variation in the concentration of potassium and/or nitrate. Twenty four black plastic containers (5dm^3) were divided into four groups. Group 1 received the basic medium containing the following components in 1 dm^3 of medium;

sodium nitrate (0,485g), calcium chloride 2-hydrate (0,524g), potassium dihydrogen phosphate (0,388g), magnesium sulphate 7-hydrate (0,935g), manganese sulphate 4-hydrate (1,0mg), zinc sulphate 7-hydrate (0,2mg), molybdic acid (0,1mg), copper sulphate 5-hydrate (0,1mg), boric acid (0,5mg), potassium iodide (0,03mg) and cobalt (II) nitrate (0,1mg). Iron was added as an iron-EDTA complex ($4 \text{ cm}^3 \cdot \text{dm}^{-1}$) prepared by mixing a solution containing iron (II) sulphate 7-hydrate (24,9g) with a solution containing ethylenediamine-tetraacetate (EDTA) (26,1g) and potassium hydroxide (16,05g) to give a final volume of 1 dm^3 . The iron solution was aerated over-night. The pH of the nutrient medium was 6,16.

In the second group the nitrate content of the medium was increased three-fold by increasing the amount of sodium nitrate added. The potassium dihydrogen phosphate of the nutrient medium of the third and fourth group of containers was replaced with tri-potassium phosphate, keeping the phosphate level of the medium constant but increasing the potassium level three-fold. The pH of the medium was decreased to 6,16 by titrating with nitric acid and the nitrate contents of these media were adjusted to the level of the basic medium and to a three-fold higher level respectively by the addition of appropriate amounts of sodium nitrate.

Six kikuyu rhizome tips (10cm long) were suspended through a

central hole in a black plastic dish covering each container. The plants were grown in a greenhouse for a period of 4 weeks at a day temperature of 32°C, and a night temperature of 25°C, followed by a growth period of 1 week at a day temperature of 20°C and a night temperature of 15°C to retard growth and facilitate nitrate accumulation. Nutrient solutions were replaced weekly. At the end of the second growth period new top growth was harvested for chemical analyses.

2.2 Sample preparation

2.2.1 Soil

Representative soil core samples (150 mm deep) were air dried in open trays, crushed, sieved (1 mm mesh) and stored for subsequent analysis of exchangeable cations, pH and total nitrogen.

2.2.2 Plants

Plant material was dried in a forced-draught oven at 100°C and milled to pass a 1 mm screen for subsequent chemical analysis.

2.3 Analytical procedures

2.3.1 Minerals in soil

2.3.1.1 Exchangeable potassium, magnesium, calcium and sodium

Reagents

Ammonium acetate. Ammonia liquor (1875 cm^3 , 25% NH_3), glacial acetic acid (1140 cm^3) and strontium chloride (75g) were added to 10 cm^3 de-ionised water. After cooling, the solution was made up to 25 dm^3 and the pH was adjusted to 7,0 by adding either ammonia or acetic acid.

Filter pulp. Filter Flock No. 122 (Schleicher & Schull) ($\pm 20\text{g}$) in de-ionised water (1 dm^3) was macerated in a Waring blender.

Cation Standards. A stock solution was prepared by dissolving potassium chloride (0,298g), sodium chloride (0,234g), calcium carbonate (2,00g), strontium chloride (3,0g), magnesium carbonate (1,690g) and concentrated hydrochloric acid (10 cm^3) in de-ionised water and making up to 1 dm^3 . Further dilutions (20 cm^3 to $100 \text{ cm}^3 \cdot \text{dm}^{-3}$) of the stock solution were made for the construction of standard curves for potassium and sodium over the range $0,2 \text{ c mol}(+) \cdot \text{dm}^{-3}$ to $1,0 \text{ c mol}(+) \cdot \text{dm}^{-3}$ and for calcium and magnesium over the range $2,0 \text{ c mol}(+) \cdot \text{dm}^{-3}$ to $10,0 \text{ c mol}(+) \cdot \text{dm}^{-3}$.

Extraction procedure

Filter pulp solution ($\pm 50 \text{ cm}^3$) was poured into a leaching column (4,5 cm x 26 cm, fitted with a 30 mm perforated ceramic disc) and sucked dry with a vacuum pump. A soil sample (10g) was leached through the column with ammonium acetate solution at a rate of about $4 \text{ cm}^3 \cdot \text{min}^{-1}$ and 250 cm^3

of leachate was collected.

Quantitative analysis

The cations in the extract were analysed using a Perkin-Elmer 2380 atomic absorption spectrophotometer.

2.3.1.2 Exchangeable phosphate

Reagents

Sulphuric acid ($0,025 \text{ mol.dm}^{-3}$). Two Merck Titrisol ampoules ($0,5 \text{ mol.dm}^{-3}$) were made up to 1 dm^3 with de-ionised water to give a $1,0 \text{ mol.dm}^{-3}$ stock solution. An aliquot of this solution was made up to 10 dm^3 with de-ionised water.

Activated carbon. Darco G 60 charcoal (500g) was washed with 50%(v/v) sulphuric acid ($2,5 \text{ dm}^3$) and rinsed with de-ionised water until the wash water had a pH value of 2,5 or higher.

Molybdovanadate reagent. Solution 1. Ammonium metavanadate (2,53g) was dissolved in hot (90°C) de-ionised water (200 cm^3). The solution was cooled, transferred to a 4 dm^3 volumetric flask and diluted with de-ionised water ($1,5 \text{ dm}^3$). Concentrated nitric acid (320 cm^3) was added to this solution with constant stirring.

Solution 2. Ammonium molybdate (46,6g) was dissolved in hot de-ionised water (1 dm^3). The solution was cooled and Wetting

agent A (Technicon Chemicals Co.) ($2,0 \text{ cm}^3$) added. Solution 2 was added to solution 1 in the volumetric flask and diluted to volume with de-ionised water.

Phosphate standard. Potassium dihydrogen phosphate (4,3929g) was dissolved in de-ionised water and made up to 1 dm^3 . A stock solution was prepared by diluting an aliquot of this solution (10 cm^3) to 1 dm^3 . Further dilutions (5 cm^3 to 25 cm^3 , 50 cm^{-3}) of the stock solution were made for the construction of a standard curve over the range 1 mg to 5 mg P. dm^{-3} .

Extraction procedure

Extraction was carried out at $25 \pm 1^\circ\text{C}$ and the flask and soil sample were allowed to stabilise at this temperature before extraction. A 200 cm^3 Erlenmeyer flask containing the soil sample (5,0g), activated carbon ($\pm 1,6\text{g}$) and $0,25 \text{ mol}.\text{dm}^{-3}$ sulphuric acid (50 cm^3 , 25°C) was shaken on a reciprocating shaker ($175 \text{ cycles}.\text{min}^{-1}$) for exactly 4 min. The extract was immediately filtered through two layers of Whatman No.541 filter paper into $4,0 \text{ cm}^3$ Elkay Auto Analyser sampling containers and sealed with Elkay stoppers.

Quantitative analysis

An automated procedure based on the reaction of phosphorus with molybdovanadate to form a phosphomolybdovanadate

complex, which is measured colorimetrically in a Technicon Auto Analyser II, was used for quantitating phosphorus in the extracts (Technicon Auto Analyser, 1972).

2.3.2 pH of soil

A soil sample (15g) in $1,0 \text{ mol.dm}^{-3}$ potassium chloride (25 cm^3) was stirred mechanically for 5 min and allowed to stand for 30 min. The sample was stirred for a further 2 min and allowed to stand for 30 min. The pH of the solution was measured with a T & C 1003 pH meter.

2.3.3 Minerals in kikuyu grass

2.3.3.1 Potassium, magnesium and calcium

Reagents

Strontium chloride solution. Strontium chloride (152,15g) was dissolved in de-ionised water and made up to 1 dm^3 .

Cation standard. Merck Titrisol standards (cation content, $1,000\text{g.dm}^{-3}$) were used. Calcium standard (250 cm^3), potassium standard (250 cm^3) and magnesium standard (50 cm^3) were diluted with distilled water and made up to 1 dm^3 . Aliquots (40 cm^3 , 80 cm^3 and 160 cm^3) of the stock solution were diluted with distilled water and strontium chloride solution (20 cm^3) added to each before making up to 1 dm^3 .

Hydrochloric acid solution. Concentrated hydrochloric acid

(200 cm³) was diluted and made up to 1 dm³ with de-ionised water.

Preparation of sample

A kikuyu grass sample (2,0g) was ashed in a glass beaker by heating overnight in a muffle furnace at 450°C. Dilute hydrochloric acid (5,0 cm³) was added and the mixture was evaporated to dryness in a boiling-water bath. A further volume of acid (5,0 cm³) was added and the beaker again heated in the water bath for about 10 min.

Strontium chloride solution (5,0 cm³) was added to the acid solution and the mixture was filtered and quantitatively washed through Whatman No. 42 filter paper into a 250 cm³ volumetric flask. The solution was made up to 250 cm³ and further dilutions (5 x for calcium, magnesium and phosphate, and 10 x for potassium) were made before analysis.

Quantitative analysis

Calcium, magnesium and potassium were analysed on a Perkin-Elmer 2380 atomic absorption spectrophotometer.

2.3.3.2 Phosphate

Reagents

Molybdovanadate reagent. Prepared as for soil phosphate

analysis (2.3.1.2).

Phosphate standard. Potassium dihydrogen phosphate (4,3929g) was dissolved in de-ionised water and made up to 1 dm³. Further dilutions (2 cm³ to 50 cm³.dm⁻³) of the stock solution were prepared for the construction of standard curves over the range 2 mg to 50 mg.dm⁻³.

Preparation of sample

Analyses were conducted on the extract prepared for potassium, magnesium and calcium analyses (2.3.3.1).

Quantitative analysis

Phosphate was quantitated on a Technicon Auto Analyser II as described for soil phosphate (2.3.1.2).

2.3.4 Nitrate nitrogen

Nitrate in kikuyu grass samples was determined by the procedure of Cataldo, Haroon, Schrader & Young (1975), which is based on the nitration of salicylic acid under highly acidic conditions and the colorimetric determination of the resulting coloured complex which absorbs maximally at 410 nm in basic (pH>12) solutions.

Reagents

Salicylic acid-sulphuric acid reagent. Salicylic acid (5,0g) was dissolved in concentrated sulphuric acid (100 cm³) and stored in a brown bottle.

Sodium hydroxide (2,0 mol.dm⁻³). Sodium hydroxide (80g) was dissolved in distilled water and made up to 1 dm³.

Nitrate Standard. Potassium nitrate (1,804g) was dissolved in distilled water and made up to 1 dm³. An aliquot (10 cm³) of this solution was diluted and made up to 100 cm³. (An aliquot (0,2cm³) of this standard solution contained 5,0 µg nitrate-N).

Extraction procedure

A kikuyu grass sample (0,2g) was added to distilled water (20 cm³) in a test-tube and incubated at 45°C for 1h with occasional shaking. After incubation the solution was centrifuged (2000 x g, 5 min, room temp.) and the supernatant used for analysis.

Chemical analysis

Salicylic acid reagent (0,8 cm³) was added to a sample of the supernatant extract (0,2 cm³) in a test-tube, mixed well and kept at room temperature for 20 min. The pH of the solution

was then raised above 12 by the addition of sodium hydroxide solution (19 cm^3), while stirring well. The test-tube was cooled to room temperature and the absorbance of the solution read at 410nm. The blank was prepared in a similar way except that the salicylic acid reagent was replaced by concentrated sulphuric acid. Standards were analysed concurrently with unknowns and the concentration of each unknown sample was calculated by applying the following equation:

$$\text{Nitrate-N content (mg\%,m/m)} = \left(\frac{\text{absorbance sample}}{\text{absorbance standard}} \right) \times \left(\frac{25000}{\text{DM\%}} \right)$$

Where DM% = dry mass % of sample

2.3.5 Total organic nitrogen, protein nitrogen and non-protein organic nitrogen

Samples were assayed for nitrogen by the macro-Kjeldahl procedure. Initially protein nitrogen was determined after precipitation with cupric hydroxide (Association of Official Agricultural Chemists, 1965). However, the lengthy procedure for the preparation of the cupric hydroxide reagent and the time-consuming filtration and washing steps reduced the appeal of this procedure. Subsequently a rapid procedure, using trichloroacetic acid (TCA) as a protein precipitant in animal feed analysis was developed in this laboratory (Marais & Evenwell, 1983). Results obtained with this procedure were comparable with the cupric hydroxide procedure.

Reagents

Trichloroacetic acid (10%,m/v). Trichloroacetic acid (50g) was dissolved in distilled water and made up to 500 cm³.

Trichloroacetic acid (2,5%,m/v). Trichloroacetic acid (25g) was dissolved in distilled water and made up to 1 dm³.

Boric acid (4%,m/v). Boric acid (400g) was dissolved in distilled water and made up to 10 dm³.

Sodium thiosulphate (8%,m/v). Sodium thiosulphate (800g) was dissolved in distilled water and made up to 10 dm³.

Sulphuric acid (0,5 mol.dm⁻³). A Merck Titrisol ampoule (0,5 mol.dm⁻³) was diluted with distilled water and made up to 10 dm³.

Sodium hydroxide (± 45%,m/v). Sodium hydroxide flakes (9kg) were dissolved in distilled water (20 dm³).

Mixed indicator. Bromo-cresol green (0,6g) was dissolved in distilled water, 0,1 mol.dm³ sodium hydroxide solution (12 cm³) was added and the mixture made up to 600 cm³ with distilled water. Methyl red (0,2g) and 0,1 mol.dm⁻³ sodium hydroxide solution (6,0 cm³) were made up to 200 cm³ with ethanol. The two indicator solutions were combined and made up to 1,6 dm³ with ethanol.

Precipitation of protein

A kikuyu grass sample (1,0g) was suspended in distilled water (15 cm^3) in a test-tube and heated in a boiling-water bath for 10 min. After cooling, cold 10% (m/v) TCA (15 cm^3) was added with mixing and the mixture was kept at about 4°C for 2h. The contents of the test-tube was filtered through Whatman No. 541 filter paper in a Buchner funnel and the retentate was washed with cold 2,5% (m/v) TCA ($\pm 50 \text{ cm}^3$).

Macro-Kjeldahl

A kikuyu grass sample (1g, for total nitrogen) or the filter paper with residue after TCA precipitation (for protein nitrogen) was transferred to a macro-Kjeldahl digestion flask. Potassium sulphate ($\pm 15\text{g}$), mercuric oxide ($\pm 0,5\text{g}$) and concentrated sulphuric acid (35 cm^3) were added and the mixture was digested in the Kjeldahl apparatus for 2h. After cooling, distilled water (350 cm^3), 8% (m/v) sodium thiosulphate (25 cm^3), two zinc pellets and 45% (m/v) sodium hydroxide (150 cm^3) were added. The solution was distilled into a 250 cm^3 Erlenmeyer flask containing 4% (m/v) boric acid (50 cm^3) and mixed indicator ($1,0 \text{ cm}^3$). The distillate ($\pm 150 \text{ cm}^3$) was titrated with $0,05 \text{ mol.dm}^{-3}$ sulphuric acid and the nitrogen content was calculated using the following equation:

$$\text{Nitrogen content (\%, m/m)} = (\text{titration volume/mass of sample})$$

2.4 Results and discussion

In experiment 1, eight 3-week and four 6-week cuts were made during the growing season. The effect of nitrogen fertilizer level upon the nitrate content over the growing season is given in Fig. 2.2. The control plots receiving no LAN fertilizer gave no yield and could not be analysed. In both the 3-week and 6-week kikuyu regrowth the nitrate content increases with increasing fertilizer-N applications. At the $100\text{kg N}\cdot\text{ha}^{-1}$ level of LAN the mean nitrate-N content during the growing season was about 60 mg% while at high LAN applications ($500\text{kg N}\cdot\text{ha}^{-1}$) the mean nitrate level increased to only about 180 mg%, which is still considered low. These results are in agreement with the findings of MacLeod & MacLeod (1974) and Reid & Strachan (1974) who described similar responses to nitrogen fertilisation for other grass species. Fig. 2.2 therefore suggests a close link between the rate of nitrate absorption from the soil and accumulation in the plant tissue.

The seasonal pattern of nitrate in the unpalatable and palatable kikuyu grass from experiment 2 is given in Fig 2.3. In the palatable plots receiving LAN fertilizer ($120\text{ kg N}\cdot\text{ha}^{-1}$) the nitrate level is also low with values less than 25 mg% for most of the growing season. In contrast, the nitrate content of the unpalatable kikuyu grass in the

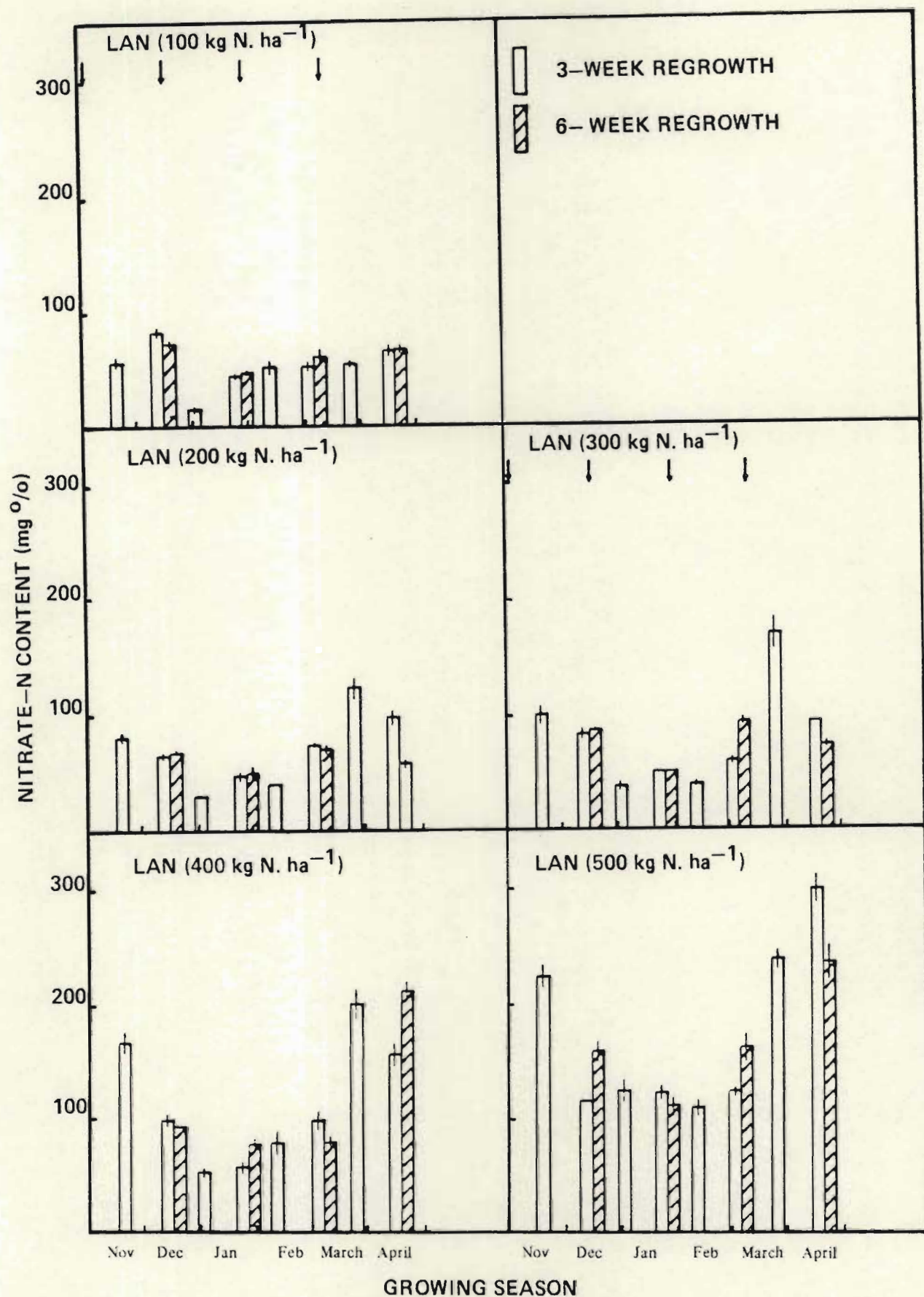


FIG. 2.2 Effect of season upon the nitrate content of a kikuyu pasture receiving different levels of nitrogen fertilizer. Arrows indicate time of fertilizer application

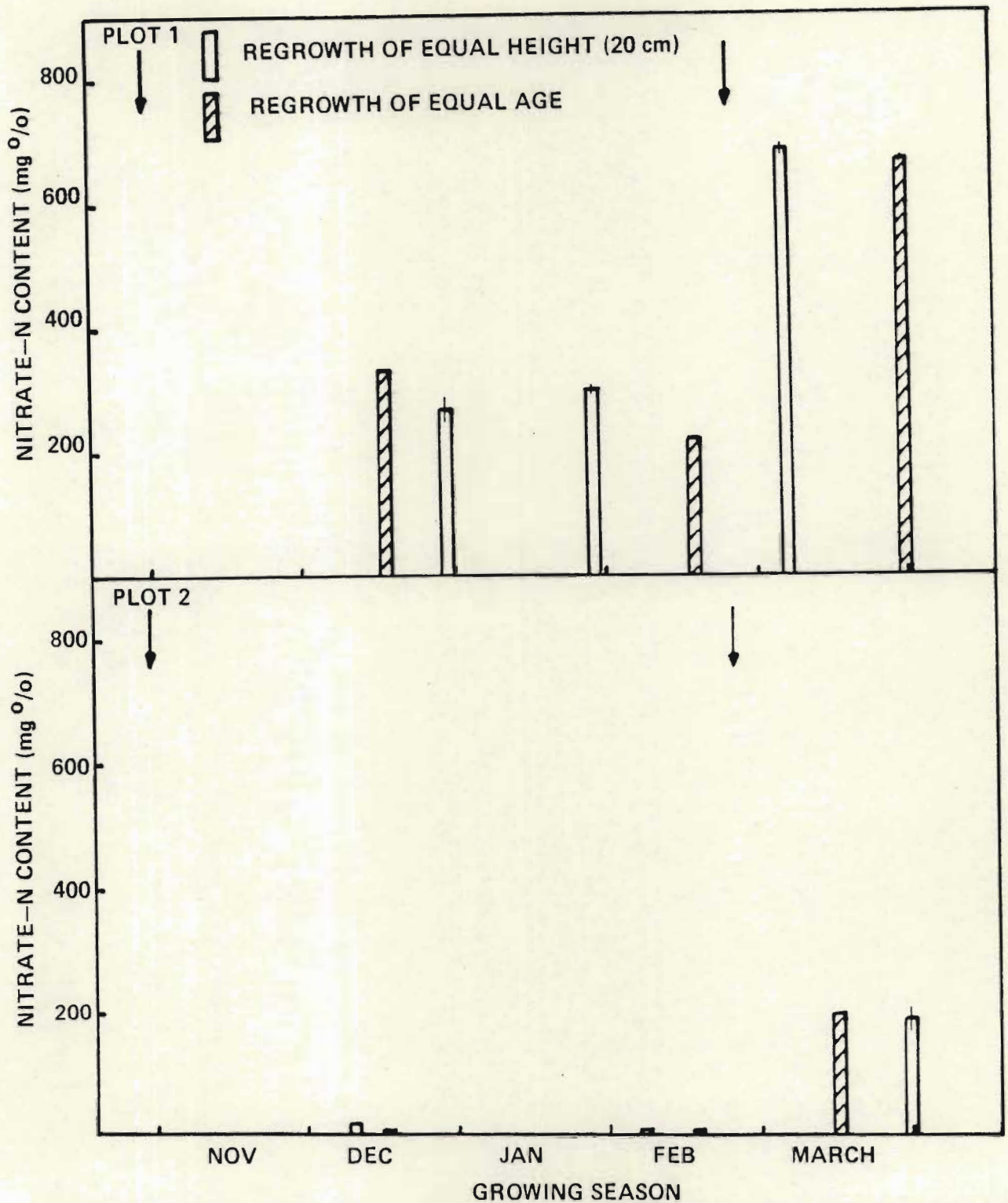


FIG. 2.3 Effect of season upon the nitrate content of unpalatable (Plot 1) and palatable (Plot 2) kikuyu grass. Arrows indicate time of fertilizer application

adjacent plots receiving the same fertilizer treatment, was above 250 mg% for most of the season and reached values of approximately 650 mg% at the end of the growing season. A similar response to nitrogenous fertilizer was obtained in experiment 3 (Table 2.1). The kikuyu tillers in plots 2 and 3 receiving an initial LAN dressing of 250 kg N.ha^{-1} , have nitrate contents of 803 mg% and 62 mg% respectively, while the nitrate-N content of the tillers in plot 1, receiving an additional LAN application ($1200 \text{ kg N.ha}^{-1}$) is 873 mg%. The results from experiments 2 and 3 seem to be in conflict with the results obtained in experiment 1, which showed a definite connection between the nitrate level in the plant tissue and the amount of nitrogen fertilizer applied to the soil.

In an attempt to resolve this problem, the mineral status of the soils from the three experimental sites was determined. The pH and soil mineral content at the onset of the experiments are given in Table 2.2.

Results indicate that the Kjeldahl nitrogen content of the soil of the high nitrate and unpalatable kikuyu grass plots is much higher than in the low nitrate kikuyu grass plots, suggesting the presence of substantial nitrogen reserves in these plots which may be derived from the faeces of grazing animals during previous seasons. This may partly explain the lack of any correlation between the level of nitrogen fertilizer applied to the old established pastures and the nitrate level in the grass. The fact that only certain areas

TABLE 2.1 Distribution of nitrate-N and macro minerals in late-season kikuyu grass tillers from experiment 3. The group of young folded leaves at the stem tip is denoted leaf 1. The older leaves are numbered individually.
 Plot 1 — high nitrate kikuyu grass, high LAN; Plot 2 — High nitrate kikuyu grass, low LAN; Plot 3 — Low nitrate kikuyu grass, low LAN

Plot	Fraction	Nitrate-N content (mg ⁰ /o,m/m)	Macro mineral content (⁰ /o, m/m)				Molar ratio K/N
			Ca	Mg	K	P	
1	Leaf 1	493 ±2	0,17	0,21	3,50	0,47	2,55
	Leaf 2	453 ±36	0,25	0,25	3,90	0,36	3,09
	Leaf 3	654 ±44	0,31	0,30	3,95	0,30	2,17
	Leaf 4	711 ±31	0,36	0,34	3,68	0,24	1,86
	Leaf 5, 6, 7	621 ±30	0,49	0,38	3,25	0,18	1,88
	Main stem	1578 ±6	0,33	0,41	4,71	0,24	1,07
	Secondary tillers	897 ±54	0,22	0,28	4,13	0,39	1,65
	Complete tiller	873	0,30	0,32	3,99	0,31	1,65
2	Leaf 1	391 ±40	0,15	0,20	3,96	0,51	3,64
	Leaf 2	471 ±36	0,23	0,25	3,78	0,37	2,88
	Leaf 3	597 ±46	0,28	0,26	4,03	0,28	2,42
	Leaf 4	593 ±58	0,33	0,32	3,80	0,24	2,30
	Leaf 5, 6, 7	510 ±5	0,45	0,39	3,51	0,16	2,47
	Main stem	1576 ±25	0,27	0,40	5,12	0,23	1,17
	Secondary tillers	888 ±26	0,22	0,29	4,56	0,41	1,84
	Complete tiller	803	0,27	0,31	4,22	0,31	1,88
3	Leaf 1	47 ±1	0,16	0,19	2,66	0,50	20,27
	Leaf 2	53 ±1	0,26	0,25	3,62	0,46	24,47
	Leaf 3	52 ±0	0,29	0,27	3,14	0,39	21,63
	Leaf 4	52 ±2	0,30	0,28	2,71	0,34	18,67
	Leaf 5, 6, 7	51 ±2	0,32	0,28	2,39	0,27	16,79
	Main stem	92 ±2	0,14	0,22	3,00	0,37	11,68
	Secondary tillers	58 ±0	0,17	0,19	3,15	0,46	19,45
	Complete tiller	62	0,22	0,24	3,01	0,41	17,38

TABLE 2.2 Soil mineral content and soil pH of experimental kikuyu grass plots

Experiment	Plot description	Soil pH	Soil Mineral content *					
			Kjeldahl N (O/o , m/m)	P (mg.kg^{-1})	K ($\text{c mol}(+)\text{kg}^{-1}$)	Ca ($\text{c mol}(+)\text{kg}^{-1}$)	Mg ($\text{c mol}(+)\text{kg}^{-1}$)	Na ($\text{c mol}(+)\text{kg}^{-1}$)
1	New pasture	4,6 \pm 0,1	0,34 \pm 0	8 \pm 0	0,86 \pm 0,09	5,67 \pm 0,21	3,83 \pm 0,08	0,03 \pm 0,01
2	1) Unpalatable pasture	4,8 \pm 0,1	0,44 \pm 1	150 \pm 5	2,24 \pm 0,10	4,13 \pm 0,20	4,55 \pm 0,31	0,02 \pm 0,00
	2) Palatable pasture	4,5 \pm 0,0	0,25 \pm 0	51 \pm 2	1,32 \pm 0,06	6,70 \pm 0,05	2,86 \pm 0,06	0,02 \pm 0,00
3	1) High nitrate kikuyu grass, high LAN	4,2 \pm 0,1	0,63 \pm 0	93 \pm 0	1,53 \pm 0,01	7,46 \pm 0,29	2,80 \pm 0,12	0,03 \pm 0,00
	2) High nitrate kikuyu grass, low LAN	4,3 \pm 0,1	0,63 \pm 0	71 \pm 2	1,31 \pm 0,01	7,21 \pm 0,06	2,69 \pm 0,03	0,04 \pm 0,01
	3) Low nitrate kikuyu	5,1 \pm 0,0	0,31 \pm 0	54 \pm 4	1,46 \pm 0,01	9,07 \pm 0,04	3,89 \pm 0,07	0,06 \pm 0,01

* Mean of 3 replicates

in the old pasture (Experiment 2) are high in nitrogen seems to point to an uneven grazing pattern and distribution of excreta in this pasture.

It must, however, be pointed out that values for Kjeldahl nitrogen in soil should be interpreted with caution as much of this nitrogen is in an organic form not directly available to the plant. However, it has been shown that microbial transformation to mineral forms (mineralisation) of these organic substances is in equilibrium with processes resynthesising organic substances (immobilisation) in the soil (Kowalenko, 1978). Furthermore, warm moist conditions enhance the mineralisation process making inorganic nitrogen available to the plant (Justice & Smith, 1962). Kikuyu grass consists of an intertwining network of rhizomes and runners from which leafy aerial tillers develop. Unless the pasture is heavily grazed old tillers tend to lodge and form a thick mat. Dead leaves and rhizomes readily accumulate in the surface layers of the soil and the warm, moist climate in which kikuyu grass thrives, creates conditions ideal for the mineralisation of organic substances. It can therefore be assumed that much of the nitrogen absorbed by kikuyu grass is derived from accumulated organic nitrogen, especially in old established pastures.

For normal growth, grasses require a soil potassium level of approximately $0,3 \text{ c mol}(+) \text{ kg}^{-1}$. Table 2.2 shows that the potassium level of all experimental plots is extremely high,

especially in the unpalatable plot. The high potassium level is probably also derived from the faeces of grazing animals during previous seasons. The potassium, magnesium, calcium and phosphate contents of the palatable and unpalatable kikuyu grass from experiment 2 and the late-season kikuyu tillers from experiment 3 are presented in Tables 2.3 and 2.1 respectively. A plant potassium content of about 2% is probably sufficient for optimum growth. Due to the high potassium level in the soil the optimum potassium content of the kikuyu grass is exceeded, especially in the high nitrate and unpalatable plots.

TABLE 2.3 Mineral content of unpalatable (Plot 1) and palatable (Plot 2) kikuyu grass from experiment 2

Mineral	Cut	Plant Mineral content(°/o, m/m)*			
		Plot 1		Plot 2	
		A	B	A	B
K	1	4,75 ±0,04	4,75 ±0,03	3,75 ±0,01	3,25 ±0,04
	2	5,25 ±0,02	4,25 ±0,02	2,75 ±0,02	2,75 ±0,01
	3	5,00 ±0,02	4,75 ±0,03	3,75 ±0,02	4,25 ±0,03
Mg	1	0,40 ±0,01	0,35 ±0,00	0,38 ±0,01	0,30 ±0,00
	2	0,38 ±0,01	0,28 ±0,01	0,30 ±0,00	0,28 ±0,01
	3	0,38 ±0,00	0,04 ±0,00	0,38 ±0,01	0,43 ±0,01
Ca	1	0,21 ±0,00	0,22 ±0,00	0,24 ±0,01	0,22 ±0,01
	2	0,22 ±0,01	0,18 ±0,01	0,24 ±0,00	0,24 ±0,01
	3	0,31 ±0,01	0,30 ±0,01	0,31 ±0,00	0,31 ±0,00
P	1	0,30 ±0,00	0,25 ±0,01	0,41 ±0,00	0,36 ±0,00
	2	0,44 ±0,01	0,36 ±0,00	0,41 ±0,00	0,42 ±0,01
	3	0,39 ±0,00	0,29 ±0,01	0,58 ±0,01	0,62 ±0,01

* Mean of 3 replicates

In experiment 1 the potassium level of the kikuyu grass does not exceed 2,3% in the high-N treatments (results not tabulated). With the exception of the 100 kg LAN treatment, the nitrate contents of the 3-week growth stage kikuyu grass at all fertilizer-N levels, showed statistically significant interactions with the potassium contents of the material. High levels of nitrate in the grass are associated with high levels of potassium.

The mean potassium values for the palatable and unpalatable kikuyu grass from experiment 2 are 3,4% and 4,9% respectively. In this experiment potassium in the plant also shows a statistically significant positive correlation with the nitrate content ($P < 0,01$).

In the late-season kikuyu tillers from experiment 3, no correlation exists between nitrate and potassium in low nitrate material and the molar ratio of potassium/nitrate in the stem fraction of these tillers is about 12. In these plants large amounts of potassium are absorbed even in the absence of high levels of nitrate. Cellular neutrality is probably achieved by the formation of carboxylates. In the high nitrate grass (plots 1 and 2) potassium is highly significantly correlated with nitrate ($P < 0,001$). In these plants potassium varies between 3% and 4% in the leaves and is about 5% in the stems. In the stems the molar ratio of potassium/nitrate approaches unity. Potassium occurs mainly in cell vacuoles (Sutcliffe, 1962), which suggests that the

high nitrate levels observed in the stems of kikuyu grass (Table 2.1, plots 1 and 2) is mainly stored in the large vacuoles of the pith parenchyma cells as counter ions for the high levels of potassium.

These results strongly suggest the involvement of potassium in the accumulation of excessive levels of nitrate in kikuyu grass. Similar ionic relations in tropical grasses have been described by Smith (1972, 1981). A further study of the effect of potassium on nitrate accumulation was undertaken by growing kikuyu tillers in defined aqueous nutrient media in which the potassium and/or nitrate levels were varied. These results are presented in Table 2.4.

TABLE 2.4 Effect of nitrate and potassium in aqueous nutrient medium upon the accumulation of nitrate and potassium in kikuyu grass tillers

Nutrient medium	Composition of plant material*	
	Nitrate (mg O/o , m/m)	Potassium (O/o , m/m)
Normal potassium normal nitrate	666 \pm 20	6,96 \pm 0,31
3 x potassium normal nitrate	838 \pm 24	7,56 \pm 0,12
3 x potassium 3 x nitrate	856 \pm 28	7,39 \pm 0,16
Normal potassium 3 x nitrate	731 \pm 37	7,31 \pm 0,14

* Mean of 6 replicates

Results show that at normal nitrate and potassium levels in the ambient medium the nitrate and potassium contents of the kikuyu tillers are 666 mg% and 6,96% respectively. A three-fold increase in the potassium level of the ambient medium results in an increase in plant nitrate and potassium of 26% and 9% respectively. At elevated (3-fold increase) nitrate levels in the ambient medium, a three-fold increase in potassium concentration increases plant nitrate by 17% while the plant potassium level remains constant. Analysis of variance showed a significant interaction ($P < 0,01$) between the potassium level of the media and the nitrate level in the kikuyu plants. The effect, however, of increased potassium levels in the media upon the potassium content of kikuyu grass is not statistically significant. The potassium content of the normal medium seems to have been sufficiently high to cause saturation level uptake of potassium and the plants did not show a further uptake response at higher ambient potassium concentrations.

Enhanced uptake of nitrate in the presence of elevated potassium levels in the ambient medium without a concomitant increase in uptake of potassium, suggests a mechanism of enhanced nitrate uptake external to the site of active absorption. These results seem to be similar to the findings of Franklin (1969, 1970) who showed that the presence of potassium in a phosphate nutrient solution substantially increased phosphate absorption, compared to the absorption of

roots pre-treated with the cation. Franklin, however, showed that divalent and trivalent cations are more effective than monovalent cations in enhancing anion absorption, while Blevins, Hiatt, Lowe & Leggett (1978) and Rufty, Jackson & Raper (1981) showed that nitrate uptake and assimilation by maize and barley seedlings is much greater with potassium nitrate in the ambient medium than with calcium or sodium nitrate.

Also, wheat seedlings treated with potassium nitrate absorbed and accumulated more nitrate and had a higher nitrate reductase activity in the roots than seedlings grown with sodium nitrate in the ambient medium (Blevins, Barnett & Frost, 1978). These results possibly indicate different absorption mechanisms in different plants.

Absorption of nitrate is not only enhanced by potassium in the ambient medium but also by potassium present in the tissue of the plant. According to the Ben Zioni-Lips model for nitrate uptake, potassium is intimately involved in the process of nitrate translocation (Ben-Zioni, Vaadia & Lips, 1971). Frost, Blevins & Barnett (1978) showed that wheat plants which have previously absorbed potassium, absorb 40% more nitrate and accumulate three times more nitrate in the shoots than plants which have been pre-loaded with sodium and calcium. According to Rufty, Jackson & Raper (1981) the presence of potassium in the plant markedly accelerated nitrate translocation out of the roots of maize seedlings.

Their observations seem to be in accordance with a proposed mechanism of co-transport of potassium and nitrate into the xylem, which is driven by a trans-membrane potassium concentration gradient, as has been proposed for potassium/sucrose co-transport out of mesophyll protoplasts (Huber & Moreland, 1981). The high potassium level in the soil and in the kikuyu grass from all experiments, therefore, seems to be an important factor stimulating nitrate absorption and accumulation if the soil nitrogen level is also high.

According to Rao & Rains (1976) nitrate uptake is influenced little by other anions in the ambient medium but the effect of cations is significant. The calcium, magnesium and sodium levels of the soils are within normal limits and would, therefore, not be a contributing cause of the observed differences in nitrate levels. Furthermore, no statistically significant interactions exist between these minerals in the plant and the nitrate content of the kikuyu grass. The pH of the soil or ambient medium has a substantial effect upon the absorption and transport of nitrate. High pH values (above pH 6) decrease nitrate uptake, while low pH values often stimulate uptake. Rao & Rains (1976) demonstrated the accumulation of nitrate in barley plants from solutions of low pH, while a decreased uptake was shown at values above pH 4. According to Table 2.2 the pH of the soils from all experimental plots is low (between 4,2 and 5,1). Nitrate absorption and accumulation is likely to be stimulated at

these pH values.

The amount of nitrate accumulated is also influenced by climatic conditions. Results presented in Fig. 2.2 show the effect of season upon the nitrate content of kikuyu grass in experiment 1 receiving different levels of nitrogen. A similar pattern for nitrate is also obtained in experiment 2 (Fig. 2.3) on the old established pasture. Meteorological data and dry matter yields for experiment 1 are given in Fig. 2.4. Results show that the vegetative growth of kikuyu grass is markedly influenced by temperature. As the temperature increases from November to February there is a general increase in growth rate as reflected in the dry matter production data. Notwithstanding the application of fertilizer-N at regular intervals during the growing season, the nitrate content drops during the period of rapid growth. As the temperature drops later in the season, the growth rate tends to diminish considerably (except for the April figure) thus decreasing the requirements for organic nitrogenous substances by meristematic tissue in the plant. This decrease in growth rate is accompanied by a rapid increase in the nitrate level of kikuyu grass. The nitrate-N level of the 3-week and 6-week kikuyu grass is negatively correlated with the mean temperature during the 3 or 6 weeks preceding defoliation respectively.

The nitrate level in plants tends to increase under conditions of water stress and the activity of nitrate

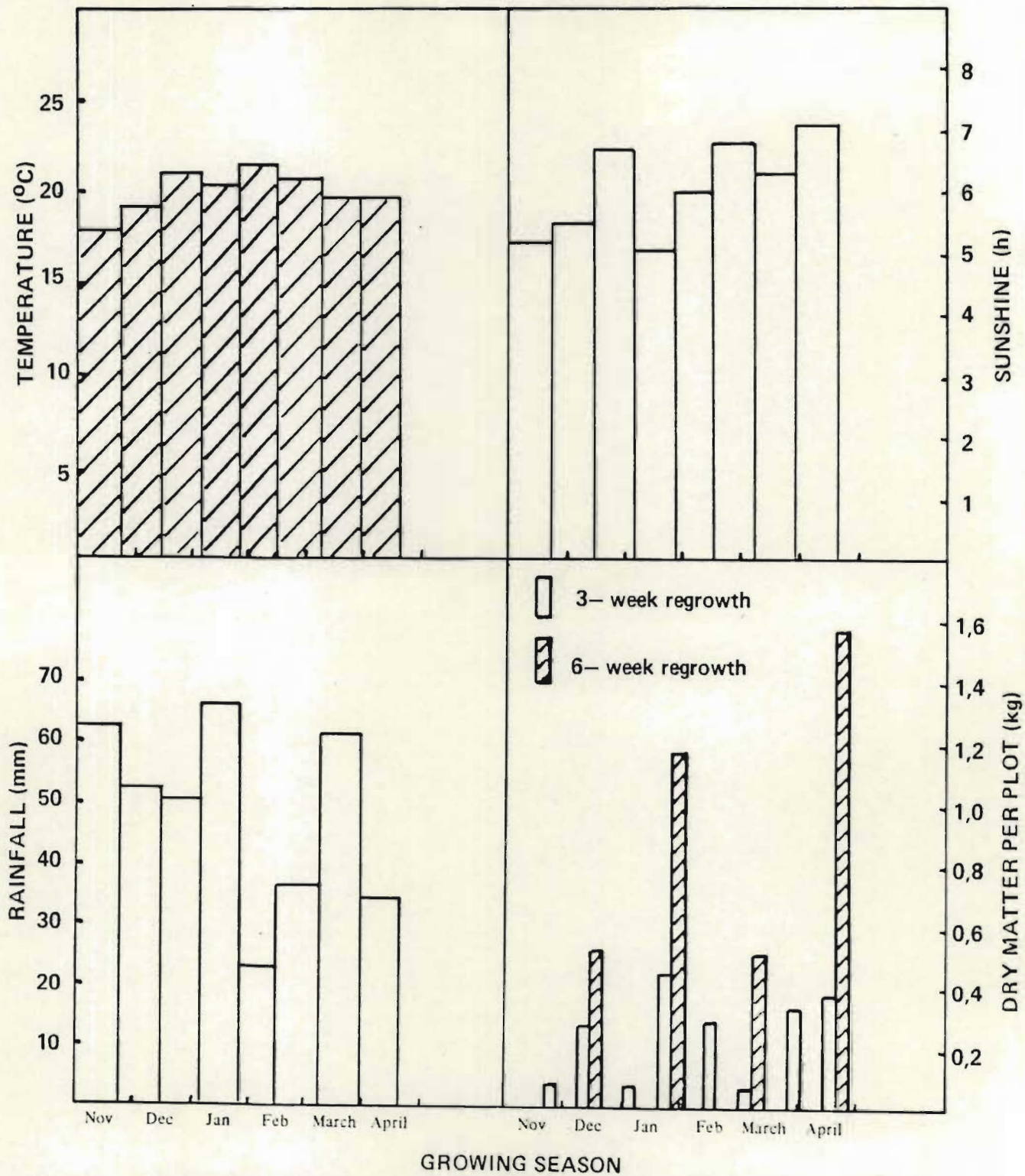


FIG. 2.4 Meteorological data and dry matter yields for experiment 1. Figures represent mean values over the 3-week period preceding cuts. Dry matter yields are mean values for all fertilizer levels

reductase has often been shown to decline under these circumstances (Hanson & Hitz, 1982). It is unlikely that conditions of severe water stress prevailed during the course of experiment 1. No significant correlation exists between rainfall and nitrate content or dry matter production, but the low rainfall figure for February and early March could have contributed to the slow growth rate during this period, while the high rainfall in January and March could have stimulated growth at the end of January and during April. These periods of rapid growth seem to be associated with a reduction in nitrate level compared to preceding periods.

High rainfall is often associated with low light intensities and light has a marked effect upon the nitrate uptake and assimilation. The process of nitrate reduction to ammonia seems to be closely linked to photosynthesis. Canvin & Atkins (1974) showed that the incorporation of nitrate into amino acids in maize, wheat and barley leaves require light. It has been proposed that NADH produced during photosynthesis is made available for nitrate reduction by a triose phosphate shuttle between chloroplasts and the cytoplasm (Beevers & Hageman, 1969; Klepper, Flesher & Hageman, 1971). Furthermore, nitrate reductase, which in maize tissue has a half life of approximately 4h, is continuously synthesised and degraded and its steady-state concentration is closely related to the energy status of the plant (Ben-Zioni, Vaadia & Lips, 1971; Aslam, Huffaker & Travis, 1973).

Under conditions of restricted light nitrate would, therefore, tend to accumulate in plant tissue. Stitzke, Croy & McMurphy (1976) have shown that under conditions of high soil nitrogen, 30% shade measurably increases the nitrate level of tall fescue grass, while 80% shading increased the nitrate-N level to 900 mg%. High light intensities during rapid growth should therefore favour low nitrate levels and the formation of organic nitrogenous substances. The rapid growth observed in experiment 1 during April (Fig 2.4) may have been facilitated by the availability of substantial amounts of photosynthate and the stimulation of nitrate reduction under conditions of ample sunshine. This seems to be substantiated by the drop in nitrate level in April compared to the previous period.

The physiological status and the stage of morphological development of the grass has a marked influence on the nitrate content of kikuyu grass. Fig. 2.5 shows the accumulation of nitrate in young kikuyu regrowth after defoliation late in the growing season (Early February). Initially (15-day regrowth, ± 10 cm) the differences in nitrate level of the plants receiving low and high levels of LAN fertilizer are large (200 and 400 mg% respectively). As the plants elongate, the nitrate levels increase and level out to between 400 mg% and 500 mg% after 33 days regrowth (23 cm), while differences between the two fertilizer levels decrease to about 50 mg%. Since the 15 day regrowth consists primarily of leaf material, the increase in nitrate content

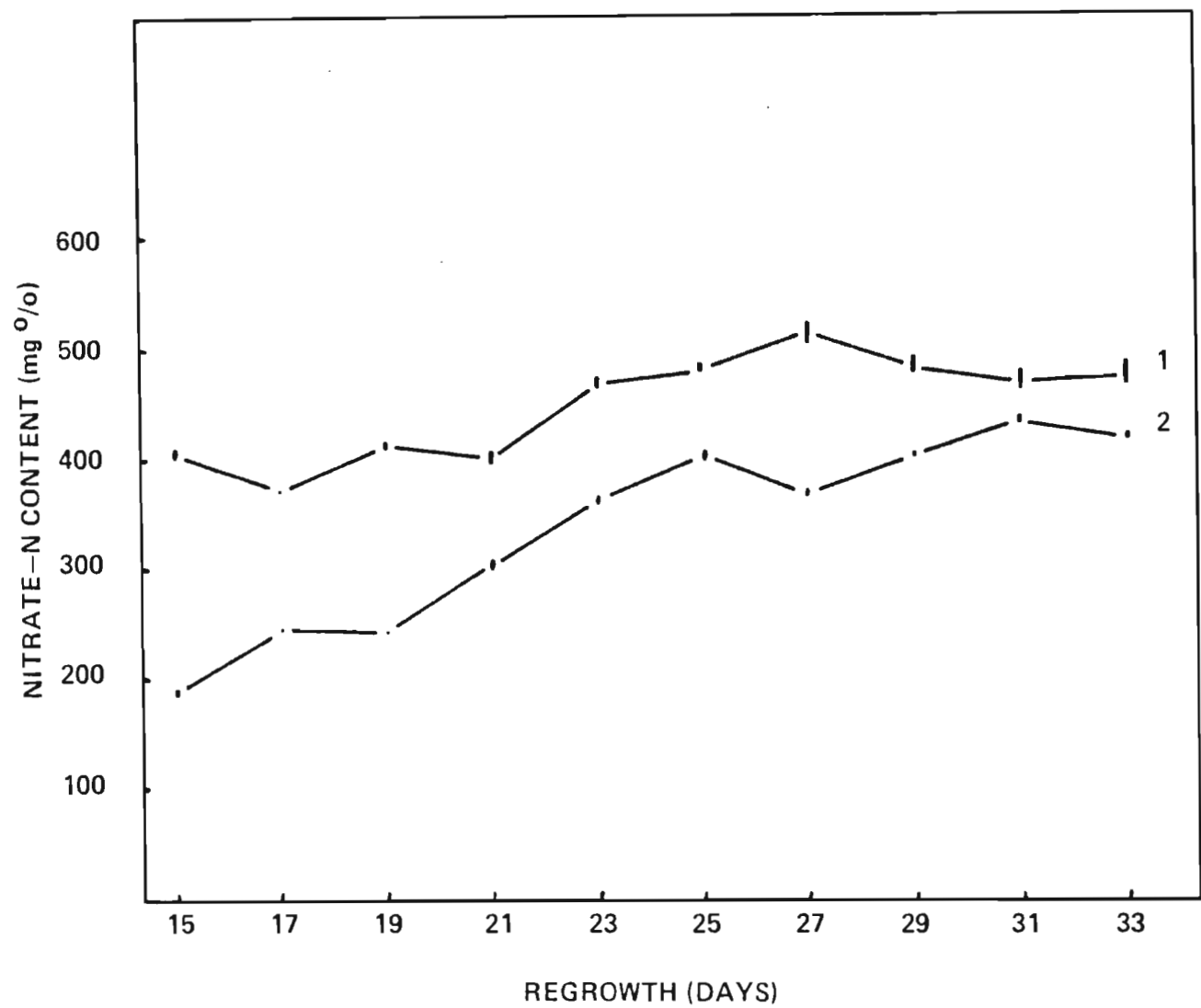


FIG. 2.5 Nitrate content of late season kikuyu regrowth. (1) Additional LAN (1200 kg N.ha⁻¹) applied at the beginning of the regrowth period (2) No additional LAN applied.

as the plants mature, could be partly due to higher levels in stem material. This is also borne out by the fact that this grass, which was cut 8 cm above ground level, had a much lower nitrate content than the complete tillers from the same plots (Table 2.1).

Table 2.1 shows that the group of young folded leaves from the high nitrate plots 1 and 2 contain almost 500 mg% and 400 mg% nitrate-N respectively, while the young leaves from the low nitrate grass from plot 3 contains less than 50 mg% nitrate-N. As the leaves mature, the nitrate content increases and again tends to decrease as the leaves become senescent (Leaf 5, 6 and 7). These results seem to be in agreement with the data presented by Darwinkel (1975) on Italian ryegrass, which showed that the leaves of younger tillers contain lower levels of nitrate than the leaves of older tillers.

Nitrate is largely localised in the stems of the plants. Smith (1973) showed that high internal concentrations of nitrate greatly reduces the influx of these ions. It seems however, that once the nitrate is deposited in storage pools in kikuyu grass, it has little regulatory effect on further absorption. In the high nitrate plants, stem tissue reaches a nitrate-N level of almost 1600 mg%, which represents more than 40% of the total nitrate in the tiller (comprising stem and leaves). In the low nitrate plants the stem contains less than 100 mg% nitrate-N and represents only about 20% of the

total nitrate-N in the tiller. Stem pith parenchyma cells seem to be the main storage depot for nitrate in members of the Gramineae. Maize plants were also shown to accumulate the major part of their total nitrate in the stem tissue (Schrader, 1978). White & Halvorson (1980) showed that toxic levels of nitrate only accumulate in Western wheatgrass and green needlegrass when the plants are in the reproductive stage when the proportion of stem material is high.

Results presented in Fig. 2.6 show the effect of season upon the total organic nitrogen, protein nitrogen and non-protein organic nitrogen contents of unpalatable and palatable kikuyu grass from experiment 2. High nitrate in kikuyu tissue is associated with high levels of these other nitrogenous substances in the grass. Significant positive correlations exist between nitrate and total organic nitrogen ($P < 0.01$), nitrate and protein nitrogen ($P < 0.01$) and nitrate and non-protein organic nitrogen ($P < 0.01$). The accumulation of nitrate in kikuyu grass, therefore, does not seem to be the result of direct inhibition of nitrate reductase by factors such as a molybdenum deficiency, but by feedback inhibition by reduced nitrogenous compounds such as amino acids and ammonia. However, some workers question the role of ammonia in the feedback inhibition of nitrate reductase. Canvin & Atkins (1974) found little effect of ammonia or nitrite on nitrate utilisation by barley leaves.

The distribution of protein-N and non-protein organic-N in

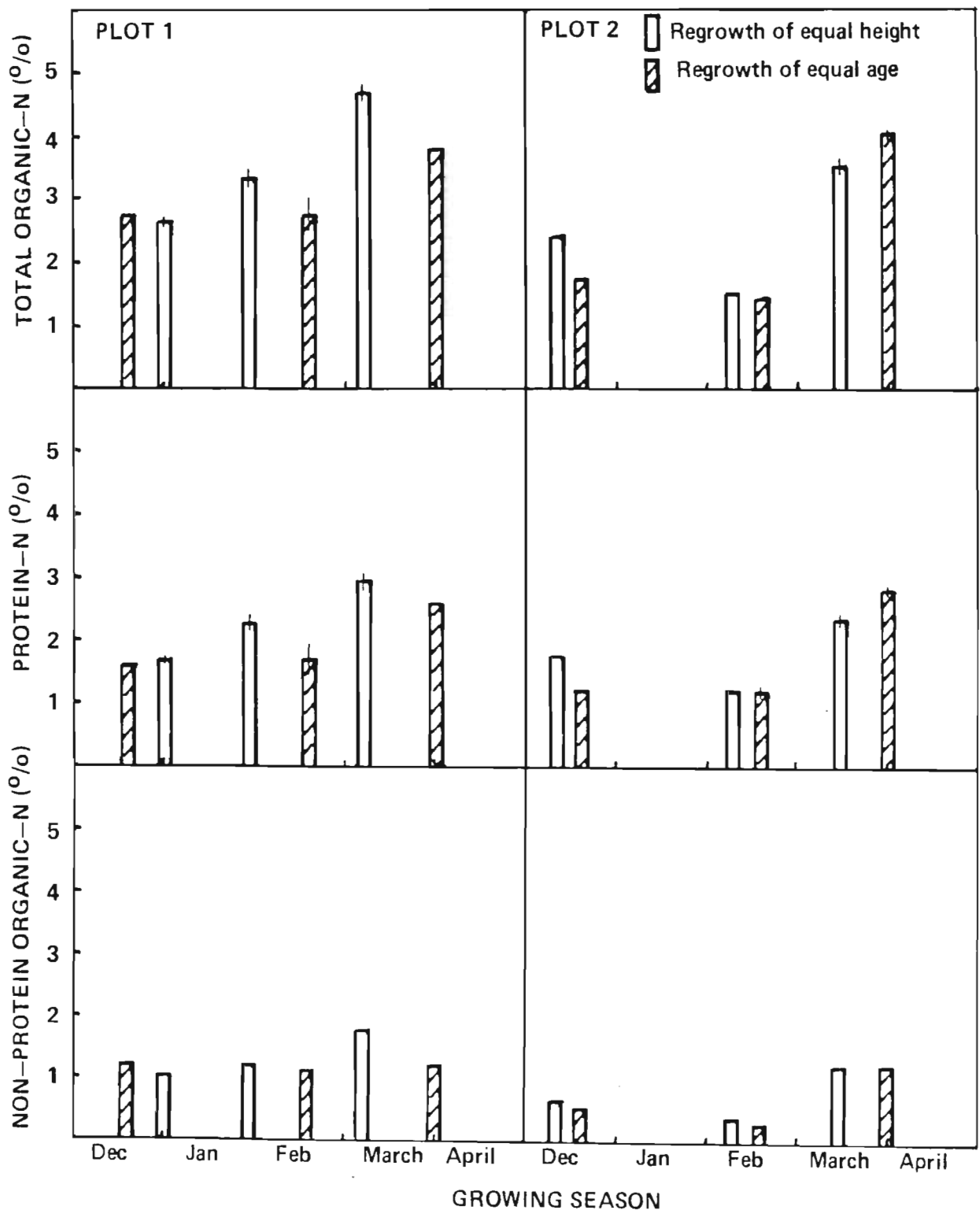


FIG. 2.6 Effect of season upon the total organic nitrogen, protein nitrogen and non-protein organic nitrogen contents of kikuyu grass. Plot 1, unpalatable kikuyu; Plot 2, palatable kikuyu

late-season kikuyu tillers is given in Table 2.5. The

TABLE 2.5 Distribution of protein-N and non-protein organic-N in late-season kikuyu grass tillers from experiment 3. The group of young folded leaves at the stem tip is denoted leaf 1. Older leaves are numbered individually. Plot 1 – high nitrate kikuyu grass, high LAN; Plot 2 – high nitrate kikuyu grass, low LAN; Plot 3 – low nitrate kikuyu grass, low LAN

Fraction	Plot 1		Plot 2		Plot 3	
	Protein-N ($\%$, m/m)	NPON ($\%$, m/m)	Protein-N ($\%$, m/m)	NPON ($\%$, m/m)	Protein-N ($\%$, m/m)	NPON ($\%$, m/m)
Leaf 1	3.34 ± 0.04	1.56 ± 0.03	2.82 ± 0.00	1.53 ± 0.04	2.23 ± 0.01	0.86 ± 0.05
Leaf 2	2.26 ± 0.06	1.16 ± 0.03	1.33 ± 0.01	1.08 ± 0.05	2.09 ± 0.03	0.66 ± 0.04
Leaf 3	2.72 ± 0.02	1.05 ± 0.01	2.51 ± 0.05	1.02 ± 0.04	1.69 ± 0.04	0.59 ± 0.01
Leaf 4	2.50 ± 0.04	1.00 ± 0.06	2.15 ± 0.05	1.03 ± 0.03	1.55 ± 0.03	0.55 ± 0.04
Leaf 5, 6, 7	2.22 ± 0.04	0.98 ± 0.04	1.97 ± 0.06	0.82 ± 0.06	1.21 ± 0.02	0.56 ± 0.03
Main Stem	1.33 ± 0.00	0.87 ± 0.06	1.27 ± 0.01	0.78 ± 0.05	0.68 ± 0.06	0.63 ± 0.03
Secondary tillers	2.59 ± 0.05	1.13 ± 0.05	2.52 ± 0.02	0.96 ± 0.05	1.43 ± 0.00	0.81 ± 0.02
Complete tiller	2.30	1.08	2.00	1.01	1.52	0.67

addition of an excessively high level of LAN to plot 1 increased the overall protein-N and non-protein organic-N contents of the kikuyu grass by about 15% and 7% respectively, while the nitrate content increased by about 9% (Table 2.1). The overall protein-N and non-protein organic-N contents of the low nitrate kikuyu grass (plot 3) are 24% and 34% lower respectively than the values of the kikuyu grass in plot 2 receiving an equal amount of fertilizer-N, while the nitrate content is 92% lower.

The highest protein-N and non-protein organic-N contents are located in the youngest folded leaves where rapid elongation takes place and tend to decrease as the leaves mature. In contrast, the nitrate-N content is lowest in the youngest leaves and tends to accumulate as the leaves mature, indicating rapid assimilation of nitrate in the youngest fast growing tissue. Stem tissue contains the lowest concentrations of protein-N and non-protein organic-N.

The results described in this chapter suggest that the high level of nitrate often observed in kikuyu pastures is the nett result of several interacting factors. High soil nitrogen seems to play a major role. In this respect, the mineralisation under hot moist conditions of accumulated organic nitrogen derived from decaying plant matter or from the faeces of grazing animals, may be of considerable significance. High potassium levels in the soil and in the plant tissue, aiding nitrate absorption, translocation and accumulation in storage pools, seem to be a further contributing factor. A decline in the requirement for assimilated nitrogenous compounds at the end of the growing season possibly leads to feedback inhibition of nitrate reductase by substances such as amino acids, while absorption of nitrate by the root system still continues.

Nitrate in grasses is primarily stored in stem tissue. In contrast to most other pasture grasses, in which the stem tips remain close to ground level and well out of reach of

the grazing animal for a large part of the growing season, kikuyu grass stems elongate continuously. Stems containing high levels of nitrate are, therefore, readily available to the grazing animal. In the next chapter the effect of high levels of nitrate on digestion in the animal will be discussed.

CHAPTER 3

THE MODE OF ACTION OF NITRATE UPON DIGESTION IN VITRO

The polysaccharides contained in the leaves and stems of various plants, constitute the main source of energy for ruminants. The structural polysaccharides of plants are not degradable by animal digestive processes and the ruminant, therefore, relies upon microbes to convert these polysaccharides to products which may be utilised by the animal. The large population of microbes inhabiting the rumen is made up primarily of obligate anaerobic bacteria and protozoa, each fermenting some component of the digest, thus producing the volatile fatty acids and microbial protein which serve as food for the ruminant.

The major fibre-digesting rumen microbes are the cellulolytic bacteria, Bacteroides succinogenes, Ruminococcus albus and Ruminococcus flavefaciens (Bryant, 1973). The cellulolytic strains of Butyrivibrio fibrisolvens and Eubacterium cellulosolvens are generally considered of less importance as cellulose digesters (Van Gylswyk & Schwartz, 1984).

In addition to cellulose, hemicellulose is also a major component of forage cell walls. Most of the cellulolytic microbes also seem to be able to degrade hemicellulose (Kock & Kistner, 1969; Coen & Dehority, 1970; Morris & Van Gylswyk,

1980). According to Dehority & Grubb (1976) and Henning (1979) xylan can be utilised as sole energy source by more than 60% of the total culturable bacteria in the rumen, but most of these bacteria only degrade hemicellulose to a limited extent in situ, in cell walls. A small number however, bring about more extensive solubilisation of hemicellulose. Among the most important of these are Bacteroides ruminicola and Butyrivibrio fibrisolvens (Dehority, 1973).

Most rumen bacteria are found associated with feed particles (Hobson & Wallace, 1982). The cellulolytic microbes in particular have been demonstrated to adhere to the plant cell walls (Akin, 1976; Latham, Brooker, Pettipher & Harris, 1978; Morris & Van Gylswyk, 1980). Furthermore, the exo-cellulases produced by these microbes also adsorb onto the substrate ensuring the production of sugars in close proximity to the bacterium and leaving the cell-free rumen fluid relatively free of cellulolytic activity (Francis, Gawthorne & Storer, 1978; Goel & Ramachandran, 1983).

The cellulolytic species seem to obtain their energy for growth from the fermentation of carbohydrates only (Bryant, 1973). The complete hydrolysis of cellulose is usually effected by the simultaneous action of three enzymes, i.e. an endo- β -1,4-glucanase, which randomly splits the glucan chains, an exo- β -1,4-glucanase, which splits off either cellobiose or glucose from the non-reducing ends of the

chains and a β -1,4-glucosidase, which cleaves cellobiose into glucose units. Some of the sugar produced is absorbed and fermented by the cellulolytic species, while the remaining carbohydrate compounds are fermented by other carbohydrate digesting species.

In addition to the carbohydrate fermenters, the rumen also hosts a large number of microbes utilising the great variety of other substances present in the rumen. Some microbes degrade proteins while others utilise compounds such as formate and succinate. The methanogenic bacteria, reduce carbon dioxide in the presence of hydrogen gas to methane. The final products of rumen fermentation by the complex of interacting microbial species is mainly acetate, propionate, butyrate, carbon dioxide, methane, ammonia and microbial cells.

Although the microbial population in the rumen is relatively stable, the populations of the various species is influenced by the nature and concentrations of available nutrients in the rumen (Therion, Kistner & Kornelius, 1982). Furthermore, toxic components found in feedstuffs, or which may arise during the fermentation process, may decrease the overall growth potential of individual organisms, thereby changing the microbial population to more resistant types (Russel, 1984). In some instances these interactions of rumen microbial populations may even be detrimental to the host animal. Changes leading to a reduction in the digestion of

structural polysaccharides, in particular, will seriously affect animal production. Such a change occurs, for example, when the rumen pH drops below pH 5, due to excessive feeding of starch-containing concentrates (Church, 1969; Dirksen, 1969).

The presence of nitrate in rumen digests in vitro has been shown to depress the digestibility of forage (Hall, Gaddy & Hobbs, 1960; Marais, 1980) notwithstanding the fact that it serves as a common terminal electron acceptor during anaerobic respiration in many microbes and can function as sole nitrogen source for certain rumen bacteria (Inderlied & Delwiche, 1973).

Anaerobic microbes obtain energy from a wide variety of chemical reactions. The energy present in an organic substrate containing reduced forms of carbon, is released by successive dehydrogenations and the reducing equivalents are thereby removed, usually in pairs and transferred to a final electron acceptor. Three main groups of bacteria are known to utilise inorganic compounds as terminal electron acceptors, i.e. those using nitrate, sulphate and carbon dioxide (Thauer, Jungermann & Decker, 1977). Considerable differences may exist between microbial species regarding the nature of participating co-factors and the intermediate and end products formed during nitrate reduction. According to Payne (1973) some nitrate reducing bacteria reduce nitrate no further than nitrite, while others can reduce it to elemental

nitrogen by a process of dissimilatory nitrate reduction or denitrification. In other species nitrous oxide is the terminal product of denitrification. With few exceptions, nitrate reductases produced by dissimilatory microbes are membrane bound (Pichinoty, 1973). A number of bacteria can reduce nitrate to ammonia, which serves as a source of nitrogen for biosynthesis. This process is known as assimilatory nitrate reduction. According to Payne (1973) assimilatory nitrate reductases do not require molybdenum as co-factor and Pichinoty (1973) showed that these enzymes are soluble and not associated with cell membranes.

The reduction of nitrate to nitrite involves the transfer of two electrons while six electrons are transferred during the conversion of nitrite to ammonia. It has frequently been claimed that the reduction of nitrite to ammonia also proceeds by a series of two-electron transfers, each catalysed by a different enzyme (Nason, 1962; Hewitt & Nicholas, 1964). However, recent evidence has shown that nitrite is completely reduced to ammonia with no free intermediates such as hydroxylamine for both the plant and bacterial enzyme (Lazzarini & Atkinson, 1961; Prakash & Sadana, 1972; Vega, Guerrero, Leadbetter & Losada, 1973).

The reduction of nitrate in the rumen has been attributed to the bacterium Veillonella alcalescens and most strains of Lachnospira multiparus, Butyrivibrio alactacidogens, Selenomonas ruminantium and Selenomonas lactilytica (Hungate,

1966). Wolin, Wolin & Jacobs (1961) showed that Vibrio succinogenes reduces nitrate to ammonia with the electron transfer reactions coupled to reactions providing energy for the cell.

Detailed studies of the mechanism of nitrate and nitrite reduction by rumen microbes have not yet been made (Allison, 1970). Available information deals mainly with the suitability of different substances to act as hydrogen donors for nitrate reduction. Dissimilatory nitrate reduction leading to the formation of nitrous oxide in mixed cultures of bovine rumen microbes, incubated in the presence of nitrate, was demonstrated by Jones (1972). Its contribution to the overall reduction of nitrate was small and assimilatory nitrate reduction leading to the formation of ammonia, was considered the major pathway for nitrate reduction. In a study of the ability of various substances to act as hydrogen donors during assimilatory nitrate reduction, Lewis (1951), using washed suspensions of rumen microbes, showed that hydrogen is the most active donor for the reduction of nitrate, nitrite and hydroxylamine. The optimum pH for nitrate and nitrite reduction was 6,5 and 5,6 respectively. Formate, succinate, lactate, citrate, glucose, malate and mannitol also acted as hydrogen donors but were less effective. Jones (1972), using whole rumen fluid showed that formate and hydrogen supported the most rapid reduction of nitrate, but nitrite reduction is much slower in the presence of hydrogen than in the presence of formate. Jones

ranked the hydrogen donors for overall effectiveness in reducing nitrate to ammonia in the following decreasing order: formate, hydrogen, glucose, lactate and succinate. The relative effectiveness of hydrogen donors is likely to be influenced by the balance of microbial species present and by competition with other hydrogen utilising reactions such as methanogenesis (Hungate, 1963) and the hydrogenation of unsaturated fatty acids (Dawson & Kemp, 1970).

In this investigation a number of experiments were conducted to establish, firstly, which nitrogenous substances present in the rumen depress digestion. Secondly, attempts were made to determine whether these substances affect the activity of the enzyme systems responsible for cellulose hydrolysis or whether they have an inhibiting effect upon the microbes as such.

Studies of this nature in the rumen in vivo are complicated by the continuous change in concentration of substances in the rumen as a result of the intake of feed and water, absorption of substances through the rumen wall and the passing of digesta from the rumen. However, the two stage in vitro procedure developed at the Grassland Research Institute, Hurley (Tilley, Deriaz & Terry, 1960; Tilley & Terry, 1963), for measuring digestibility in feed samples, provides a suitable tool for a controlled study of the effect of these nitrogenous compounds on the digestion of feeds.

In this procedure digestion is conducted in a sealed container in which rumen conditions are simulated in respect of temperature, anaerobic conditions, constitution of salivary buffer and pH. Furthermore, the volume of buffer used is sufficiently large relative to the substrate mass to minimise product inhibition. This technique yields results which closely resemble those from rumen digestion in vivo. For example, it has been shown that the dry matter and organic matter digestibilities in vitro correlate well (correlation coefficients in the order of 0,95 to 0,99) with in vivo results (Alexander, 1969; Minson & McLeod, 1972).

3.1 Materials and methods

3.1.1 Digestibility in vitro

The method is based upon the two-stage procedure described by Tilley & Terry (1963), subsequently modified by Minson & McLeod (1972). The grass sample is digested initially under conditions simulating rumen fermentation, followed by an acid pepsin digestion to solubilise the protein in the sample.

Reagents

Salivary buffer solution. Buffer A. Sodium bicarbonate (16,46g), anhydrous di-sodium hydrogen phosphate (6,21g), potassium chloride (0,96g), sodium chloride (0,78g), magnesium sulphate (0,20g), glucose (1,54g) and urea (1,54g) were dissolved in distilled water (1680 cm³) in a flask calibrated at 1680 cm³ and 2100 cm³.

Buffer B. Calcium chloride (5,3g) was dissolved in distilled water and made up to 100 cm³.

Preparation of salivary buffer-rumen fluid solution. Buffer B (1,68 cm³) was added to Buffer A (1680 cm³) in the calibrated flask and warmed on a hot plate to 39°C. While maintaining this temperature, the pH of the buffer was adjusted to 6,9 by bubbling carbon dioxide through the solution.

Rumen fluid was obtained from South African Mutton Merino wethers maintained on a daily ration of lucerne hay (2,5kg) and whole maize (100g) plus ad lib access to a salt mixture consisting of dicalcium phosphate - sodium chloride, 1:1 (m/m). Immediately prior to the daily feeding rumen fluid was withdrawn using an adaptation of the suction strainer technique described by Raun & Burroughs (1962). The technique used differed from that of Raun & Burroughs in that the rumen fluid was withdrawn under anaerobic conditions directly into the flask containing the salivary buffer solution (maintained at 39°C) by means of an electric vacuum pump. Rumen fluid was drawn into the flask up to the 2100 cm³ mark. The probe strainer, which had large holes (5,0 mm), was covered with nylon mesh cloth (1,0 mm) to effect filtration.

Analytical procedure

Salivary buffer-rumen fluid solution (50 cm³) was dispensed

into a 100 cm³ polythene screw-capped bottle containing the dried grass sample (0,5g). The bottle was flushed with carbon dioxide and sealed to ensure anaerobic conditions. For standard digestibility determinations in vitro, bottles were incubated at 39°C for 48h (shaking twice daily), while the digestion period was varied from 0 to 72h for digestion time studies. After incubation, the clear supernatant liquid was removed from the bottle by suction through a sintered glass filter stick. Acid pepsin solution (50 cm³) was dispensed into the bottle and incubated at 39°C for a further 48h for all determinations. After incubation the residue in the bottle was allowed to settle and the clear supernatant was removed by suction through the filter stick. The residue was washed with water (50 cm³), allowed to settle for 30 min and the clear supernatant removed. The washing procedure was repeated twice before the residue was transferred to a 100 cm³ glass beaker of known mass. The beaker was dried at 105°C and the mass of the residue determined. A blank without grass was treated similarly and the digestibility of the grass in vitro was calculated by means of the following equation:

$$\text{Dry matter digestibility (\%,m/m)} = 100(C - F + F.\text{blank})/C$$

Where, C = dry mass of sample

F = dry mass of residue

F.blank = dry mass of residue of blank.

3.1.2 Total non-structural carbohydrates (TNC)

Non-structural carbohydrates were analysed as reducing sugars after complete hydrolysis to monosaccharides by means of a carefully controlled acid hydrolysis procedure (Marais, 1979). The method was developed for the rapid determination of non-structural carbohydrates in pasture grasses and compares favourably with earlier enzymic procedures (Marais, 1969). The reducing sugars formed during hydrolysis were determined quantitatively by the modified Nelson-Somogyi method previously described (Marais, DeWit & Quicke, 1966).

Reagents

Copper reagent. Solution 1. Sodium carbonate (30g, anhydrous), sodium bicarbonate (20g), potassium-sodium tartrate (15g) and sodium sulphate (180g, anhydrous) were dissolved in distilled water and made up to 1 dm³.

Solution 2. Sodium sulphate (45g, anhydrous) and copper sulphate (5,0g) were dissolved in distilled water and made up to 250 cm³. Immediately before use, 4 volumes of Solution 1 were mixed with 1 volume of Solution 2.

Arsenomolybdate reagent. Ammonium molybdate (25g) was dissolved in about 400 cm³ distilled water and concentrated sulphuric acid (21 cm³) was carefully added. Sodium arsenate heptahydrate (3,0g) was dissolved in distilled water (±25 cm³), added to the acidic ammonium molybdate solution and made up to 500 cm³. The resulting solution was incubated at

37°C for 48h and stored in a glass-stoppered brown bottle.

Reducing sugar standard. Glucose (0,6667g) was dissolved in distilled water and made up to 200 cm³.

Sulphuric acid (0,1 mol.dm⁻³). A Merck Titrisol ampoule (0,05 mol.dm⁻³) was diluted with distilled water and made up to 500 cm³.

Sulphuric acid (0,05 mol. dm⁻³). 0,1 mol. dm⁻³ sulphuric acid (250 cm³) was diluted with distilled water and made up to 500 cm³.

Hydrolysis of non-structural carbohydrates

Sulphuric acid (10cm³) was added to a sample of dried and milled kikuyu grass (0,3g), in a test-tube, and the mixture was heated for exactly 55 min in a boiling-water bath. The tube was immediately cooled to room temperature and its contents were quantitatively transferred to a 250 cm³ volumetric flask and made up to volume. A blank containing 0,05 mol.dm⁻³ sulphuric acid (10 cm³) only and a standard containing reducing sugar (5,0 cm³) and 0,1 mol. dm⁻³ sulphuric acid (5,0 cm³) were treated similarly.

Chemical analysis

An aliquot of the dilute hydrolysate (3,0 cm³) was mixed well with copper reagent (3,0 cm³) in a test-tube and the tube was heated in a boiling-water bath for exactly 20 min. After

cooling to room temperature arsenomolybdate reagent ($2,0\text{ cm}^3$) was added. The tube was agitated on a whirlimixer until bubble formation ceased and the colour was allowed to develop for 1,5h. The coloured solution was quantitatively transferred to a 200 cm^3 volumetric flask and made up to volume with distilled water. The absorbance was read against a blank at 750 nm in a Beckman DB spectrophotometer and the TNC content calculated using the following equation:

$$\text{TNC content (\%, m/m)} = (\text{absorbance sample/absorbance standard}) \times (555,555/\text{DM}\%)$$

Where DM% = dry mass % of sample

3.1.3 Assay for nitrite nitrogen

Nitrite was determined by the method described by Nicholas & Nason (1957). It is based on the reaction of nitrite with a primary amine in an acidic medium to form a diazonium salt which is coupled to an aromatic amine to yield a red AZO dye, the concentration of which can be determined colorimetrically.

Reagents

Sulphanilamide reagent. Sulphanilamide (2,0g) was dissolved in distilled water (150 cm^3) in a 200 cm^3 volumetric flask and made up to volume with concentrated hydrochloric acid.

n-Naphthylethylenediamine dihydrochloride (NEDD) reagent.

NEDD (0,04g) was dissolved in ethanol (200 cm^3) and kept in a dark bottle.

Zinc acetate solution. Zinc acetate (0,033g) was dissolved in distilled water and made up to 100 cm^3 .

Nitrite standard. Potassium nitrite (0,1520g) was dissolved in distilled water and made up to 500 cm^3 . An aliquot ($2,0\text{ cm}^3$) of this solution was diluted with distilled water and made up to 100 cm^3 .

Procedure

Interfering substances were removed by adding zinc acetate solution ($0,2\text{ cm}^3$) to a rumen fluid sample (5 cm^3), mixing well and centrifuging ($2000 \times g$, 5 min, 20°C). Rumen fluid supernatant ($0,2\text{ cm}^3$), distilled water ($4,8\text{ cm}^3$) and sulphanilamide reagent ($2,5\text{ cm}^3$) were mixed well. NEDD reagent ($2,5\text{ cm}^3$) was added, mixed well and the colour allowed to develop for 15 min. A standard was prepared containing nitrite solution ($0,5\text{ cm}^3$), distilled water ($4,5\text{ cm}^3$), sulphanilamide reagent ($2,5\text{ cm}^3$) and NEDD reagent ($2,5\text{ cm}^3$). The absorbance of the solution was read in a Beckman DB spectrophotometer at 540 nm against a blank consisting of rumen fluid supernatant ($0,2\text{ cm}^3$), distilled water ($4,8\text{ cm}^3$), sulphanilamide reagent ($2,5\text{ cm}^3$) and ethanol ($2,5\text{ cm}^3$).

The nitrite content was calculated using the following

equation:

$$\text{Nitrite-N } (\mu\text{g.cm}^{-3}) = (\text{absorbance sample} / \text{absorbance standard}) \times 2,6$$

3.1.4 Volatile fatty acid (VFA) analysis

Volatile fatty acids were determined in aqueous medium by gas chromatography, using a method described in a manufacturers catalogue (Supelco Inc., 1975).

Reagents and equipment

VFA Standards. The following fatty acids were measured out and dissolved in distilled water (1 dm^3):

<u>VFA</u>	<u>Concentration (m mol.dm^{-3})</u>
Acetic Acid	17,473
Propionic acid	6,702
Isobutyric acid	1,345
Butyric acid	1,359
Isovaleric acid	0,455
Valeric acid	0,460

Gas chromatograph

A Hewlett Packard Model 5790 gas chromatograph equipped with

a hydrogen flame detector was used. A glass column (1,7 m x 3,0 mm i.d.) with a replaceable pre-column (6,0 cm), for the accumulation of non-volatiles, was packed with 10% SP 1000/1% phosphoric acid on 100/120 Chromosorb W-AW (Supelco Inc., Bellefonte, Pennsylvania). The high purity nitrogen carrier gas was adjusted to a flow rate of 30 cm³ per min. The inlet, column and detector temperatures were maintained at 250, 160 and 200°C respectively.

Procedure

Metaphosphoric acid (1 cm³ of 25%, m/v) was added to an aliquot of rumen fluid (5,0 cm³), the solution was mixed well, allowed to stand for 30 min and centrifuged (2000 x g, 10 min, room temp.). A sample (0,001 cm³) of the supernatant was injected into the gas chromatograph, the peaks were integrated and the results expressed as $\mu\text{mol.cm}^3$.

3.1.5 Ammonia Nitrogen

Ammonia was determined by the phenol-hypochlorite method using sodium nitroprusside as catalyst (Weatherburn, 1967).

Reagents

Phenol-nitroprusside reagent. Phenol (5,0g) and sodium nitroprusside (0,25g) were dissolved in distilled water and

made up to 500 cm³. The reagent was stored at 4°C.

Alkaline hypochlorite reagent. Sodium hydroxide (2,5g) and sodium hypochlorite (4,2 cm³) were dissolved in distilled water and made up to 500 cm³. The reagent was stored at 4°C.

Hydrochloric acid solution ($\pm 0,1 \text{ mol.dm}^{-1}$). Concentrated hydrochloric acid (1,39 cm³) was diluted in distilled water and made up to 500 cm³.

Ammonium sulphate standard. Ammonium sulphate (2,832g) was dissolved in distilled water and made up to 1 dm³. An aliquot (5,0 cm³) was diluted with distilled water and made up to 100 cm³.

Preparation of sample

Strained rumen digest (1,0 cm³) was diluted with 0,1 mol.dm⁻³ hydrochloric acid (5,0 cm³) and an aliquot (0,1 cm³) of this solution was used for analysis.

Analytical procedure

Phenol-nitroprusside reagent (5,0 cm³) was added to an aliquot (0,1 cm³) of acidified strained rumen digest in a test-tube and the contents of the tube were mixed well. Alkaline hypochlorite reagent (5,0 cm³) was added, mixed in well and the mixture was allowed to react for exactly 1h before the absorbance of the solution was read at 625 nm in a Beckman DB spectrophotometer against a blank containing no

ammonia.

3.1.6 Assay for cellulase activity

The assay is based upon the analysis of reducing sugars formed during hydrolysis of the substrate, carboxymethyl cellulose or acid swollen cellulose (Krishnamurti & Kitts, 1969).

Reagents

Acetate Buffer ($0,1 \text{ mol.dm}^{-1}$, pH6,0). Anhydrous sodium acetate (0,820g) was dissolved in distilled water (100 cm^3). The pH of the acetate solution was adjusted to 6,0 by the dropwise addition of a solution consisting of 18 mol.dm^{-3} acetic acid ($0,56 \text{ cm}^3$) in distilled water (100 cm^3).

Carboxymethylcellulose substrate. Carboxymethylcellulose sodium salt (BDH, low viscosity) (0,4g) was dissolved in toluene saturated water (100 cm^3).

Acid swollen (Walseth) cellulose substrate (Tansey, 1971). Sigmacell type 20 microcrystalline cellulose (15g) was slowly added to 85% ortho-phosphoric acid while stirring vigorously to prevent the formation of lumps. The suspension was stirred for 2h, diluted to 1 dm^3 with distilled water and filtered through five layers of cheese cloth over two layers of Whatman No. 1 filter paper in a Buchner funnel. The swollen

cellulose was suspended in distilled water (1 dm^3), suction filtered as before and resuspended in 2% (m/v) sodium bicarbonate (250 cm^3). The suspension was homogenised and stored for 12h. The suspension was filtered as before, washed with distilled water (2 dm^3), resuspended in distilled water (500 cm^3), centrifuged and the precipitate homogenised in distilled water ($2,5 \text{ dm}^3$). The homogenate was stored at 4°C .

Standard glucose solution. Glucose (0,06g) was dissolved in distilled water and made up to 1 dm^3 . An aliquot ($1,5 \text{ cm}^3$) was used for analysis.

Reagents for sugar analysis. See 3.1.2.

Analytical procedure

Cellulose substrate ($2,0 \text{ cm}^3$) was added to the crude enzyme (0,02g) or to the enzyme extract ($1,0 \text{ cm}^3$) in sodium acetate buffer ($2,0 \text{ cm}^3$) and the digest was incubated for 4h at 39°C . A blank containing no enzyme was included. After hydrolysis the reaction was stopped by rapid cooling, followed by centrifugation ($1000 \times g$, 4°C , 10 min). The reducing power of a sample ($1,0 \text{ cm}^3$) of the supernatant, was determined by the modified Nelson-Somogyi procedure described in 3.1.2 and the enzyme activity was expressed as $\mu \text{ mol}$ of glucose liberated per mg of crude enzyme per min.

3.1.7 Assay for xylanase activity

The assay is based upon the procedure described by Taiz & Honigman (1976).

Reagents

Acetate buffer ($0,05 \text{ mol.dm}^{-3}$), pH 5,0). Anhydrous sodium acetate ($0,410\text{g}$) was dissolved in distilled water (100 cm^3). The pH of the acetate solution was adjusted to pH 5,0 by the dropwise addition of a solution consisting of 18 mol.dm^{-3} acetic acid ($0,28 \text{ cm}^3$) in distilled water (100 cm^3).

Xylan buffer solution. Larchwood xylan (Sigma)(50 mg) was dissolved in $0,5 \text{ mol.dm}^{-3}$ sodium hydroxide ($1,0 \text{ cm}^3$) by grinding with a glass rod in a test-tube. The syrupy solution was diluted with water ($8,0 \text{ cm}^3$) followed by the addition of $0,5 \text{ mol.dm}^{-3}$ acetic acid ($1,0 \text{ cm}^3$).

Standard xylose solution

Xylose ($0,05\text{g}$) was dissolved in distilled water and made up to 1 dm^3 . An aliquot ($1,5 \text{ cm}^3$) was used for analysis.

Reagents for sugar analysis. See 3.1.2.

Analytical procedure

Xylan buffer ($2,0 \text{ cm}^3$) was added to the enzyme ($0,02\text{g}$) or to

the enzyme extract ($0,8 \text{ cm}^3$) and the digest was incubated for 2h at 39°C . After hydrolysis the reaction was stopped by rapid cooling, followed by centrifugation ($1000 \times g$, 4°C , 10 min). The reducing power of a sample ($0,2 \text{ cm}^3$) of the supernatant was determined by the modified Nelson-Somogyi procedure described in 3.1.2 and the enzyme activity was expressed as μmol of xylose produced per mg of crude enzyme per min.

3.1.8 Assay for β -glucosidase activity

The β -glucosidase activity was determined by the spectrophotometric measurement of the amount of p-nitrophenol liberated from p-nitrophenyl- β -D-glucoside by the enzyme under defined conditions.

Reagents

Acetate buffer ($0,05 \text{ mol.dm}^{-3}$), pH 5,0). See 3.1.7.

Sodium bicarbonate solution. Sodium bicarbonate (1,25g) was dissolved in distilled water (500 cm^3).

p-Nitrophenyl- β -D-glucoside reagent.

p-Nitrophenyl- β -D-glucoside (Sigma) was dissolved in sodium acetate buffer ($0,05 \text{ mol.dm}^{-3}$, pH 5,0).

Analytical procedure

p-Nitrophenyl- β -D-glucoside reagent ($0,8 \text{ cm}^3$) was added to the enzyme extract ($0,2 \text{ cm}^3$) and the digest was incubated for 15 min. The reaction was quenched by the addition of sodium bicarbonate solution ($4,0 \text{ cm}^3$) and the absorbance was read at 401 nm. The enzyme activity was expressed as the number of μ mol of p-nitrophenol released per min per cm^3 of enzyme solution.

3.1.9 Preparative flat-bed isoelectric focusing

The net electrical charge of a protein molecule is dependent upon the number of positive charges due to associated amino groups relative to the number of negative charges due to dissociated carboxylic groups of the side chains of the amino acids constituting the protein. Since association and dissociation of amino and carboxylic groups are pH dependent there is a particular ambient pH, known as the isoelectric point at which the net electrical charge on the protein will be zero. If a protein is placed in a pH gradient which embraces the isoelectric point of the protein and an electrical potential is applied along the gradient, the protein will migrate along the pH gradient losing net electrical charge until it reaches a position in the gradient corresponding to its isoelectric point. At this point of zero charge the protein will become stationary. Isoelectric focusing makes use of this principle to separate proteins of

different isoelectric points into distinct narrow bands in a gel.

Apparatus

For preparative electrofocusing, use was made of the LKB 2117 Multiphor apparatus (LKB-Produkter AB, Bromma, Sweden).

Analytical procedure

The procedure used was that described in the LKB application Note No.198. The carbohydrase enzyme complex (0,2g), Bio Gel P-150 (5,0g) and distilled water 95 (cm³) were mixed into a slurry. LKB "Ampholine" (5,0 cm³) was added to form a linear, pH gradient over the required pH range within an electric field. The gel slurry was gently stirred and poured onto the tray held in a level position, to form an even layer. A small fan was mounted above the tray and the gel suspension was evaporated with a light stream of air until the water loss reached 38,9% of the initial slurry mass.

The tray was transferred onto the cooling plate of the apparatus and the anode wick, soaked in 1 mol.dm⁻³ sodium hydroxide, was placed in position. Current was applied via electrodes in the lid of the apparatus by an LKB 2103 Power Supply for 19h at 6°C. (Initial voltage 0,25 kV and current 15 mA, final voltage 0,65mV and current 6,0 mA).

The gel bed was fractionated with a fractionating grid and the gel sections were transferred to eluting columns and eluted with 25 m mol.dm⁻³ sodium phosphate buffer, pH 6,0 (5,0 cm³). A sample of the eluate was used for the analysis of enzyme activity.

3.1.10 Measurement of redox potential

When one substance is oxidised, releasing electrons, another substance simultaneously takes up the electrons and is reduced. These reversible oxidation and reduction processes can, therefore, be defined in terms of electron migrations between chemical compounds and can be measured as voltage by means of an inert noble metal electrode connected to a reference electrode (Jacob, 1970).

Electrodes

Measuring electrode. The measuring electrode consisted of a polished platinum rod (diameter 1,0 mm) encased in a glass tube (100 mm x 8,0 mm). The tip of the platinum rod protruded 30 mm from the end of the glass tube.

Reference electrode. A standard calomel electrode was used as reference.

Calibration of electrode

Although the measured potential is independent of the size of

the measuring electrode, exact measurements can only be obtained if the electrode surface is polished and flawless. Furthermore, the redox potential is measured, in principle, against the standard hydrogen electrode (potential = 0) as reference. However, in practice it is more convenient to measure redox potential against a standard reference electrode characterised by a constant potential which, in the case of the calomel electrode, amounts to 241 mV at 25°C.

The only reliable method for calibrating the electrodes is to measure the potential of a well-defined solution in which a redox equilibrium exists (Kjaergaard, 1977). Kjaergaard recommends the use of a saturated solution of quinhydrone at 25°C as standard. The redox potential of the quinhydrone electrode is expressed by the following equation, due to Linnet (1970):

$$\text{Redox potential (quinhydrone)} = 699 - 59,1 \cdot \text{pH}$$

Measuring procedure

Both redox electrodes were mounted through holes in a rubber stopper which fitted the glass digestion vessels used in these experiments. During measurements the screw cap of the digestion vessel was replaced under anaerobic conditions, with the rubber stopper and the electrodes. The digest was gently stirred by means of a magnetic stirrer and the most negative reading obtained within 2 min was recorded. These values were corrected for the electrodes used.

3.1.11 Viable counts of rumen bacteria

The procedure is based upon the enumeration of rumen bacteria grown on selective solid culture media under conditions which closely resemble those existing in the rumen, as described by Kistner (1960).

Anaerobic conditions were maintained by boiling the water used for media, to remove dissolved oxygen, and by displacing the air in the culture vessels with oxygen-free gas mixtures under positive pressure. Cysteine and sodium sulphide were included in the media to maintain a low redox potential. Redox potential was monitored with the redox indicator, indigo carmine (Redox potential - 125mV, pH 7,0).

The special gas mixtures used, 98% carbon dioxide + 2% hydrogen and 98% nitrogen + 2% hydrogen (Special Gas Division, Afrox Limited, Germiston) were passed through "Deoxo" catalytic gas purifier cartridges (Engelhard, New Jersey, USA) to remove traces of oxygen. The gas mixtures were conducted through metal or butyl rubber tubing (Exo Rubber Ltd., Teddington, England) to minimise the re-entry of oxygen by diffusion through the walls of rubber tubing. Cultures and media were transferred by means of syringes, the dead volume of which had been cleared of air. Sterile conditions were obtained by autoclaving, or in the case of heat labile substances, by passing through Milli-Stak GS 0,22 μ m bacterial filters (Millipore, Massachusetts, USA).

Reagents

Mineral solution I. Dipotassium hydrogen phosphate (3,0g) was dissolved in distilled water (500 cm³).

Mineral solution II. Sodium chloride (6,0g), ammonium sulphate (6,0g), potassium dihydrogen phosphate (3,0g), calcium chloride (0,6g) and magnesium sulphate (1,23g) were dissolved in distilled water (500 cm³).

Cysteine hydrochloride-sodium sulphide solution. Cysteine (1,25g) and sodium sulphide (1,25g) were dissolved in boiled, nitrogen purged 0,2 mol. dm⁻³ sodium hydroxide solution (100 cm³) under anaerobic conditions.

Sodium bicarbonate-glucose solution. Sodium bicarbonate (10,617g) and D-glucose (0,83g) were dissolved in distilled water (100 cm³). The solution was purged with nitrogen (containing 2% hydrogen) to remove dissolved air.

Diluting solution. Mineral solution I (37,5 cm³), mineral solution II (37,5 cm³) and 0,05% (m/v) indigo carmine solution (10,0 cm³) were added to boiled de-ionised water (915,0 cm³) and boiled for 2 min to dispel air. While purging the solution with carbon dioxide (containing 2% hydrogen), cysteine hydrochloride (0,5g) and sodium bicarbonate (6,37g) were added and allowed to dissolve.

Ball-milled cellulose suspension. Shredded Whatman No. 541 filter paper (12g) in de-ionised water (600 cm^3) was placed in a ball mill (capacity 1 dm^3) and milled with a mixed charge of porcelain balls for about 72h at 57 revolutions per min. The ball-milled cellulose suspension was centrifuged ($1000 \times g$ for 10 min) and a portion (300 cm^3) of the supernatant discarded. The cellulose and remaining supernatant were made up to 400 cm^3 with de-ionised water.

Culture medium for total microbes. Bacto-agar (10g), cellobiose (0,25g), xylan (0,25g), soluble starch (0,25g), mineral solution I ($18,75\text{ cm}^3$), mineral solution II ($18,75\text{ cm}^3$), particle free rumen fluid (200 cm^3) and 0,05% (m/v) indigo carmine solution ($5,0\text{ cm}^3$) were dissolved in de-ionised water by boiling for 20 min. The solution was made up to 460 cm^3 with de-aerated water, purged with nitrogen (containing 2% hydrogen) for 15 min, autoclaved for 20 min and cooled to 47°C . Filter sterilised solutions of cysteine/sodium sulphide (10 cm^3) and sodium bicarbonate/D-glucose (30 cm^3) were introduced and mixed well.

Culture medium for xylanolytic microbes. Bacto-agar (10g), xylan ($15\text{g}/200\text{ cm}^3$ dist. H_2O), mineral solution I ($18,75\text{ cm}^3$), mineral solution II ($18,75\text{ cm}^3$), particle-free rumen fluid (200 cm^3) and 0,05% (m/v) indigo carmine solution ($5,0\text{ cm}^3$) were boiled for 20 min to dissolve. The solution was made up to 460 cm^3 with de-aerated water, purged with

nitrogen (containing 2% hydrogen) for 15 min, autoclaved for 20 min and cooled to 47°C. Filter sterilised solutions of cysteine/sodium sulphide (10 cm³) and sodium bicarbonate/D-glucose (30 cm³) were introduced and mixed well.

Culture medium for cellulolytic microbes. A mixture of Bacto-agar (10g), ball-milled cellulose suspension (200 cm³), mineral solution I (18,75 cm³), mineral solution II (18,75 cm³), particle-free rumen fluid (200 cm³), 0,05% (m/v) indigo carmine solution (5,0 cm³) and distilled water (17,5 cm³) was boiled for 20 min to ensure that the soluble constituents were dissolved. The solution was made up to 460 cm³ with de-aerated water, purged with nitrogen for 15 min, autoclaved for 20 min and cooled to 47°C. Filter-sterilised solutions of cysteine/sodium sulphide (10 cm³) and sodium bicarbonate/D-glucose (30 cm³) were introduced and mixed well.

Procedure for making viable counts.

The rumen digest was homogenised with an Ultra Turrax, homogeniser (type TP 18/10, Ganke & Kunkel, West Germany) under anaerobic conditions to dislodge bacteria from solid plant particles. A sample (1,0 cm³) of rumen fluid was injected through the rubber stopper of a sterile oxygen-free 28 cm³ McCartney bottle containing an aliquot (9,0 cm³) of diluting solution, by means of a 2 cm³ sterile syringe with an oxygen-free dead volume. By means of similar transfers a series of ten-fold dilutions were made. Depending upon the

expected microbial count an aliquot ($1,0 \text{ cm}^3$) of an appropriate dilution was transferred to a sterile oxygen-free McCartney bottle, sealed with a rubber stopper and containing the culture medium ($9,0 \text{ cm}^3$) kept at 47°C . An aliquot ($1,0 \text{ cm}^3$) of the inoculated culture medium was transferred in a similar way to a sterile oxygen-free Astell roll bottle at 47°C fitted with a rubber stopper. The Astell roll bottle was immediately spun in an Astell apparatus (Astell Laboratory Service Co. Ltd., London) to form a thin layer of growth medium on the inside of the bottle.

The Astell bottles were incubated upside down in an incubator at 39°C . The incubation period depended upon the type of bacteria cultured. Visible colonies of xylanolytic and total microbes developed after 24 to 36h, while cellulolytic microbes required an incubation period of 3 to 4 weeks. The colonies were counted under low magnification and the results were expressed as number of bacteria per cm^3 of rumen fluid.

3.1.12 Determination of methaemoglobin

Methaemoglobin was determined by the photometric method described by Evelyn & Malloy (1938). The absorbance spectrum of methaemoglobin exhibits a characteristic shoulder at 630 nm. When potassium cyanide is added to the methaemoglobin the absorbance at 630 nm is decreased and the shoulder is completely lost as the methaemoglobin is converted to cyanomethaemoglobin. The change in absorbance at this wavelength is proportional to the concentration of

methaemoglobin in the blood.

Reagents

Potassium phosphate buffer ($0,01 \text{ mol.dm}^{-3}$, pH 6,6)

Potassium dihydrogen phosphate (1,361g) was dissolved in distilled water ($\pm 900 \text{ cm}^3$) and titrated with a concentrated potassium hydroxide solution to pH 6,6. The solution was made up to $1,0 \text{ dm}^3$.

Acetic acid solution. Glacial acetic acid (12 cm^3) was made up to 100 cm^3 with distilled water.

Potassium cyanide solution. Potassium cyanide (10g) was dissolved in distilled water and made up to 100 cm^3 . Within 1h of the time of use, a neutralised solution of potassium cyanide was prepared by mixing equal volumes of the potassium cyanide and acetic acid solutions.

Procedure

Fresh whole blood ($0,1 \text{ cm}^3$) was added to $0,01 \text{ mol.dm}^{-3}$ sodium phosphate buffer (10 cm^3) of pH 6,6. The solution was allowed to stand for 5 min and the absorbance (L1) was read against a water blank in a spectrophotometer at 630 nm. Neutralised potassium cyanide ($0,05 \text{ cm}^3$) was added to the solution and the absorbance (L2) was read at the same wavelength after 2 min. The concentration of methaemoglobin, expressed as grams

per 100 cm³ of blood, was calculated using the following equation, due to Evelyn & Malloy (1938):

$$\text{Methaemoglobin (\%, m/v)} = 100(L_1 - L_2)/2.77$$

3.2 Study of the nitrogenous substances depressing digestion in vitro

Some of the work reported in this section has been published elsewhere (see Marais, 1980).

3.2.1 Distinction between the effects of nitrate and other soluble nitrogenous substances upon digestion

The effect of nitrate and other non-protein organic nitrogenous (NPON) substances upon digestion has received little attention. An experiment was therefore conducted to determine the effect of these components on the digestibility in vitro of kikuyu grass.

Kikuyu grass samples containing a high level (670 mg%, m/m) and a low level (10 mg%, m/m) of nitrate nitrogen were extracted with water to remove any nitrate and soluble non-protein organic nitrogen present. These samples were subjected to digestion in vitro, with or without the addition of nitrate-N in the form of potassium nitrate (670 mg%, m/m) to both the original high and low nitrate kikuyu grass samples. Results were compared with those obtained on

unextracted material (Table 3.1).

TABLE 3.1 Effect of nitrate nitrogen and non-protein organic nitrogen (NPON) upon digestion in vitro (48h) of kikuyu grass samples

Treatment prior to digestion	Dry matter digested <i>in vitro</i> (°/o, m/m) *	
	High nitrate kikuyu grass (670 mg °/oN, m/m)	Low nitrate kikuyu grass (10 mg°/o N, m/m)
Unextracted kikuyu grass	65,0 ± 0,4	73,1 ± 0,3
Nitrate and NPON extracted with H ₂ O	73,2 ± 0,2	73,5 ± 0,2
Extracted N replaced with KNO ₃ (670 mg °/o N, m/m)	64,6 ± 0,3	63,7 ± 0,6

* Mean of 6 determinations

Results presented in Table 3.1 show that the extraction of soluble substances, such as nitrate and NPON from the high nitrate kikuyu grass, increases the dry matter digestibility in vitro by 12,6%, while the effect on the low nitrate grass is negligible. Since the observed increase in digestibility can be prevented by the addition of nitrate, equivalent to the amount extracted, it would appear that nitrate or a product formed from nitrate during digestion, has a depressing effect on digestion in vitro, while NPON appears to have no detrimental effect upon digestion. However, the possibility that NPON and nitrate both have a non-additive effect on digestion in vitro is not excluded. These results are qualitatively in agreement with the findings of Hall, Gaddy & Hobbs (1960) who showed that the digestibility in vitro of Sudan forage is decreased by more than 50% if the

sample is supplemented with 50 mg nitrate nitrogen per 100g of forage.

3.2.2 Effect of nitrate upon digestion

Tilley & Terry (1963) state that the process of fibre digestion is complete by the end of a 48h digestion period in vitro. In order to establish whether nitrate (or its conversion products) exerts its effect on the rate of digestion and whether the observed depression of digestion could be overcome by increasing the digestion time, an experiment was conducted in which the normal 48h digestion period was extended.

The McDougall saliva buffer (McDougall, 1948) used in these experiments is capable of maintaining the pH of the digest within the limits 6,7 to 6,9 under normal circumstances (Tilley & Terry, 1963). Changes in pH of the digest could, however, bring about considerable changes in digestibility (Alexander, 1969). In view of these findings it was therefore also necessary to establish whether the observed reduction in digestion could be a pH response due to the aqueous extraction of the grass material and the addition of nitrate.

Kikuyu grass samples, containing a high level (670 mg%, m/m) of nitrate-N were pre-extracted with water. In half of the samples extracted nitrate was replaced with an equivalent amount of nitrate in the form of potassium nitrate and the

rate of digestion was followed over a period of 72h. The pH of the digest was recorded at the end of each digestion period. Results obtained are presented in Fig. 3.1 and Table 3.2.

TABLE 3.2 Effect of extracting the plant material with water and adding nitrate back to the digest upon the pH of the rumen digest *in vitro*

Treatment prior to digestion <i>in vitro</i>	pH of digest at different times of digestion *				
	0h	6h	24h	48h	72h
Unextracted kikuyu grass	6,87 ±0,01	6,90 ±0,02	6,87 ±0,01	6,84 ±0,01	6,79 ± 0,01
Kikuyu grass pre-extracted with H ₂ O	6,83 ±0,01	6,91 ±0,01	6,83 ±0,00	6,77 ±0,02	6,76 ± 0,01
Extracted N replaced with KNO ₃ (670 mg n, m/m)	6,86 ±0,01	6,89 ±0,01	6,90 ±0,01	6,93 ±0,01	6,80 ± 0,02

* Mean of 3 replicates

Results presented in Fig 3.1 show that the digestion in vitro of high nitrate kikuyu grass is not complete after 48h. Initially the grass with a high level of nitrate has a slower rate of digestion than water extracted grass, and the effect is most marked after about 24h. If digestion is continued for longer than 24h, the rate of digestion of the high nitrate

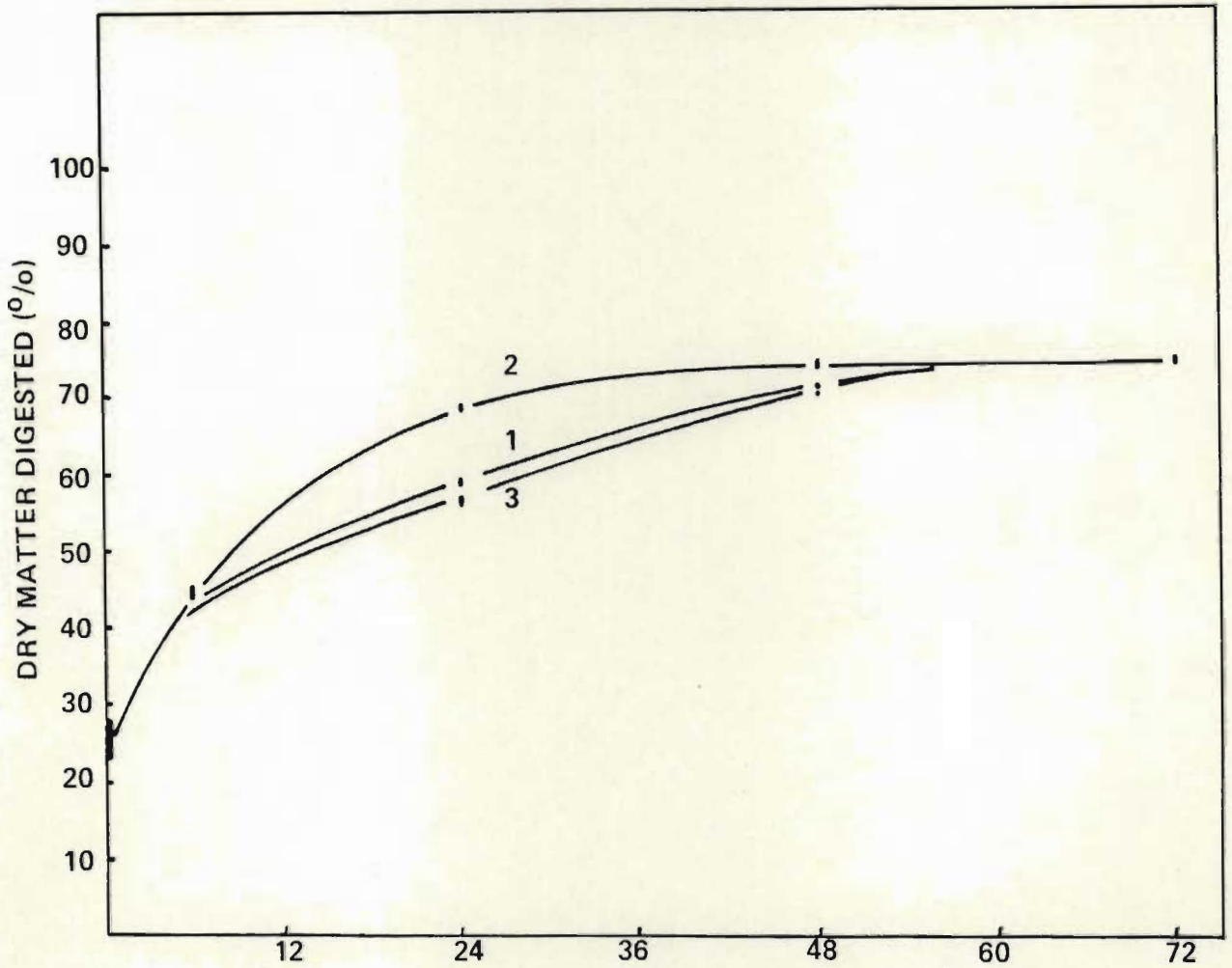


FIG. 3.1 Effect of nitrate in kikuyu grass upon the rate of digestion *in vitro*. (1) High nitrate kikuyu grass (670 mg O/o , m/m); (2) Kikuyu grass pre-extracted with water (low nitrate); (3) Pre-extracted kikuyu grass supplemented with potassium nitrate (670 mg O/o , m/m)

samples becomes higher than that of the water extracted samples, but complete digestion of the high nitrate samples is only achieved after about 72h. These results suggest that the factor(s) responsible for the decrease in digestion exerts its effect within the first 24h of digestion.

Upon replacing extracted nitrate with an equivalent amount of potassium nitrate, the suppression of digestion becomes similar to that in the high nitrate sample, substantiating the results given in Table 3.1.

The pH of the digest remains remarkably constant. Results in Table 3.2 show a decrease of only about 0,07 pH units over the digestion period of 72h. The different treatments have little influence on the pH of the digest and the observed changes in digestion is therefore not due to pH effects.

In a subsequent study the effect of increasing nitrate concentrations upon digestion in vitro was investigated. The nitrate content of kikuyu material with a low nitrate content (24mg% N, m/m) was adjusted to different levels over the range 50 to 800 mg% (m/m) nitrogen by the addition to the material of appropriate amounts of potassium nitrate prior to digestion.

The dry matter digested after 24h and 48h was calculated and the results are presented in Fig. 3.2. These results show that low nitrate concentrations (<100 mg% N, m/m) stimulate

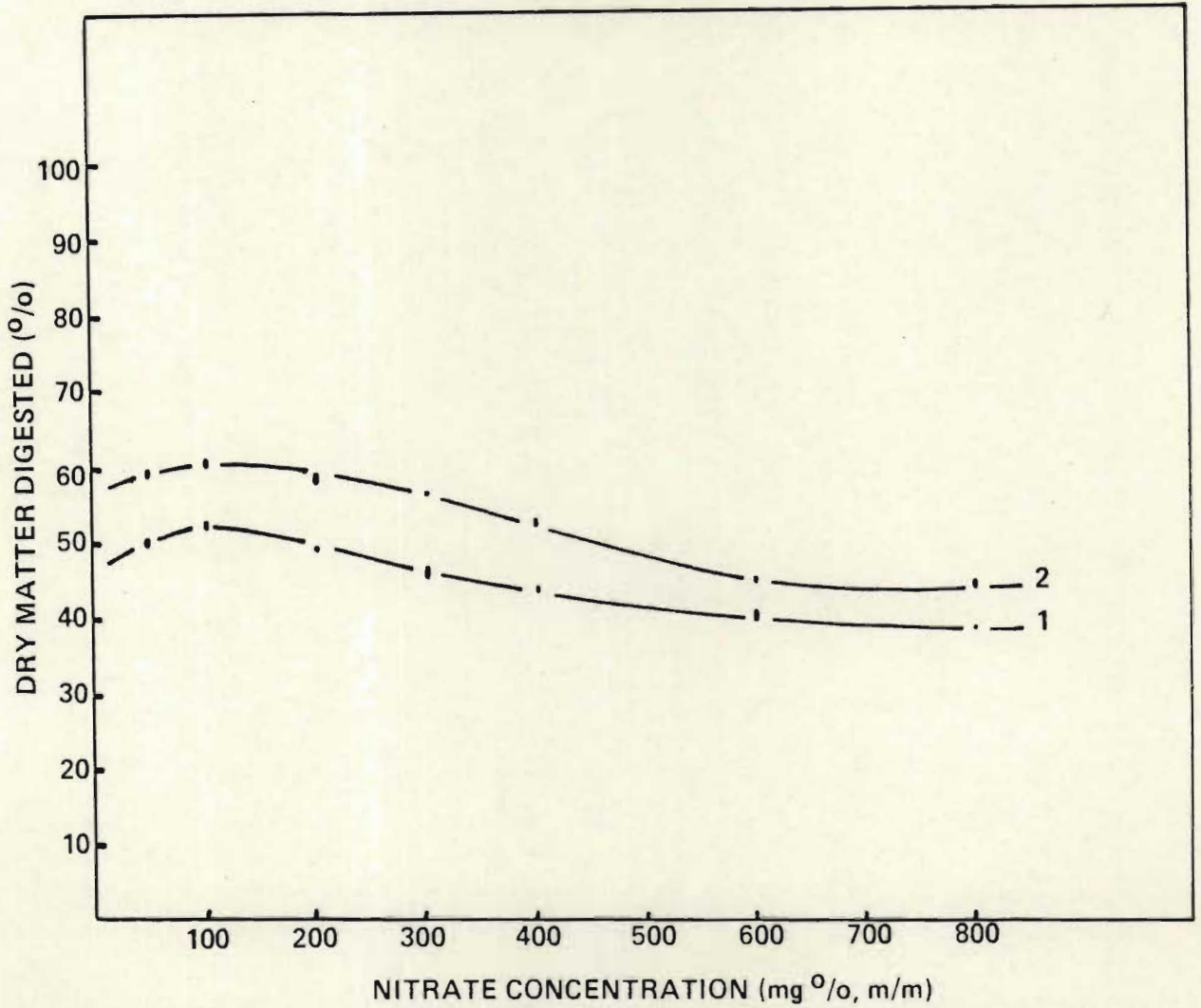


FIG. 3.2 Effect of nitrate concentration upon the amount of dry matter digested *in vitro*.
(1) Digestion time 24 hours; (2) Digestion time 48 hours

digestion in vitro, possibly by acting as a nitrogen source for bacterial growth. Optimum stimulation occurs at a nitrate content of about 100 mg% N (m/m) while higher nitrate levels cause a decrease in digestion. At 800 mg% N (m/m) the reduction in digestion is approximately 26%. The digestion curves obtained as a response to the addition of nitrate are similar at both digestion times.

3.2.3 Assimilatory metabolism of nitrate in vitro

The assimilatory metabolism of nitrate was followed over a 48h digestion period in vitro. Prior to digestion the nitrate content of kikuyu material was adjusted to 600 mg% N (m/m), by the addition of potassium nitrate. Changes in nitrate, nitrite and ammonia levels expressed as $\mu\text{g.cm}^{-3}$ of the digest, were determined. Results are presented in Fig. 3.3.

Results show that nitrate decreases from an initial concentration of about $65\mu\text{g.cm}^{-3}$ to about $5\mu\text{g.cm}^{-3}$ after 48h. Nitrite production reaches a peak of about $35\mu\text{g.cm}^{-3}$ after 18h and then rapidly decreases to 0 after 36h. The McDougall salivary buffer used in these digestion experiments contained a considerable amount of urea which readily gave rise to ammonium ions during digestion. The ammonia level, therefore, remains high (between 400 and $500\mu\text{g.cm}^{-3}$) and the ammonia derived from reduced nitrite could not be distinguished from this high background level.

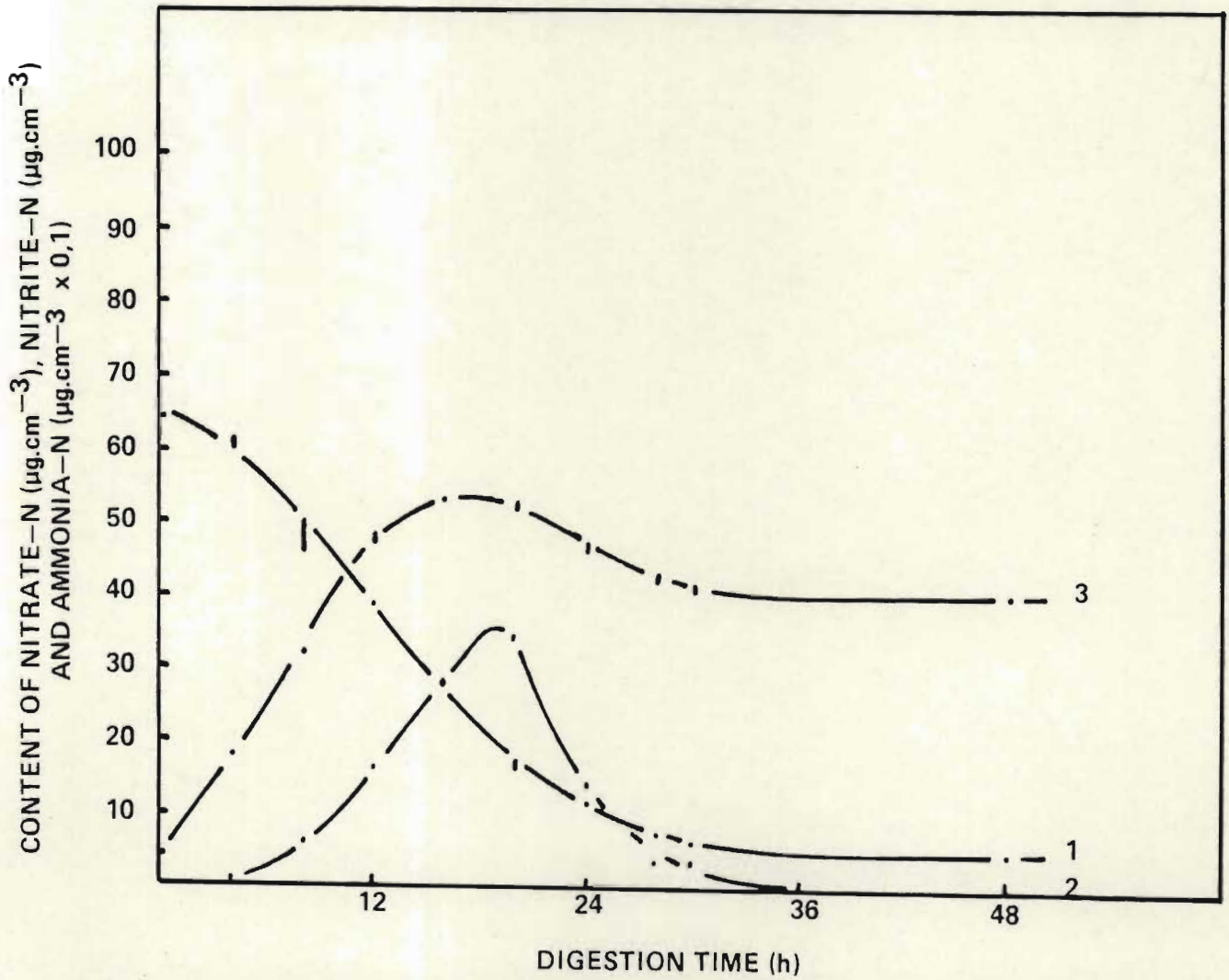


FIG. 3.3 Assimilatory metabolism of nitrate during digestion *in vitro*. (1) Nitrate; (2) Nitrite; (3) Ammonia

The accumulation of nitrite in the digest depends upon the relative activities of nitrate and nitrite reductases. The relative rates of the reactions catalysed by these enzymes seem to be affected by proteins and readily digestible carbohydrates in the digest. Nitrate and nitrite disappearance from the rumen was enhanced by feeding sheep with glucose and maize (Emerick, Embry & Seerley, 1965). Takahashi (1980), using washed suspensions of rumen microbes, showed that the presence of starch lowers the level of nitrite accumulating during digestion in vitro, while the addition of fibrous material, prepared from either grass or clover, increases both nitrate reduction and the nitrite level of the digest. Takahashi observed similar changes in the rumen in vivo. Following intra-ruminal administration of sodium nitrate, higher levels of nitrite accumulate in the rumen of animals fed a ration low in total digestible nutrients (TDN) than in a ration high in TDN (Takahashi, Masuko, Endo, Dodo & Fujita, 1980). This could be due to a pH effect since Takahashi, Takatsu, Imai, Suzuki & Fujita (1983) showed that the increased accumulation of ruminal nitrate caused by an increase in digestible crude protein in the ration of Suffolk Down wethers, is associated with a rise in rumen pH. A study of the effect of pH on the reduction of nitrate and nitrite, using washed suspensions of rumen microbes, showed that nitrate reduction is optimal at pH 6,6 while optimal nitrite reduction occurs at pH 5,8 (Takahashi, Masuda & Miyagi, 1978). Tillman, Sheriha & Sirny (1965) showed that under pH conditions favouring nitrate reduction

(pH 6,5) nitrite rapidly increased in the rumen fluid and blood of sheep.

In order to study the effect of nitrate upon digestion at constant pH in the presence of varying amounts of non structural carbohydrates and proteins, a number of grasses of different chemical composition was subjected to digesion in vitro.

The total non-structural carbohydrate content, the total organic nitrogen content and the nitrate content of nine different grass species were determined. By the addition of nitrate in the form of potassium nitrate, the nitrate level in the material was adjusted to either 100 or 600 mg% N (m/m) and the samples were subjected to digestion in vitro for a period of 24h. The dry matter digested and the percentage reduction in digestion, as a result of increasing the nitrate content from 100 to 600 mg% N (m/m), were calculated. Results are presented in Table 3.3.

Results show that the decrease in digestibility in vitro (24h), due to the increase in nitrate content, ranges from 26% in kikuyu grass to 2,8% in Lolium perenne. Other constituents of the grass, therefore, considerably modify the effect of nitrate at constant pH. The digestion of sub-tropical grasses is affected more severely by nitrate than that of temperate grasses. Results suggest that the presence of non-structural carbohydrates and protein reduces

TABLE 3.3 Effect of nitrate upon the digestion *in vitro* of grass species with different chemical compositions. TNC = total non-structural carbohydrates; TON = total organic nitrogen

Grass Species	TNC content ($^{\circ}/_{\circ}$, m/m)	TON content (mg $^{\circ}/_{\circ}$, m/m)	Dry matter digested in 24 hours at different nitrate levels *		Reduction in digestibility ($^{\circ}/_{\circ}$, m/m)
			100 mg $^{\circ}/_{\circ}$ N(m/m)	600 mg $^{\circ}/_{\circ}$ N(m/m)	
<i>Pennisetum clandestinum</i>	8,1 \pm 0,1	1669 \pm 11	40,8 \pm 0,8	30,2 \pm 0,2	26,0
<i>Eragrostis curvula</i>	5,9 \pm 0,1	2336 \pm 19	41,8 \pm 0,4	31,2 \pm 0,9	25,4
<i>Aristida junciformis</i>	4,7 \pm 0,1	789 \pm 5	20,0 \pm 0,3	15,8 \pm 0,5	21,0
<i>Paspalum dilatatum</i>	5,1 \pm 0,2	2379 \pm 14	46,5 \pm 0,6	36,9 \pm 0,9	20,7
<i>Cynodon sp.</i>	7,4 \pm 0,4	2064 \pm 32	47,5 \pm 0,5	40,7 \pm 0,5	14,1
<i>Festuca arundenacea</i>	12,7 \pm 0,1	2645 \pm 77	65,1 \pm 0,5	59,3 \pm 0,5	8,9
<i>Dactylis glomerata</i>	9,0 \pm 0,0	4235 \pm 60	59,4 \pm 0,9	59,4 \pm 0,9	8,9
<i>Lolium multiflorum</i>	11,2 \pm 0,1	4485 \pm 131	62,4 \pm 1,3	59,8 \pm 0,6	4,2
<i>Lolium perenne</i>	13,1 \pm 0,1	3973 \pm 11	66,9 \pm 0,5	65,0 \pm 0,6	2,8

* Mean of 8 determinations

the suppressing effect of nitrate on digestion. The reduction in digestibility is negatively correlated with the total non-structural carbohydrate content ($r=0,761$) and the total organic nitrogen content ($r=0,671$) of the grasses. The r values are significant at the 1% level.

In order to establish whether the difference in response to nitrate in different grasses is associated with differences in the conversion of nitrate to nitrite, an experiment was conducted in which the nitrate contents of a kikuyu grass sample and Dactylis glomerata sample were adjusted to 320 mg% N (m/m) or 920 mg% N (m/m) by the addition of appropriate amounts of potassium nitrate. These samples were subjected to digestion in vitro and the nitrite content of the digest was monitored over a period of 36h.

Results presented in Fig 3.4 show a higher level of nitrite in kikuyu grass than in Dactylis glomerata for the same initial level of nitrate in the grass. In both instances the pH remained constant during digestion. Low levels of nitrate (300 mg% N, m/m) in the grasses result in nitrite levels in the digest of less than $10 \mu\text{g.cm}^{-3}$. Tripling the concentration of nitrate in the grasses causes an eight-fold increase in nitrite concentration of the digest for both grasses.

In the present study, small day to day variations in the nitrite level of digests have been observed under identical conditions of digestion in vitro. The reason for these variations is not clear but could be due to the rumen fluid inoculum used. These observations seem to be in agreement with the findings of Tilley & Terry (1963) and Minson (1981) who ascribed variations in results of standard analyses of digestibility in vitro to fluctuations in the inoculum used

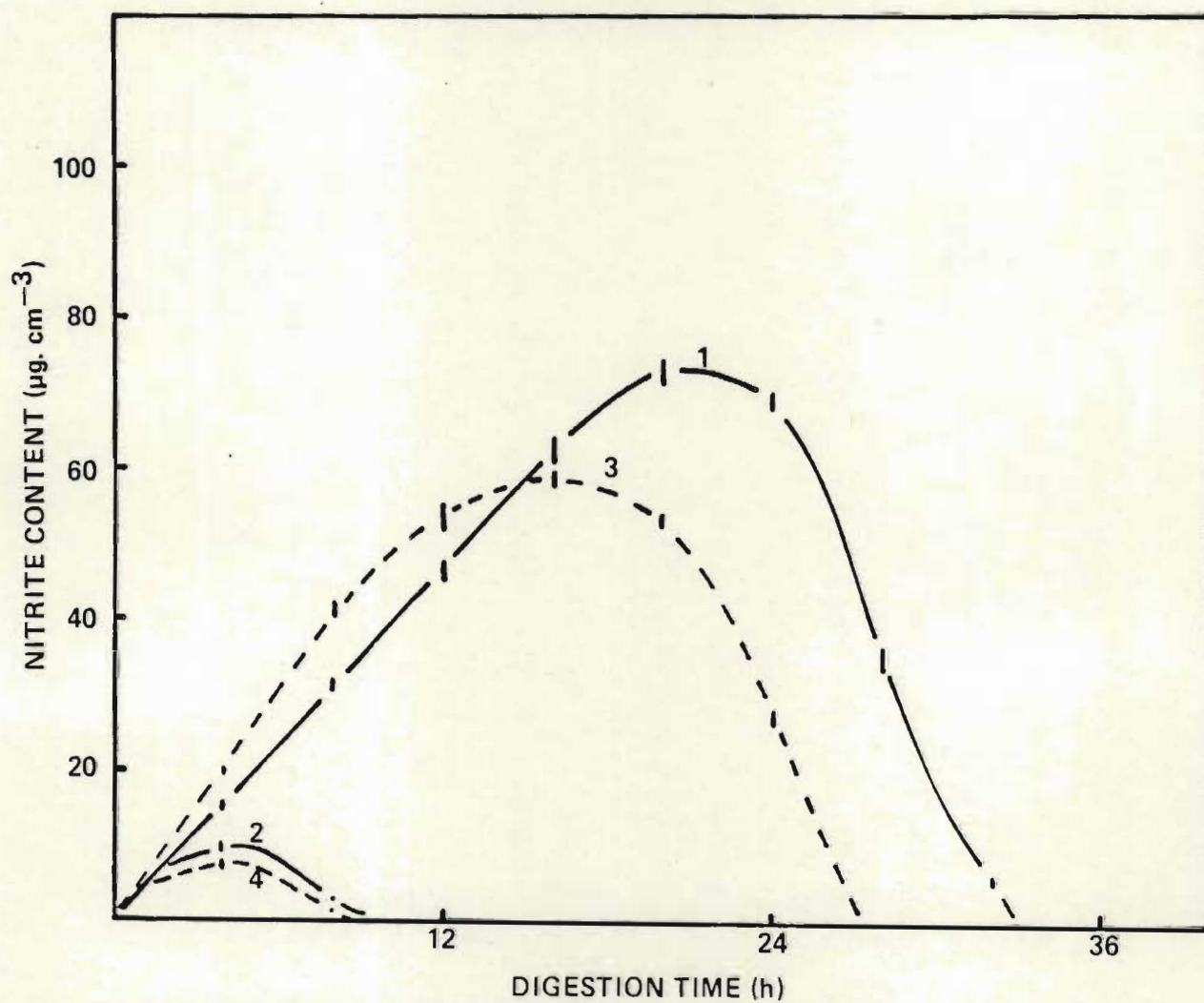


FIG. 3.4 Nitrite formation as a function of the type of grass digested and the nitrate level of the grass. (—) Kikuyu; (----) *Dactylis glomerata*; (1,3) 900 mg % nitrate; (2,4) 300 mg % nitrate

for these determinations. Diurnal variations in ruminal contents (Leedle, Bryant & Hespel, 1982) and the sporadic intake of water by the donor sheep, could influence microbial numbers. Miyazaki, Okamoto, Tsuda, Kawashima & Uesaka (1974) showed that cellulose digestion in vitro is a function of the microbial concentration in the rumen microbial inoculum. Lowering the microbial concentration results in a drop in digestibility of cellulose.

3.2.4 Distinction between the effects of nitrate, nitrite and ammonia upon digestion

Previous experiments show that the factor(s) responsible for decreased digestion, exerts its effect within the first 24h of digestion. During this period reduction products of nitrate, such as nitrite and ammonia, are also present in the digest and could be responsible for the observed reduction in digestibility. In order to distinguish between these compounds low nitrate (50 mg% N, m/m) kikuyu grass samples were adjusted to 600 mg% (m/m) nitrate-N by adding potassium nitrate, while other samples received nitrite-N (600 mg%, m/m) in the form of potassium nitrite or ammonium-N (600 mg%, m/m) in the form of ammonium sulphate. These samples were subjected to digestion in vitro for 24h and 48h and the results of this experiment are presented in Table 3.4

TABLE 3.4 Effect of nitrate, nitrite and ammonia upon digestion in vitro of kikuyu grass samples. Ammonia was added as ammonium sulphate while the nitrate and nitrite were added as potassium salts

Digestion time (h)	Addition (600 mg O/o N,m/m)	Dry matter digested (O/o , m/m)*	Reduction in digestibility (O/o m/m)
24	Nitrate	24,0 \pm 0,4	32
	Nitrite	19,4 \pm 0,6	45
	Ammonia	34,7 \pm 0,6	2
	Control	35,3 \pm 1,0	
48	Nitrate	35,7 \pm 1,4	39
	Nitrite	34,2 \pm 0,3	41
	Ammonia	57,6 \pm 0,3	1
	Control	58,4 \pm 0,4	

* Mean of 5 determinations

Results show no effect of ammonium ions upon digestion. Deviation from the control values at both 24h and 48h is small, but the addition of both nitrate and nitrite markedly lowers the digestibility of the kikuyu grass after digestion for 24h and 48h. The effect of nitrite upon digestion is much more severe than the effect of nitrate. Nitrite lowers digestibility by 45% and 41% after 24h and 48h respectively, while nitrate causes a reduction of 32% and 39% after 24h and 48h digestion respectively. The observed effect of nitrate on digestion however, could entirely or partly be due to its conversion to nitrite during digestion. In order to distinguish between the effects of nitrate and nitrite upon digestion, an experiment was designed in which the conversion

of nitrate to nitrite during digestion was prevented by the addition of tungstate, an inhibitor of nitrate reductase.

Assimilatory nitrate reduction leading to the formation of ammonia, seems to be the major pathway of nitrate metabolism in the rumen (Jones, 1972). According to Payne (1973) only bacterial dissimilatory nitrate reductase requires molybdenum as co-factor, while the assimilatory enzyme functions without molybdenum. These results suggest only a limited requirement for molybdenum, if any, by nitrate reducing rumen microbes. Tillman, Sheriha & Sirny (1965), on the other hand, demonstrated a marked dependence upon molybdenum for nitrate reduction by mixed rumen micro organisms. In the absence of molybdenum in the diet, the blood nitrate level of sheep was three times higher and the nitrite level lower than in the presence of molybdenum. Subsequently Inderlied & Delwiche (1973) and Ruoff & Delwiche (1977) showed that nitrate reduction in the bacterium, Veillonella alcalescens, isolated from sheep rumen, displays some characteristics of a dissimilatory nitrate reduction system and has a requirement for molybdenum, but also resembles an assimilatory system in that nitrate reduction is coupled with a nitrite-reducing ammonia assimilating pathway. According to Hungate (1966) it is doubtful whether Veillonella alcalescens is quantitatively significant in cattle. The presence of high levels of nitrate in the rumen, however, may stimulate growth of these microbes. Little is known about the molybdenum requirements of other nitrate reducing microbes in the rumen.

Molybdenum and tungsten are similar elements, both belonging to group VI T of the periodic table. As a result of this close similarity, tungsten can replace molybdenum in nitrate reductase, resulting in the loss of most of the enzyme activity (Taniguchi & Itagaki, 1960). Tungsten has been used as a prophylactic against nitrite toxicity by inhibiting the conversion of nitrate to nitrite in animals with a high nitrate intake. (Korzeniowski, Geurink & Kemp, 1980a and b).

In the present investigation tungsten was used to distinguish between the effect of nitrate and nitrite upon the digestion of kikuyu grass. In order to determine whether complete inhibition of nitrite formation can be achieved, low nitrate (100 mg% N, m/m) and high nitrate (600 mg% N, m/m) kikuyu grass samples were spiked with sodium tungstate (2,0 m mol. dm^{-3}) and subjected to digestion in vitro. The disappearance of nitrate and the formation of nitrite in the digest were followed over a period of 36h. Results presented in Fig. 3.5 show a considerable reduction in the conversion of nitrate to nitrite in the presence of tungstate. The continued disappearance of nitrate suggests a partial inhibition of nitrate reductase only. These results are in agreement with the findings of Prins, Cline-Theil, Malestein & Counotte (1980) who showed that tungstate prevents the synthesis of an active nitrate reductase in nitrite reducing strains of the rumen bacterium Selenomonas ruminantium subsp. lactilytica and Veillonella parvula J803, but did not inhibit active

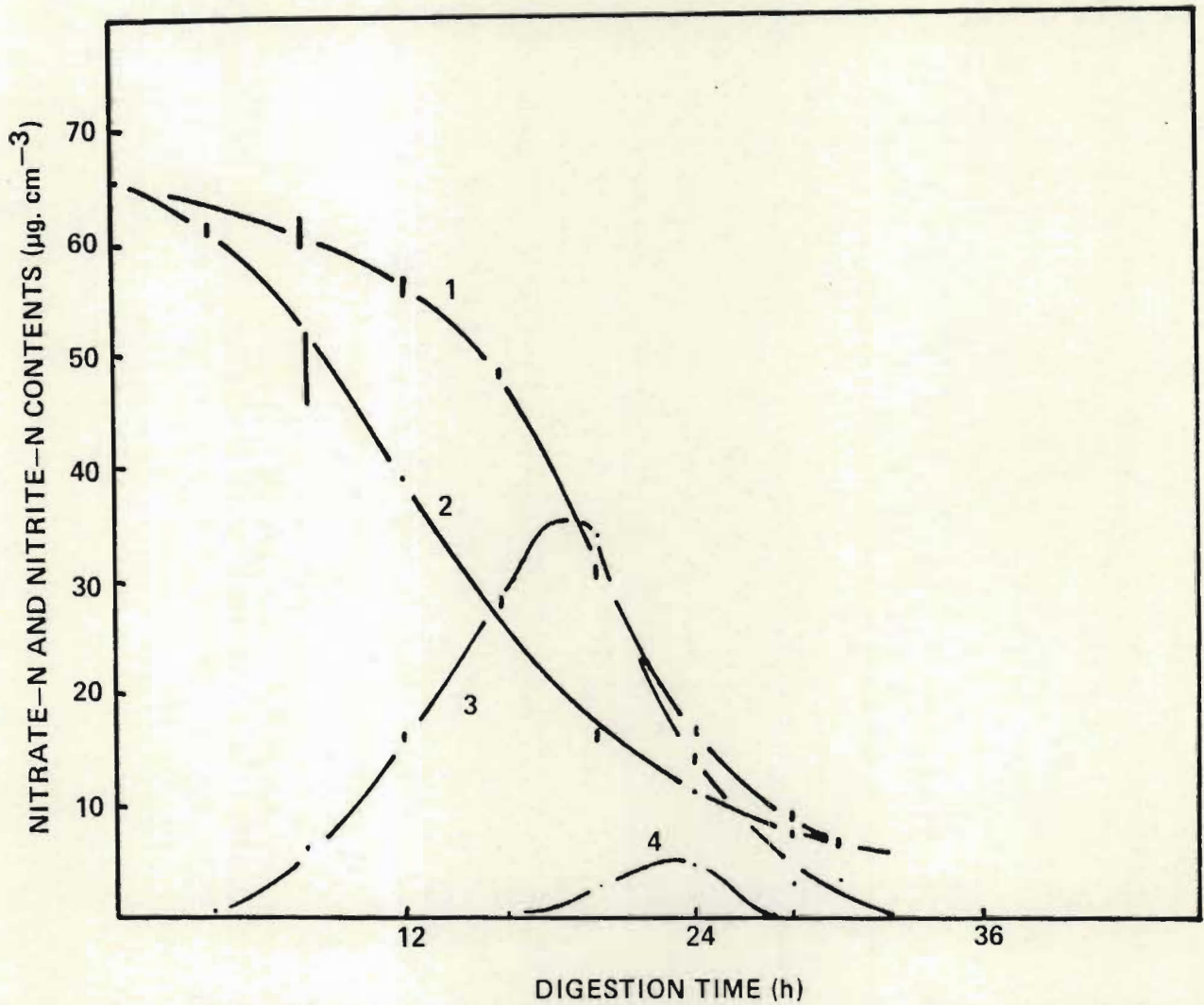


FIG. 3.5 Effect of tungstate upon the reduction of nitrate *in vitro*. Tungstate concentration of digest—2,0m mol. Nitrate—N content of grass — 600 mg ⁰/o. (1) Nitrate content of digest in presence of tungstate; (2) Nitrate content of digest in absence of tungstate; (3) Nitrite formation in absence of tungstate; (4) Nitrite formation in presence of tungstate

enzyme already formed. It may also point to the presence in the rumen digest of other nitrate reducing systems not dependent upon molybdenum.

In the absence of tungstate, the nitrite level in the digest peaks at a concentration of about $35 \mu\text{g}.\text{cm}^{-3}$ after 18h. The decrease in nitrate reduction rate changes the relative rates of nitrate and nitrite reduction sufficiently to reduce the accumulation of nitrite to a level of only $5 \mu\text{g}.\text{cm}^{-3}$ in the digest after 22h.

In a similar experiment the effect of a reduced level of nitrite upon digestion in vitro was studied. Low nitrate (100 mg% N, m/m) kikuyu grass samples and samples with the nitrate level adjusted to 600 mg% N (m/m), were subjected to digestion in vitro for periods of 24h and 48h in the absence and presence of tungstate ($10,0 \text{ m mol}.\text{dm}^{-3}$). Results from this experiment (Table 3.5) show a similar effect on nitrate and nitrite metabolism and indicate that even a tungstate concentration of $10,0 \text{ m mol}.\text{dm}^{-3}$ does not completely inhibit the reduction of nitrate. After digestion for 24h the nitrate level is reduced to approximately $14 \mu\text{g}.\text{cm}^{-3}$ both in the presence and absence of tungstate. Nevertheless, nitrite accumulation is drastically reduced in the presence of tungstate.

TABLE 3.5 Effect of tungstate upon the digestion *in vitro* of kikuyu grass containing a high level of nitrate (600 mg O/o N, m/m). The tungstate concentration of the digest was adjusted to $10,0 \text{ m mol. dm}^{-3}$

Digestion time (h)	Nitrate adjusted to 600 mg O/o N, (m/m)	Addition of tungstate ($500 \mu \text{ mol}$)	Nitrate content ($\mu \text{g.cm}^{-3}$)	Nitrite content ($\mu \text{g.cm}^{-3}$)	Dry matter digested (O/o , m/m)*
24	—	+	$5,8 \pm 0,2$	0,0	$40,6 \pm 0,2$
	—	—	$5,4 \pm 0,1$	0,0	$37,6 \pm 0,7$
	+	+	$14,6 \pm 0,2$	$0,1 \pm 0,0$	$39,3 \pm 0,4$
	+	—	$13,2 \pm 0,8$	$9,4 \pm 0,1$	$31,4 \pm 0,5$
48	—	+	$7,6 \pm 0,2$	0,0	$57,4 \pm 0,7$
	—	—	$6,3 \pm 0,2$	0,0	$56,4 \pm 0,4$
	+	+	$8,6 \pm 0,2$	0,0	$56,9 \pm 0,5$
	+	—	$7,6 \pm 0,2$	0,0	$46,7 \pm 0,8$

* Mean of 4 determinations

Results further show that by increasing the nitrate content of the grass to 600 mg% (m/m), the digestibility is reduced by approximately 6 digestibility units after 24h and by about 10 digestibility units after 48h. In the presence of tungstate the effect of nitrate upon digestion is eliminated. These results suggest that the reduction in digestibility is caused by the nitrite in the digest. Nitrite may therefore either have a direct effect upon digestion or it may give rise to some substance other than ammonia, which affects digestion.

3.2.5 Adaptation of rumen microbes to nitrate in the digest

Several workers have shown that rumen fluid obtained from sheep which are adapted to feed containing nitrate, could reduce nitrate more rapidly than rumen ingesta of sheep on a nitrate free diet, thus indicating adaptation of the rumen microbiota to increased nitrate intake (Jamieson, 1959; Sinclair & Jones, 1964; Farra & Satter, 1971). If the microbial population is capable of adjusting to a more rapid metabolism of nitrate, it may also be able to adapt to overcome the adverse effect of nitrate upon digestion. This, however, has not been investigated by these workers.

In a study of this phenomenon, the nitrate content of the donor sheep ration (lucerne hay), was gradually increased to 200 mg% N (m/m) over a period of two months by spraying the feed with a potassium nitrate solution. This animal provided the source of nitrate-adapted ingesta used in an in vitro digestibility experiment on kikuyu material containing either 200 or 670 mg% (m/m) nitrate nitrogen. The pH of the ingesta was 6,75 compared to 6,73 of the control sheep.

Results presented in Fig. 3.6 indicate that the effect of nitrate, in lowering the digestibility of kikuyu grass, is not influenced by the degree to which the rumen fluid inoculum has been adapted to nitrate. Increasing the concentration of nitrate fed to the donor sheep to 400 mg%

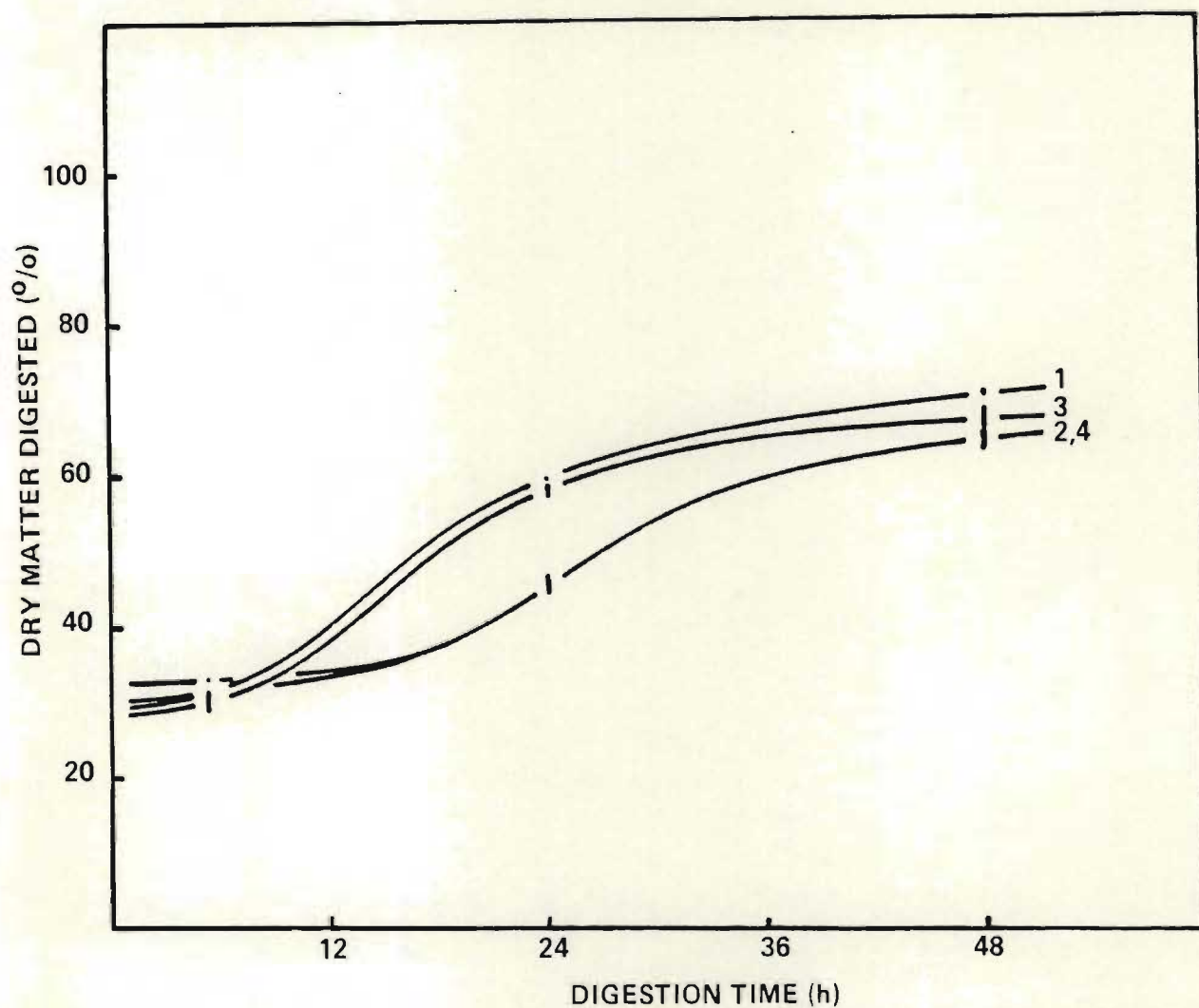


FIG. 3.6 Effect of rumen fluid inocula from nitrate adapted sheep upon digestion *in vitro* of kikuyu grass containing high (670 mg⁰/o N) and low (200 mg⁰/o N) levels of nitrate. (1) Nitrate adapted inoculum, low nitrate digest, (2) Nitrate adapted inoculum, high nitrate digest; (3) Non-adapted inoculum, low nitrate digest; (4) Non-adapted inoculum, high nitrate digest

(m/m) in further experiments and determining the digestibility in vitro of kikuyu material containing an identical amount of nitrate, gave similar results.

In order to substantiate these results on donor sheep fed kikuyu grass and to study further the adaptation of rumen microbes to nitrate in the feed, six 20 month old Merino wethers with a mean live mass of 42,5 kg were over-wintered on maize silage and then transferred to a low nitrate (67 mg% N, m/m) kikuyu grass pasture for one month. After an adaptation period in metabolic pens for 10 days on chopped low nitrate (67 mg% N, m/m) kikuyu grass, supplemented with an ad lib. salt lick (dicalcium phosphate - sodium chloride, 1:1, m/m), the animals were switched to a high nitrate (490 mg% N, m/m) kikuyu grass for 8 days. The nitrate intake was 0,12g N.kg LM.⁻¹day⁻¹. On day 8 the nitrate intake of the sheep was doubled by introducing a solution (200 cm³) containing potassium nitrate (4,5g N) into the rumen in two equal quantities 1h and 2h after feeding. Rumen fluid was collected three times during the adaptation period and three times during the 8 days after switching to the high nitrate kikuyu grass. Collection was done before the morning feeding using the procedure described in 3.1.1. The rumen fluid was used as inoculum for a digestibility study in vitro on a standard kikuyu grass sample in the presence and absence of nitrate. Rumen fluid samples were also collected at 2-hourly intervals for the analysis of nitrate and nitrite. Heparinised jugular blood samples (5,0 cm³) were collected

simultaneously for the determination of methaemoglobin. Results presented in Table 3.6 show that although considerable day-to-day variation in the in vitro results exists, there is no indication of a change in sensitivity of the microbes towards nitrate with time.

TABLE 3.6 Effect of the rumen inoculum from nitrate-adapted and unadapted sheep upon the digestion *in vitro* for 24 hours of kikuyu grass containing low and high levels of nitrate

Inoculum adaptation period (days)	Digestion <i>in vitro</i> (°/o, m/m)*		
	Low nitrate kikuyu grass 100 mg°/o (m/m)	High nitrate kikuyu grass 600 mg °/o (m/m)	Difference
-4	50,5 ± 0,4	46,6 ± 1,4	3,9 ± 1,3
-2	50,9 ± 0,7	42,2 ± 1,8	8,7 ± 2,0
0	55,3 ± 1,2	49,7 ± 1,9	5,5 ± 1,1
+2	54,1 ± 0,5	49,0 ± 1,6	5,1 ± 1,8
+6	53,0 ± 1,1	45,2 ± 1,8	7,8 ± 1,1
+8	53,4 ± 0,4	47,3 ± 1,8	6,1 ± 1,5

* Mean values using the inocula from 6 sheep

These results substantiate the results presented in Fig. 3.6 using rumen fluid from a donor sheep on lucerne hay.

A study was made of the extent to which the rumen population of the low nitrate kikuyu grass fed sheep could adapt to metabolise nitrate to nitrite by following the change in rumen nitrite content after feeding high nitrate kikuyu grass. Results presented in Fig. 3.7 indicate that although

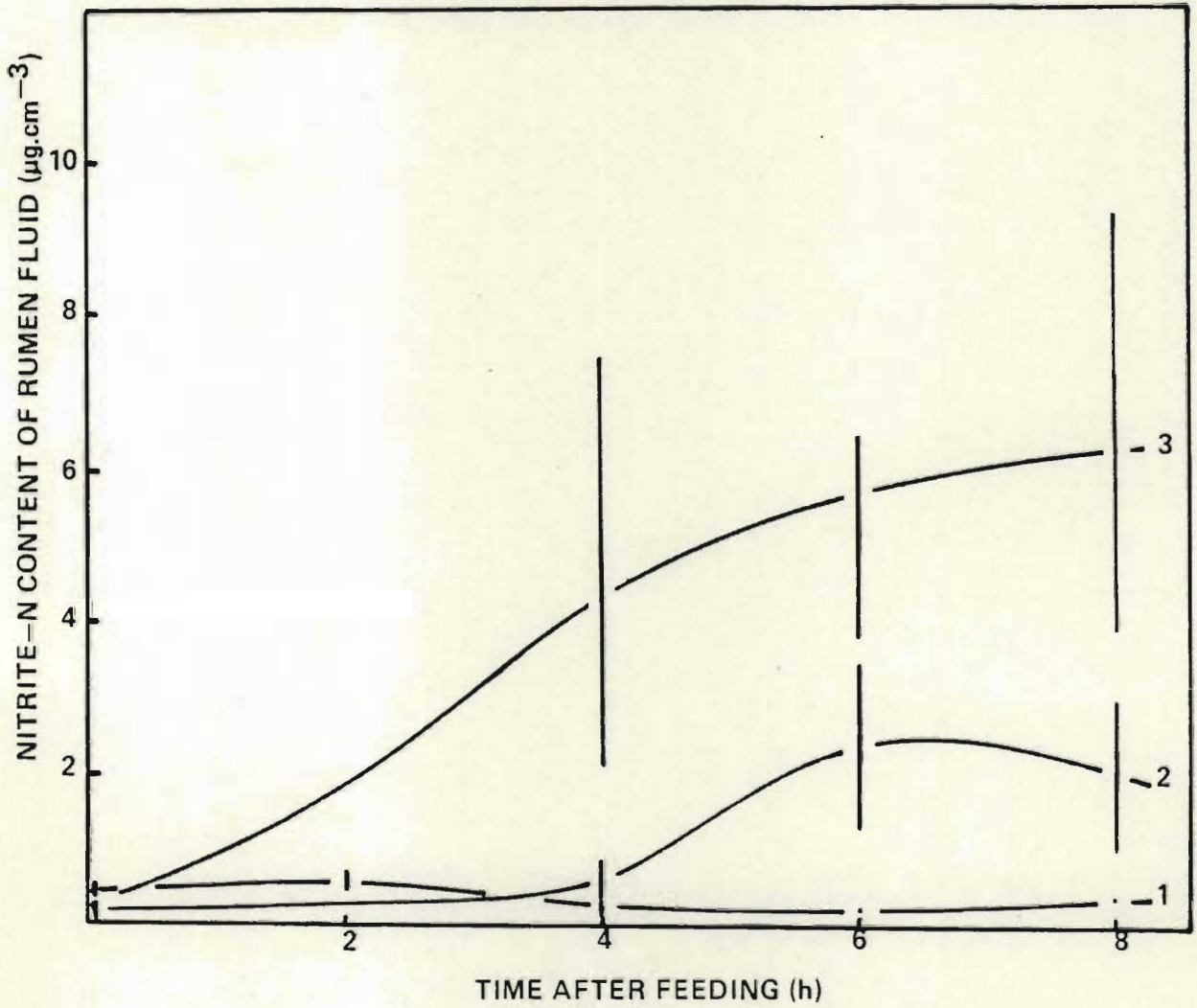


FIG. 3.7 Ability of the rumen microbes in non-adapted sheep to reduce nitrate to nitrite. (1) First day of feeding high nitrate (490 mg⁰/o N, m/m) kikuyu grass; (2) Third day of feeding high nitrate kikuyu grass; (3) Seventh day of feeding high nitrate kikuyu grass

considerable variation exists between sheep, a steady daily increase in the nitrite level occurs, changing from $0,4 \mu\text{g N.cm}^{-3}$ 8h after feeding on day one to $6,6 \mu\text{g N.cm}^{-3}$ for the same time on day seven.

These results are in agreement with the findings of Nakamura, Yoshida, Nakamura & Horie (1976), Nakamura, Tada, Shibuya, Yoshida & Nakamura (1979) and Nakamura, Tada, Saito, Yoshida & Nakamura (1981) who showed that during the first few days of high nitrate ($\pm 0,16\text{g N.Kg}^{-1}.\text{day}^{-1}$) feed intake by non-adapted sheep, the amount of nitrite formed in the rumen liquor was small and no symptoms of methaemoglobinaemia could be detected, but continued feeding of high nitrate feed over a period of seven days caused a gradual increase in the level of nitrite in the rumen. Allison & Reddy (1984) observed a similar initial increase in nitrite in the rumen fluid, but found that with a lower nitrate intake ($0,04\text{g N.Kg}^{-1}.\text{day}^{-1}$) the rumen microbes are able to adapt to completely metabolise both nitrate and nitrite within six days.

During the present investigation 20 month old Merino wethers were also kept on a high nitrate pasture for two months. After a further adaptation period in metabolic pens for 10 days on chopped high nitrate (550 mg\% N, m/m) kikuyu grass the daily fluctuation in nitrite content of the rumen fluid and the methaemoglobin content of the blood were determined. No nitrite could be detected in the rumen fluid and no

elevated levels of methaemoglobin in the blood could be observed.

These results suggest the rapid selection in the rumen digest of microbial groups capable of reducing nitrate and nitrite. Allison & Reddy (1984) recently showed that nitrate reducers comprised 18,2% of the total viable bacteria from nitrate adapted sheep compared to 1,8% in non-adapted sheep.

The introduction into the rumen of 4,5g of nitrate nitrogen in the form of potassium nitrate during the present investigation, considerably increased the nitrite level in the rumen (Fig.3.8). Results show an increase from $0 \mu\text{g.cm}^{-3}$ before feeding to over $20 \mu\text{g.cm}^{-3}$ 5h to 9h after feeding, indicating rapid conversion of nitrate to nitrite. After administration, the nitrate content decreased from $40 \mu\text{g.cm}^{-3}$, 5h after feeding, to $29 \mu\text{g.cm}^{-3}$, 9h after feeding.

The rumen seems to have a higher capacity to reduce nitrate than to reduce nitrite even in adapted or partly adapted sheep. The introduction into the rumen of a readily available form of nitrate nitrogen must have exceeded considerably the reduction capacity of the nitrite reducers, consequently leading to the accumulation of nitrite.

Analysis of methaemoglobin in the blood of the sheep on the high nitrate (490 mg% N, m/m) kikuyu grass only, was less than 0,2g in 100 cm^3 blood during the experimental period (0

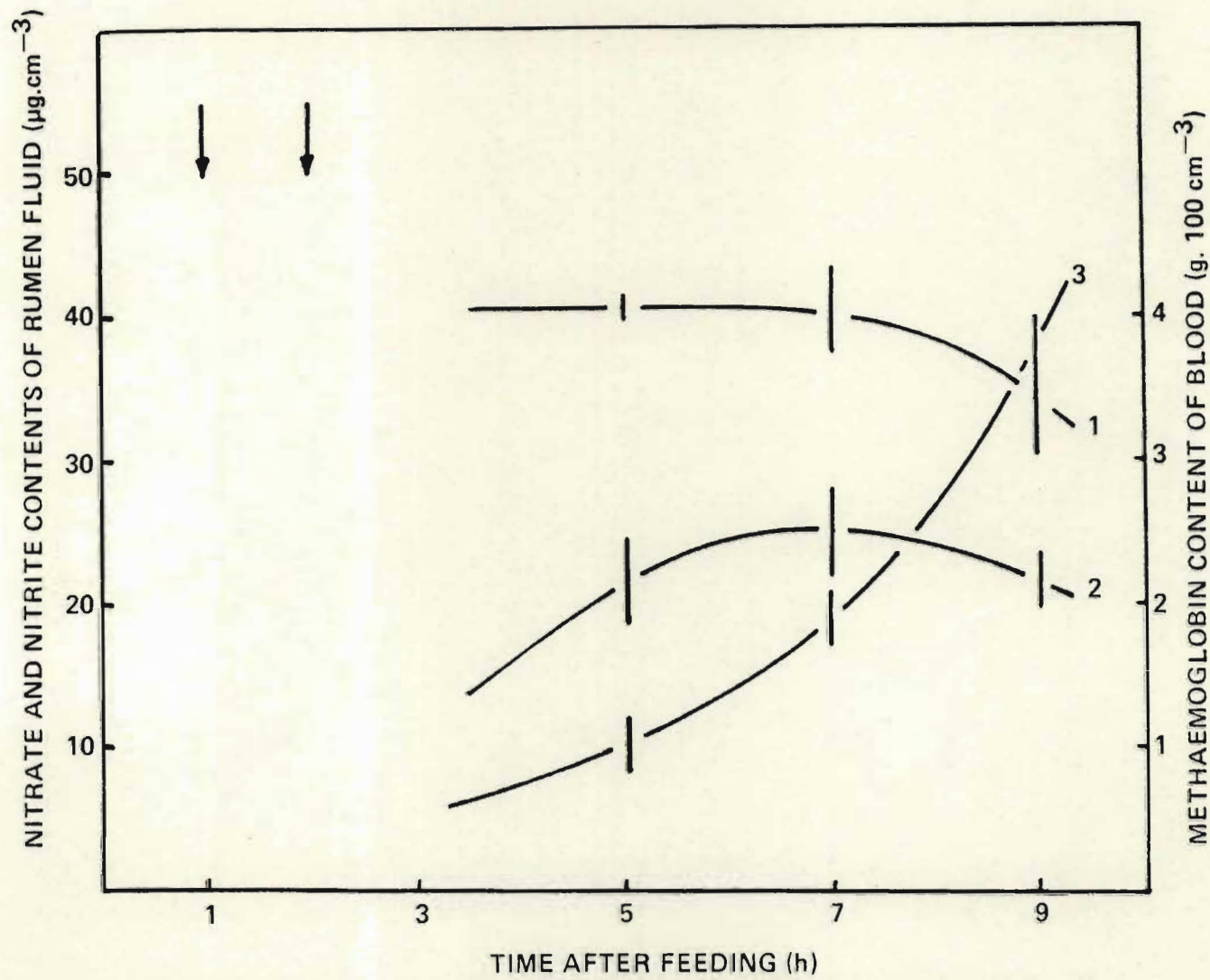


FIG. 3.8 Effect of high levels of nitrate upon nitrite formation in the rumen and upon methaemoglobin formation in the blood of sheep. (1) Nitrate; (2) Nitrite; (3) Methaemoglobin; Arrows indicate times of nitrate administration

to 8h after feeding), however, the additional introduction into the rumen of nitrate in the form of potassium nitrate resulted in a rapid increase in methaemoglobin in the blood from 1,0g in 100 cm³ blood 5h after feeding to 3,8g in 100 cm³ blood 9h after feeding (Fig.3.8), indicating rapid absorption of nitrite through the rumen wall into the blood. These animals suffered severely from methaemoglobinaemia but survived the treatment. In experiments where the methaemoglobin content in the blood rose to 5,9g in 100 cm³ blood, animals died rapidly. Similar results were obtained by Kemp, Geurink, Haalstra & Malestein (1977).

Available information, therefore, shows that the microbial population of sheep not adapted to nitrate in the diet can, within a week or two, increase their ability to metabolise both nitrate and nitrite due to a rapid increase in the numbers of nitrate metabolising microbes. The capacity to reduce nitrate seems to remain higher than the reduction capacity for nitrite. The accumulation of nitrite, therefore, depends upon the rate of release of nitrate into the rumen and the reduction capacity of the nitrite reducers. The release of nitrate from kikuyu grass seems to be relatively slow. Kikuyu grass with a nitrate content of about 500 mg% fed to nitrate adapted sheep does not lead to the accumulation of nitrate in the rumen. The further introduction into the rumen of nitrate in a readily available form, exceeded the reduction capacity of the nitrite reducers and led to the accumulation of nitrite.

3.3 Effect of nitrite upon the activity of rumen carbohydrases

3.3.1 Effect of nitrite upon cellulose and starch digestion in vitro

Digestion of a feed sample involves primarily the solubilisation of the structural carbohydrate components of the sample. It is, therefore, assumed that the observed effect of nitrite upon the dry matter digestibility of the grass, is caused by a reduction in the hydrolysis of cellulose and hemicellulose in the sample. Support for this assumption is given by Hall, Gaddy & Hobbs (1960) who showed that the digestion in vitro of pure cellulose (Solca-floc) by washed suspensions of rumen microbes is reduced by high levels of nitrate.

An experiment was conducted to determine the effect of nitrite, formed during digestion, upon the rate of cellulose and starch digestion in vitro. Whatman No.1 filter paper, ground to pass a 1mm sieve, and starch (Merck) were used as substrate. Nitrate (500 mg% N, m/m) was added to half of the samples, while the remaining samples were supplemented with an equivalent amount of nitrogen in the form of urea. The rate of digestion was followed over a period of 72h.

Results (Fig. 3.9) show that nitrite, formed during

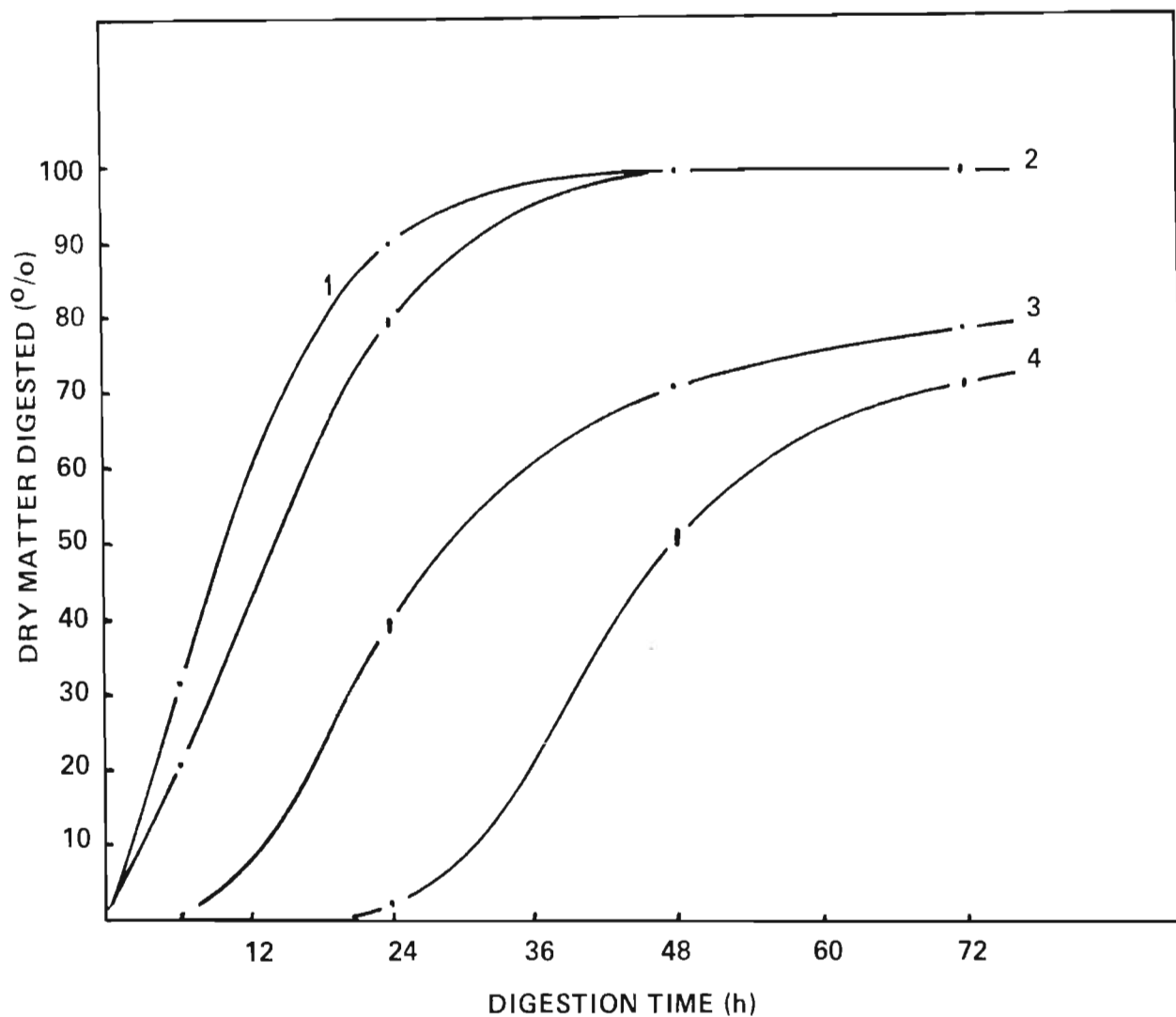


FIG. 3.9 Effect of nitrite upon microgranular cellulose powder (Whatman CC31) and maize starch (BDH) digestion in vitro. Digestion procedure as for kikuyu grass samples. (1) Starch + urea (500 mg% N, m/m); (2) Starch and nitrate (500 mg% N, m/m); (3) Cellulose + urea (500 mg% N, m/m); (4) Cellulose + nitrate (500 mg% N, m/m)

fermentation, does not only affect cellulose digestion, but also suppresses the digestion of starch. In the presence of nitrate, cellulose digestion commences only after about 24h of digestion, the time required for the complete metabolism of nitrite.

3.3.2 Studies on the isolation of cellulases from a cellulose digest in vitro

A decrease in digestibility of cellulose in the presence of nitrite, is due to a reduction in either the activity of the cellulase(s) or to the amount of cellulase(s) present in the digest. An investigation of these possibilities would be facilitated by the availability of pure cellulase preparations isolated from the in vitro digests. However, Francis, Gawthorne & Storer (1978) showed that 90% of the cellulase activity in whole rumen contents, is adsorbed onto the plant material being digested, while 7% is associated with the bacteria and a further 3% is located in the cell-free rumen fluid.

These workers succeeded in extracting some activity from the squeezed rumen solids by means of 20m mol.dm^{-3} sodium phosphate buffer at pH 6,9, while Gawthorne (1979) used 50 m mol.dm^{-3} phosphate as extractant at the same pH. Krishnamurti & Kitts (1969), in turn, recommend 100 m mol.dm^{-3} acetate buffer at pH 5,5 for extraction. In a previous study conducted in this laboratory on the influence of ruminant

salivary buffer salts upon the in vitro microbial digestion of forages (Dennison & Marais, 1980), it was shown that both very low buffer concentrations (below 50% of artificial saliva buffer) and very high concentrations (above 125% artificial saliva Buffer) cause a rapid decline in the digestion of grasses. It was tentatively concluded that the observed effect might be a consequence of an effect of ionic strength on the binding of cellulases to cellulose. It was subsequently established that the observed effect was indeed an effect of the buffer upon cellulase activity. If the low activity is further due to the inability of the enzyme to bind to the substrate at low ionic strengths, solutes such as water could possibly be used to isolate cellulases from the solid substrate. To test this hypothesis, cellulose powder (Whatman, Microgranular CC31)(100g) and ground filter paper (Whatman No.1)(100g) were digested in vitro (5 dm^{-3} final volume) for a period of 28h. The digest was centrifuged ($2250 \times g$, 10 min) and the supernatant was discarded. The cellulose residue was extracted initially with $50 \text{ mmol} \cdot \text{dm}^{-3}$ phosphate buffer, pH 6,0 ($4 \times 250 \text{ cm}^3$) followed by extraction with distilled water ($4 \times 250 \text{ cm}^3$). The combined extracts were dialysed (Visking, 2°C , 48h against dist. H_2O) and freeze dried. The activity of the crude enzyme extracts and the activity remaining on the cellulose residue were determined as described in 3.1.6. Results are presented in Table 3.7.

TABLE 3.7 Extraction of cellulases from digests of pure cellulose filter paper and Whatman microgranular cellulose powder (C31) by means of phosphate buffer (50 m mol. dm^{-3} , pH 6,0) and distilled water. Activity expressed as μ moles glucose released per minute from CM-cellulose substrate by the total enzyme extract or residue (See 3.1.6)

Fraction	Cellulose powder		Filter paper	
	Activity (μ mol. min $^{-1}$)	Activity (%)	Activity (μ mol.min $^{-1}$)	Activity (%)
Buffer extract	141 \pm 8	7,2	57 \pm 4	0,7
Water extract	184 \pm 1	9,4	65 \pm 4	0,9
Cellulose residue	1 643 \pm 79	83,5	7 246 \pm 199	98,3

Results show that only 7,2% and 0,7% of the total activity in the residual cellulose powder and filter paper respectively are extracted with 50 m mol. dm^{-3} phosphate buffer. Subsequent water extraction removes a further 9,4% and 0,9% of the activity of the cellulose powder and filter paper respectively. Water, therefore, seems to be a better extractant of cellulases than phosphate buffer, but the bulk of the activity (>80%) remains adsorbed onto the insoluble substrate.

The higher concentration extracted from the cellulose powder can possibly be attributed to the finer consistency of the cellulose powder. The enzyme extracted also seems to be

particle bound. Protein analysis (Kjeldahl nitrogen x 6,25) and readily hydrolysable carbohydrate analysis (reducing sugars released after hydrolysis with sulphuric acid ($0,05 \text{ mol.dm}^{-3}$, 100°C , 150 min) were done on the crude enzyme obtained by water extraction of the cellulose powder digest. Results show that the crude enzyme consists of only about 43% protein and approximately 11% readily digestible carbohydrates.

Attempts were made to purify the crude enzyme extracted from the cellulose by means of ammonium sulphate precipitation. Only 70% of the enzyme mass could be recovered after precipitation and the recovered enzyme exhibited only 43% of the activity of the original crude extract.

The pH optimum for cellulase activity of the crude enzyme was determined over the range pH 5,0 to pH 7,0 using a sodium phosphate buffer (50 m mol.dm^{-3}) over the range, pH 6,5 to 7,0, and a sodium acetate buffer (50 m mol.dm^{-3}) for pH values below 6,5.

Results (Fig 3.10) indicate an optimum cellulase activity at pH 6,0. Considerable variation in pH for optimum cellulase activity has been reported in the literature. According to Gawthorne (1979) the carbohydrase complex extracted from a sheep rumen digest by means of phosphate buffer (50 m mol.dm^{-3} , pH 6,9) has a pH optimum towards native cellulose of 6,9 at 49°C . Leatherwood (1965) reported a pH optimum

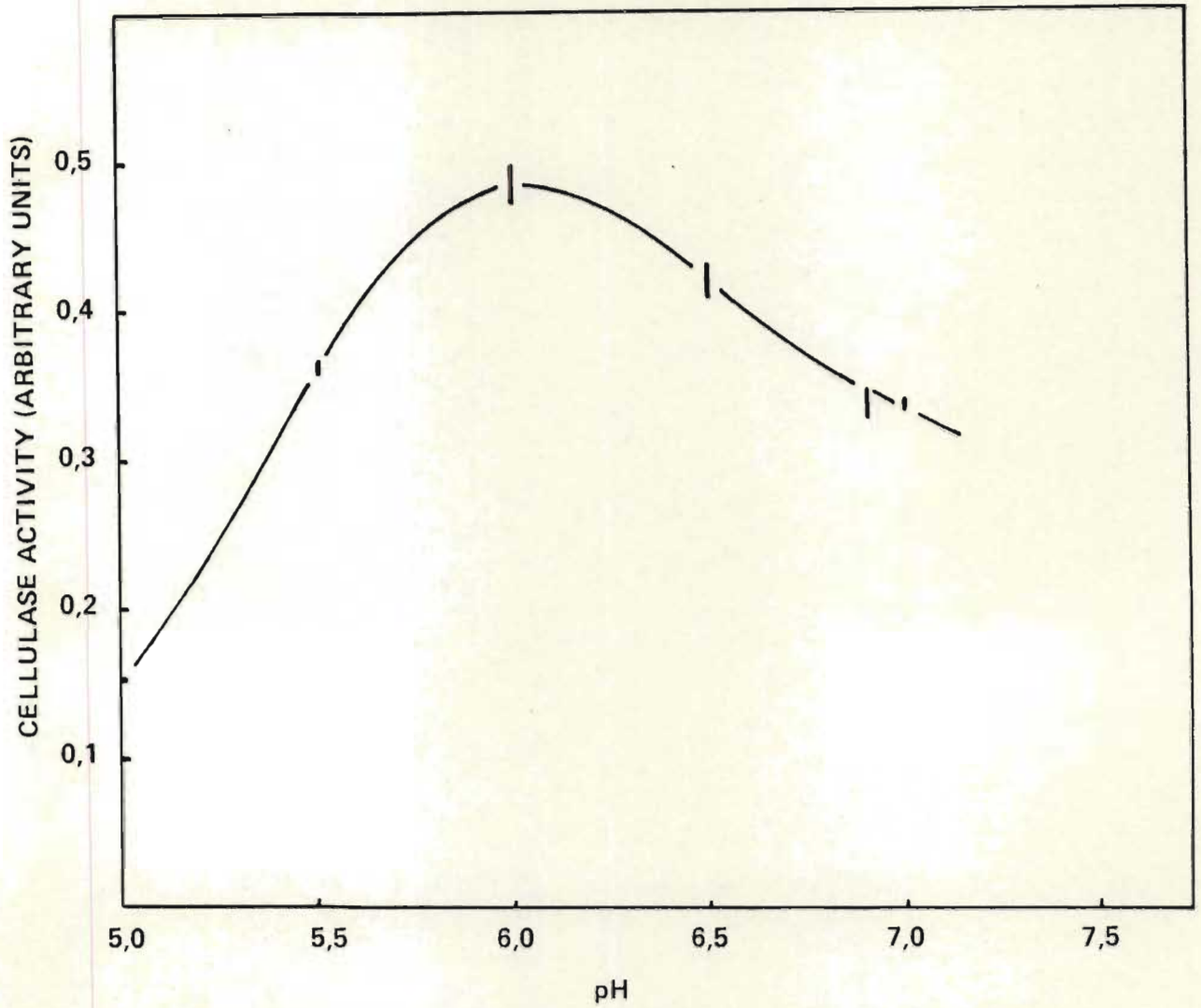


FIG. 3.10 Optimum pH for rumen cellulase activity. Substrate, acid swollen cellulose; pH 5.0–6.0, acetate buffer (50m mol.dm^{-3}); pH 6.5–7.0, phosphate buffer (50m mol.dm^{-3}); 39°C

between 5,5 and 6,0 for the cellulases produced by Ruminococcus albus, as measured by changes in viscosity of carboxymethylcellulose, and values ranging between pH 5,3 and 6,8 if the assay is based on reducing sugars produced from carboxymethylcellulose or swollen cellulose. Gill & King (1957) obtained optimum activity of free cellulases in rumen fluid at pH 6,5 if measured as changes in viscosity of carboxymethylcellulose.

The observed differences in pH optimum reported by different authors, are likely due to the relative abundance of different components of the cellulase complex present in the digest, the nature of the substrate used in the assay and whether viscosity or reducing power is used as the criterion of activity.

In addition to cellulases the crude enzyme preparation may also contain other enzymes. Gawthorne (1979) showed that the phosphate buffer extracted cellulase exists as a complex with a relative molecular mass of 400000. He subsequently showed that apart from cellulase, xylanase and β -glucosidase, the complex also exhibits amylase, β -galactosidase, β -glucuronidase, sucrase, alkaline phosphatase, lipase and esterase activity (J.M. Gawthorne, pers. comm.*).

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Veterinary Science, Murdoch University,
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Attempts in this laboratory to separate the different enzymes in the complex by means of gel filtration and polyacrylamide gel electrophoresis (PAGE) were unsuccessful due to the refractory rheological properties of the concentrated carbohydrase complex, preventing the applied sample from penetrating the gel in the column. Isoelectric focusing on BioGel P-150, however, seemed to show some promise for the isolation of the different enzyme moieties in the complex. The crude enzyme preparation was subjected to preparative isoelectric focusing using an ampholine range of pH 3,0 to pH 10,0 as described in 3.1.9. The activity of cellulase, xylanase and β -glucosidase was determined on the different gel fractions and the results are presented in Fig. 3.11. Most of the activity of all three enzymes was concentrated in the pI range of 4,1 to 5,2.

Attempts were made to further resolve these peaks by separating the crude enzyme in a gel with ampholine ranging from pH 3,5 to 5,0. Results (Fig. 3.12) show a slight improvement on the separation over a wider pH range, but it became clear that complete isolation of the enzymes using isoelectric focusing would be unlikely.

In the absence of a successful isolation technique, the absorptive properties of the enzyme complex was exploited by considering the washed grass residue after digestion as the crude cellulase enzyme in subsequent studies to establish the

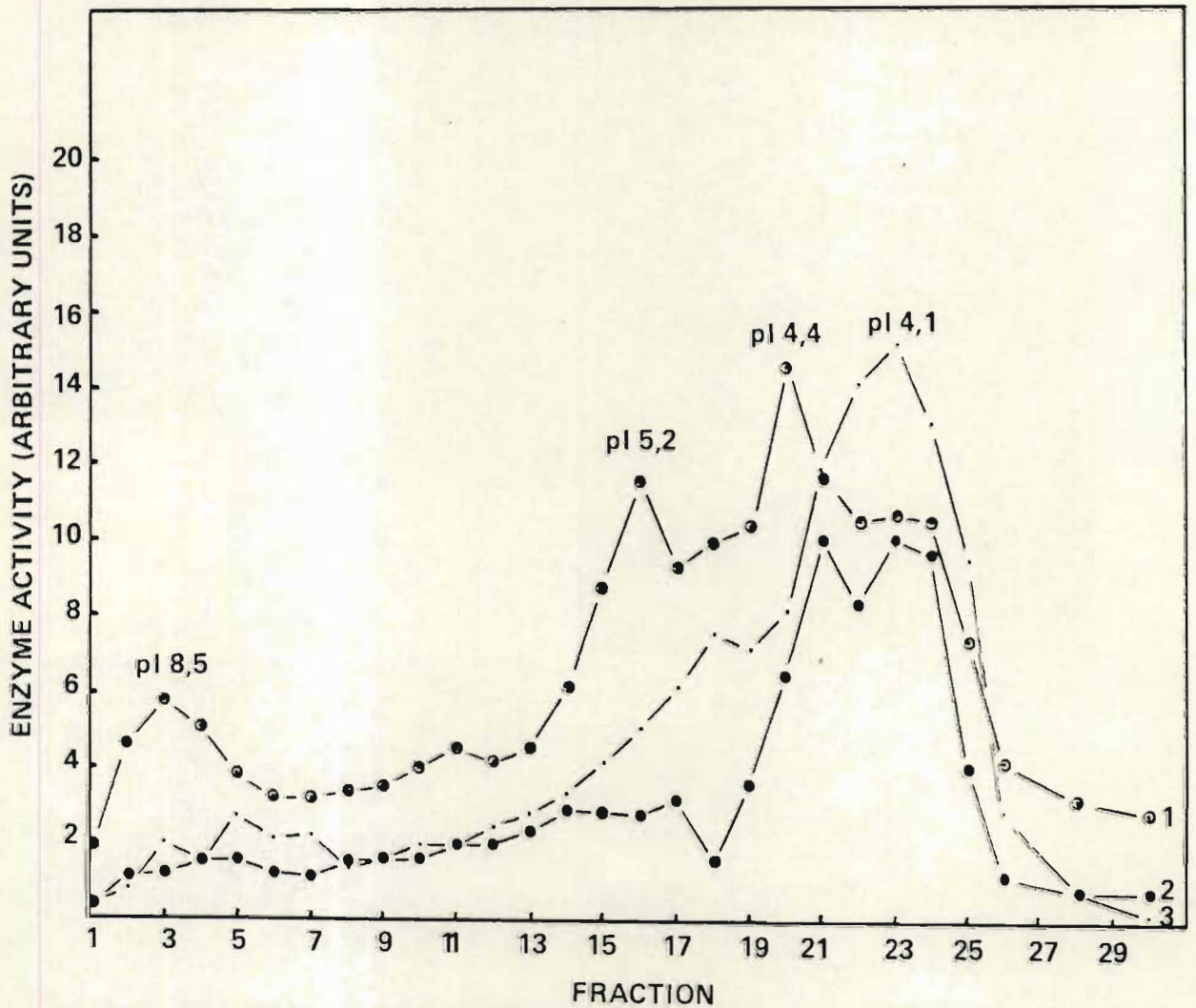


FIG. 3.11 Preparative isoelectric focusing (pH range 3–10) on Bio Gel P–150 of the hydrolytic enzymes from a water extract of the rumen microbial digest of filter paper cellulose. (1) Xylanase; (2) β -glucosidase; (3) Cellulase

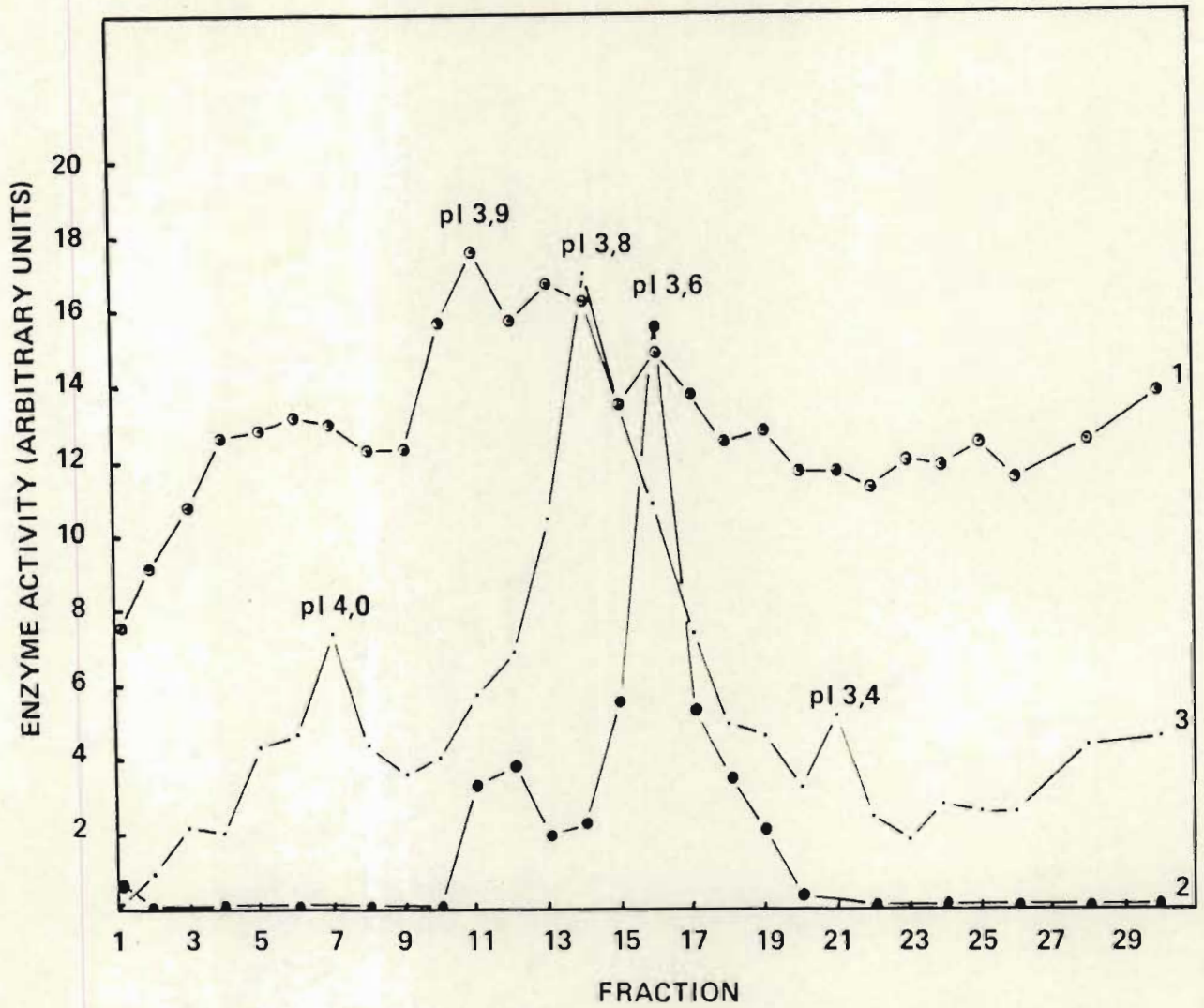


FIG. 3.12 Preparative isoelectric focusing (pH range 3.5–5.0) on Bio Gel P-150 of the hydrolytic enzymes from a water extract of the rumen microbial digest of filter paper cellulose. (1) Xylanase; (2) β -glucosidase; (3) Cellulase

mode of action of nitrite upon cellulose digestion.

3.3.3 Effect of nitrate, nitrite and ammonia upon the activity of the crude cellulase preparation.

In order to determine whether nitrite possibly directly affects cellulase activity, the activity in the washed grass residue after digestion in vitro was determined in the absence and presence of $40 \mu\text{g. cm}^{-3}$ nitrite nitrogen. The effect of nitrite was compared with that of nitrate and ammonia. (Table 3.8).

TABLE 3.8 Effect of nitrite, nitrate and ammonia upon cellulase activity.
Nitrate and nitrite added as potassium salts and ammonia as ammonium sulphate

Nitrogenous substance ($40 \mu\text{g N.cm}^{-3}$)	Cellulase activity * ($\mu\text{mol.mg}^{-1}\text{.min}^{-1}$)
Nitrate	$0,46 \pm 0,01$
Nitrite	$0,45 \pm 0,02$
Ammonia	$0,43 \pm 0,02$
Control	$0,45 \pm 0,02$

* Mean of 6 determinations

From the results presented in Table 3.8 it is clear that neither nitrite, nitrate or ammonia has any direct effect upon cellulase activity.

3.3.4 End product inhibition of rumen cellulases

The end products of cellulose digestion are inhibitors of cellulase activity. Smith, Yu & Hungate (1973) showed that concentrations of cellobiose as low as 0,01% in the ambient medium are inhibitory and that concentrations of 1,0% almost completely inhibit the cellulase activity of Ruminococcus albus, although not affecting enzyme synthesis. Glucose, in turn, is generally less inhibitory, but 0,5% in the medium reduces cellulase activity by 61%. Under normal circumstances these sugars are readily metabolised by the rumen microbial population and do not accumulate in the digest (Hungate, 1966).

Enzymes produced by anaerobes are generally more susceptible to inactivation under aerobic conditions than those of aerobic bacteria (Morris, 1975). Groleau & Forsberg (1981) and Forsberg & Groleau (1982) showed that while the cellulase produced by Bacteroides succinogenes is particularly stable and retains its activity even during prolonged periods of incubation at 39°C, β -glucosidase is readily inactivated, especially on exposure to air or to merthiolate, a sulphydryl inhibitor. Loss of activity can be prevented by the addition of dithiothreitol (which protects reduced sulphydryl groups) in an anaerobic atmosphere. In the presence of air the protective effect of dithiothreitol is only transient, possibly due to the dissipation of its reducing power on exposure to oxygen.

The possibility therefore exists that nitrite, which is an oxidising agent, could exert its effect on cellulase activity by inhibiting enzymes sensitive to oxidation, such as β -glucosidase, thus favouring the accumulation of inhibitory end products of cellulose digestion. In order to check this hypothesis, kikuyu grass containing low (100 mg%, m/m) and high (600 mg%, m/m) levels of nitrate-N was subjected to digestion in vitro for 20h. The digest was centrifuged and a sample (95 cm³) of the supernatant was clarified with barium hydroxide and zinc sulphate solutions ($\pm 0,36 \text{ mol.dm}^{-3}$) in equivalent proportions to remove components of large molecular mass. A sample (15 cm³) of the clarified sample was electrodialysed to remove charged substances of low molecular mass (Macpherson, 1946). The electrodialysed sample containing the neutral sugar fraction was freeze-dried and re-dissolved in a small volume (1,0 cm³) of water. The sugar extract was separated by means of descending paper chromatography using ethyl acetate-acetic acid - water (9:2:2) as solvent. Spots were revealed by spraying the chromatogram with an aniline phosphate reagent (Marais, 1966) and heating the chromatogram in an oven for 15 min at 100°C.

No glucose or cellobiose could be detected on the chromatogram of the low and high nitrate digests indicating the rapid metabolism of simple sugars by the microbial population, even in the presence of nitrite. End product inhibition of cellulase activity in digests containing

nitrite, therefore, does not seem to be a factor affecting cellulase activity. The possibility, however, exists that the oxidising properties of nitrite could be responsible for the inactivation of other enzyme systems essential for microbial growth.

3.4 Effect of nitrite upon the rumen microbial population

3.4.1 Effect of nitrite upon microbial growth and carbohydrase production

Nitrite has been widely used as an additive in the meat processing industry (Perigo & Roberts, 1968), particularly as a growth inhibitor of Clostridium perfringens and Clostridium botulinum, but little is known of its effect upon the rumen microbial population.

To study its effect on rumen microbes, low nitrate (100 mg% N, m/m) and high nitrate (600 mg% N, m/m) kikuyu grass samples were subjected to digestion in vitro and the change in microbial numbers followed over a period of 24h. By making use of selective growth media (3.1.11) a distinction could be drawn between cellulolytic, xylanolytic and total microbial populations. After removal of samples for microbial counts and for the analysis of nitrite content, the digest was centrifuged (2250 x g, 10 min, 4°C) and the supernatant discarded. The grass residue was washed twice with cold

distilled water and centrifuged, followed by freeze-drying of the residue. The cellulase and xylanase activity of the residue were determined as described in 3.1.6 and 3.1.7.

The change in total viable counts over the 24h digestion period is given in Fig. 3.13. Results show a rapid initial increase in counts reaching peak values after 8h, followed by a gradual decrease as the substrate becomes growth limiting. In the low nitrate grass microbial numbers peak at about $210 \times 10^9 \cdot \text{cm}^{-3}$ and in the high nitrate grass at $90 \times 10^9 \cdot \text{cm}^{-3}$, a reduction of 57%. In this experiment nitrite peaked after about 8h at a level of approximately $10 \mu\text{g} \cdot \text{cm}^{-3}$.

The change in xylanolytic counts over the digestion period is shown in Fig. 3.14. The xylanolytic bacterial numbers increase rapidly, peak after a digestion period of 8h and then decrease as substrate limits growth. In the low nitrate grass xylanolytic counts peak at almost $50 \times 10^8 \cdot \text{cm}^{-3}$. High nitrate levels cause an almost 30% decrease in xylanolytic count after 8h digestion.

The xylanase activity of the digest is given in Fig. 3.15. These results show a gradual increase in enzyme activity in the low nitrate digest from an initial value of $0,79 \mu \text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ to approximately $4,0 \mu \text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ after 24h. The xylanase activity of the high nitrate digest generally follows a similar pattern, but the activity is considerably lower. The biggest difference ($\pm 43\%$) in xylanase

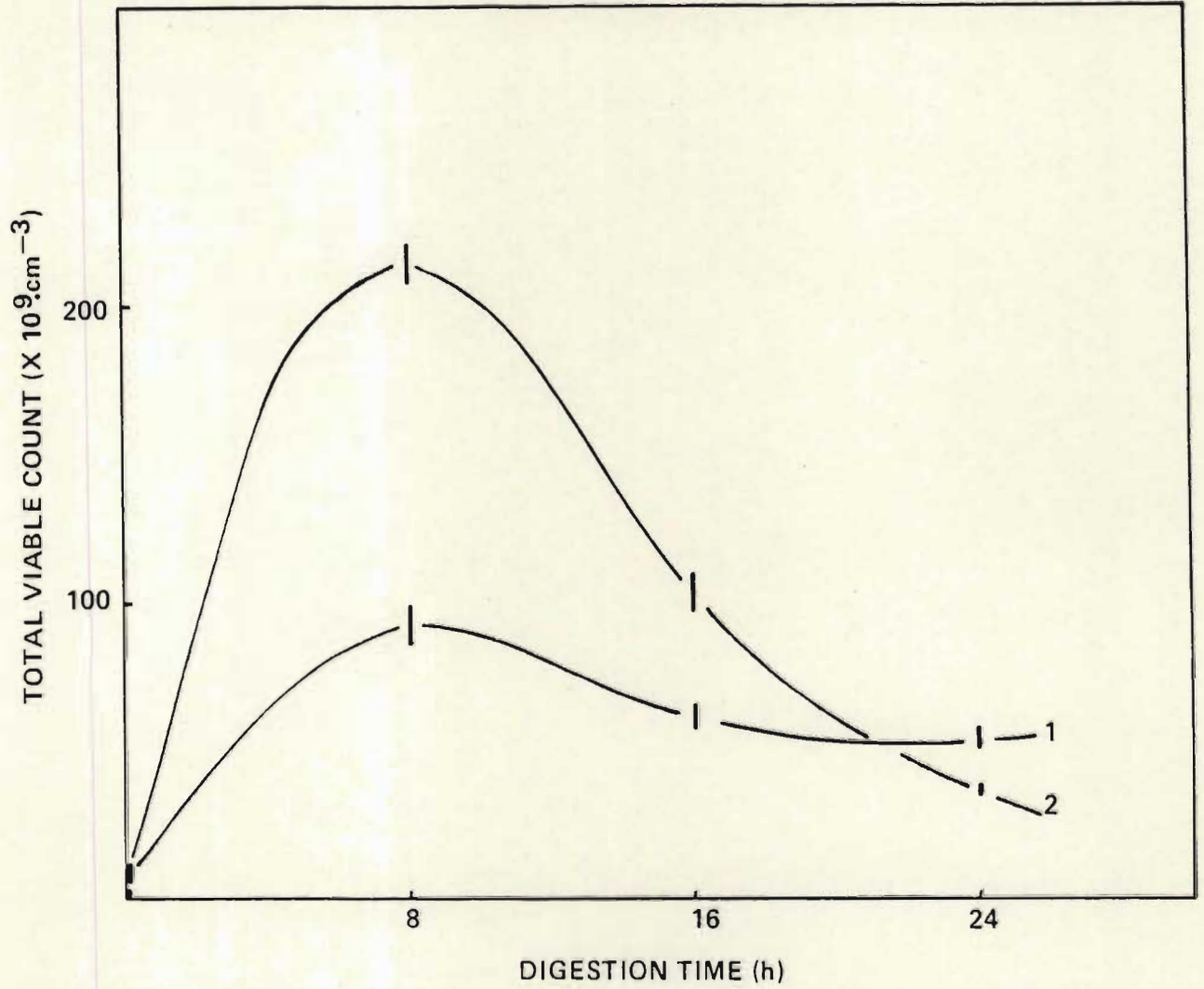


FIG. 3.13 Effect of nitrite upon the total viable bacterial count during digestion *in vitro*.
 (1) High nitrate kikuyu grass (600 mg⁰/o N, m/m); (2) Low nitrate kikuyu grass (100 mg⁰/o N, m/m)

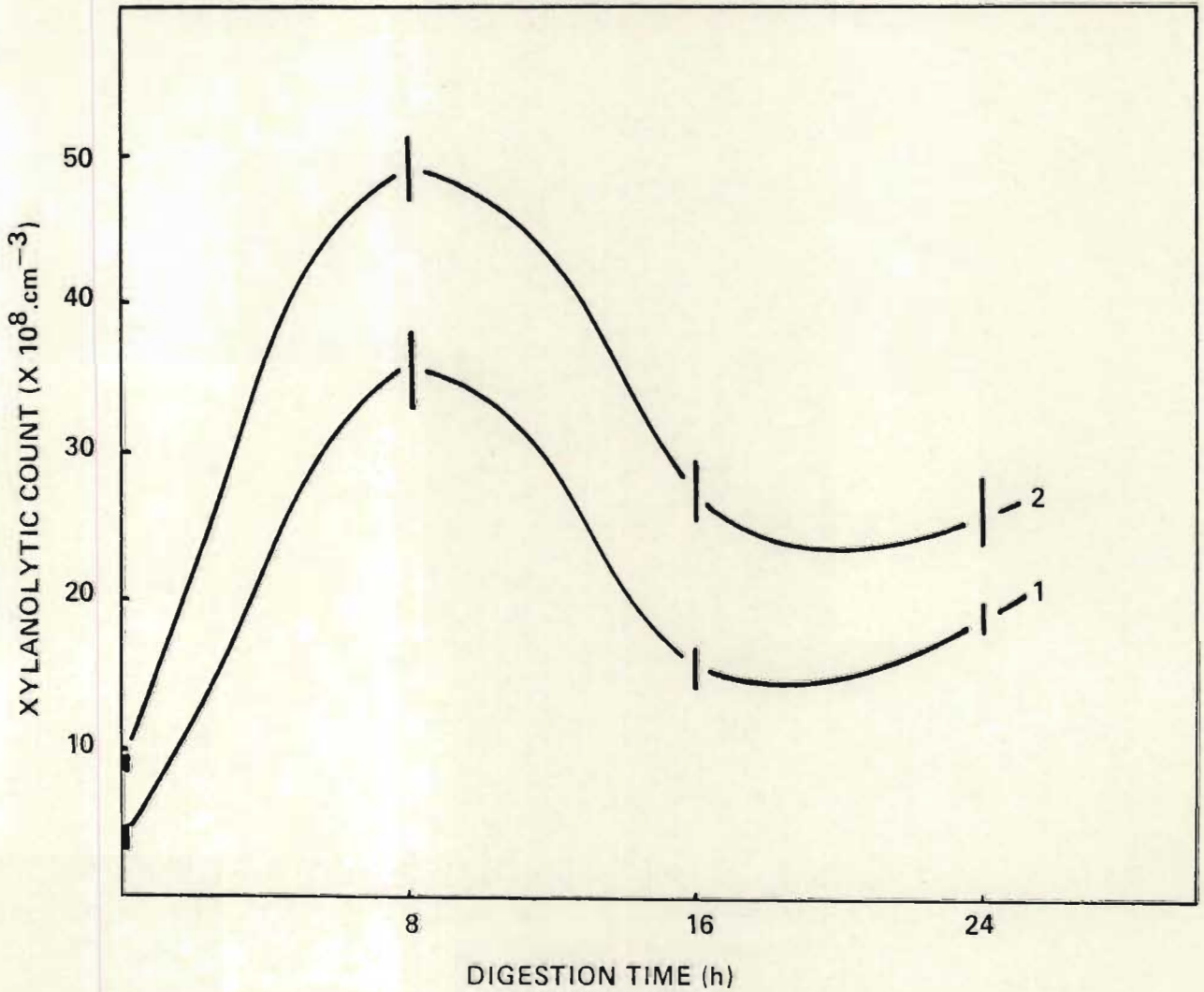


FIG. 3.14 Effect of nitrite upon the xylanolytic bacterial count during digestion *in vitro*.
(1) High nitrate kikuyu grass (600 mg⁰/o N, m/m); (2) Low nitrate kikuyu grass (100 mg⁰/o N, m/m)

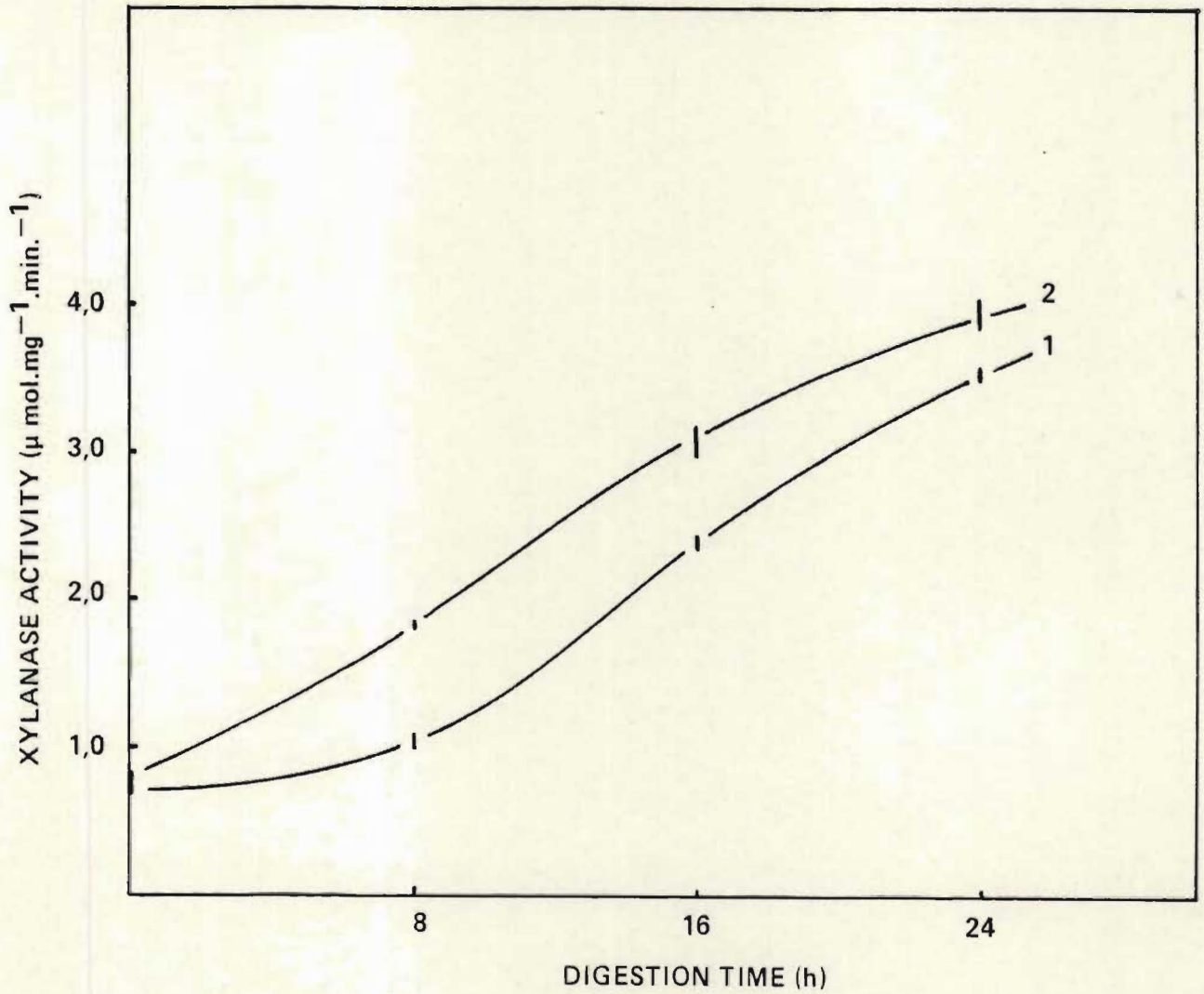


FIG. 3.15 Effect of nitrite upon the xylanase activity of the digest. (1) High nitrate kikuyu grass (600 mg⁰/o N, m/m); (2) Low nitrate kikuyu grass (100 mg⁰/o N, m/m)

activity between the high and low nitrate digest occurs after 8h digestion and corresponds with peak values in nitrite content.

Results presented in Fig. 3.16 show the decrease in xylanolytic microbial count and xylanase activity in the presence of nitrate over the digestion period. The decrease in xylanase activity largely parallels the decrease in numbers of xylanolytic microbes. Furthermore, it was found that the percentage decrease in enzyme activity over the digestion period was smaller than the percentage decrease in microbial numbers. These results suggest that nitrite affects microbial growth but does not directly affect xylanase synthesis.

Fig. 3.17 shows the change in cellulolytic counts in the high and low nitrate digest. In the low nitrate digest the cellulolytic microbes increase from an initial population of about $30 \times 10^4 \text{ cm}^{-3}$ to about $50 \times 10^4 \text{ cm}^{-3}$ after approximately 8h and then decrease gradually. In the high nitrate digest the cellulolytic counts drop immediately to about $18 \times 10^4 \text{ cm}^{-3}$ after 8h digestion, followed by a slow increase in numbers. The immediate drop in microbial numbers of the high nitrate digest, giving a count 64% lower than the low nitrate digest at 8h digestion, indicates a much higher sensitivity to nitrite of the cellulolytic microbes than of the xylanolytic microbes. Since it is generally assumed that most cellulolytic microbes also degrade xylan (Morris & Van

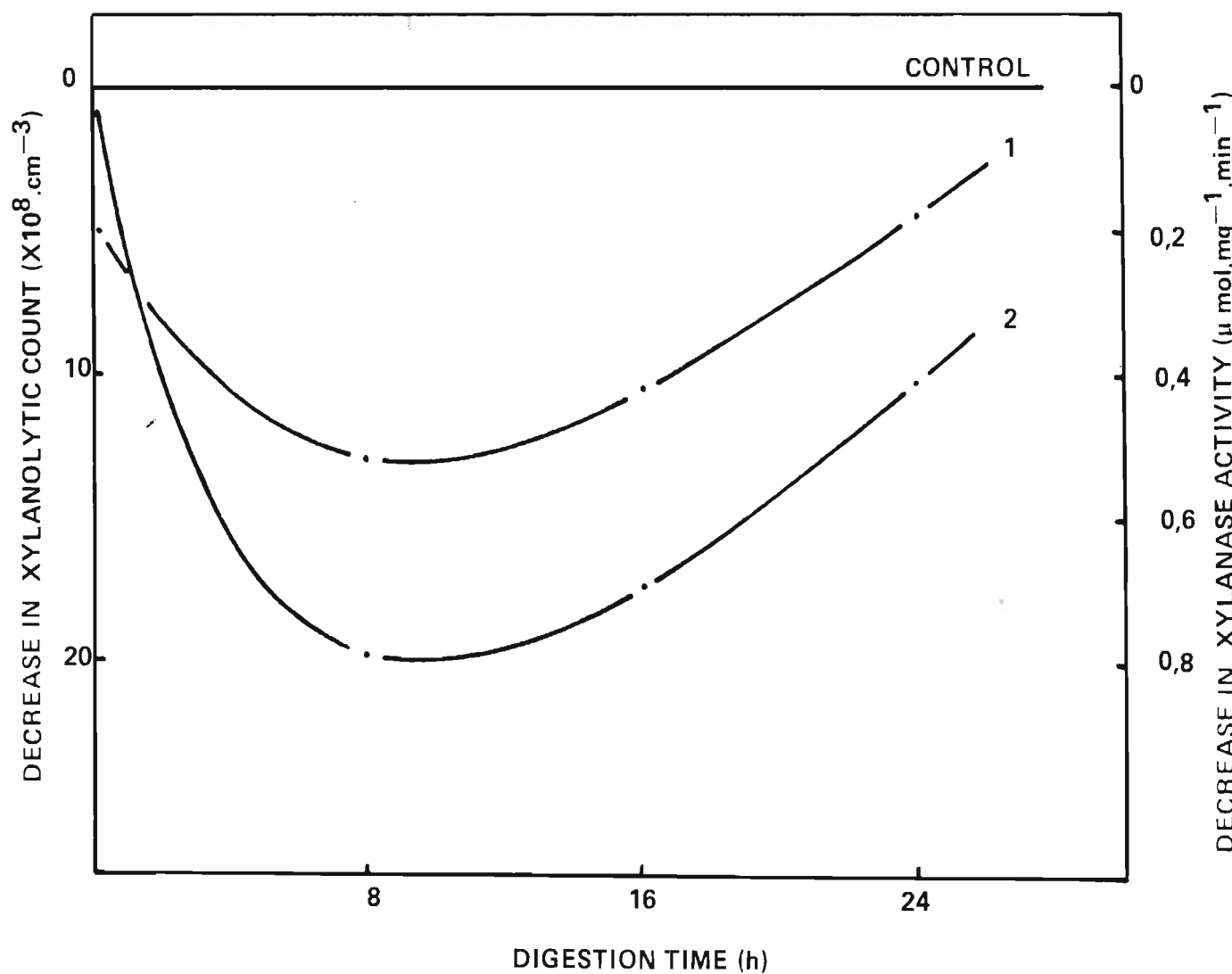


FIG. 3.16 Change in xylanolytic microbes and xylanase activity of a high nitrate kikuyu grass digest (600 mg% N, m/m) over the digestion period, relative to the low nitrate control digest (100 mg% N, m/m). (1) Xylanolytic count; (2) Xylanase activity

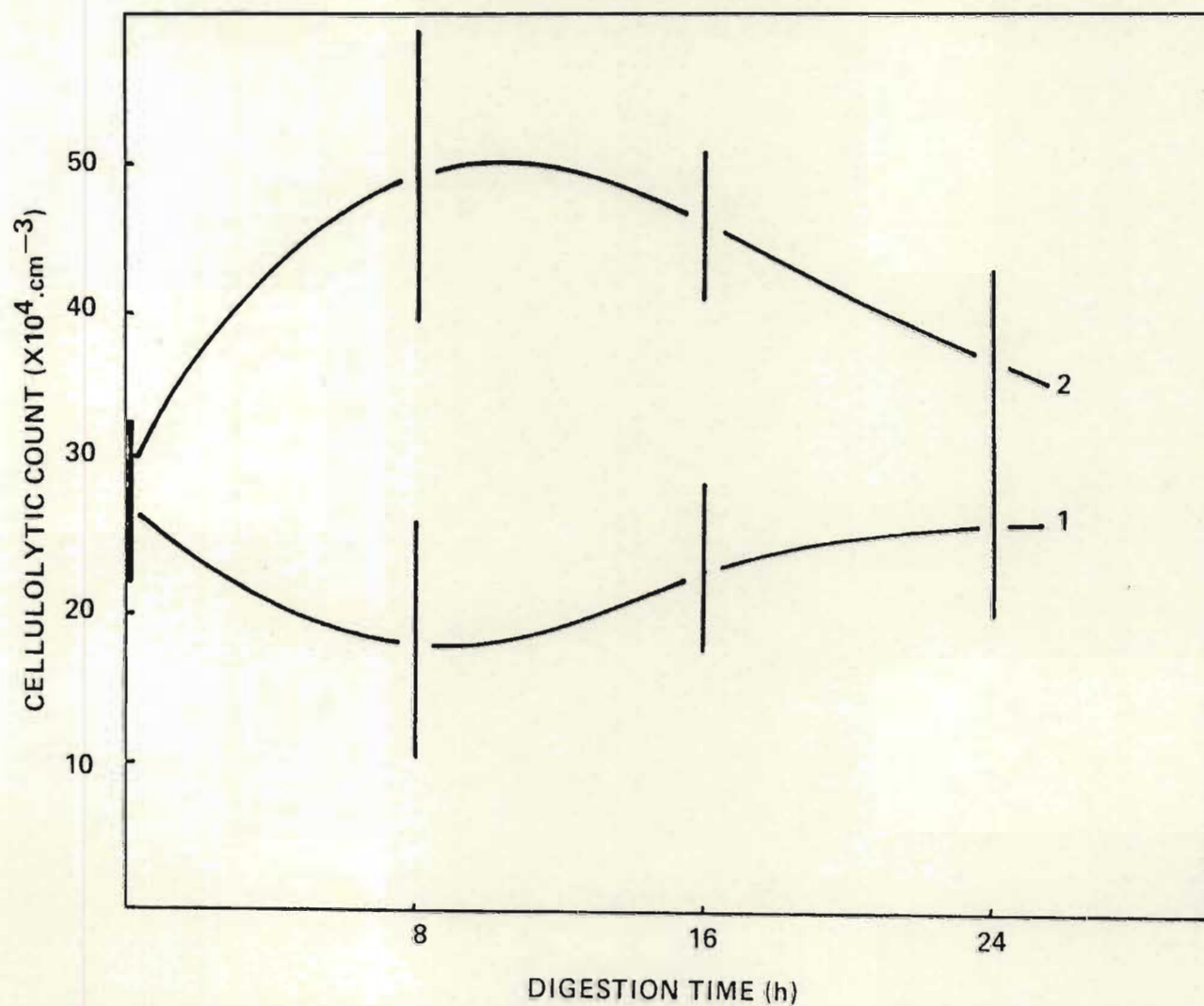


FIG. 3.17 Effect of nitrite upon the cellulolytic count during digestion *in vitro*. (1) High nitrate kikuyu grass (600 mg^O/o N, m/m); (2) Low nitrate kikuyu grass (100 mg^O/o N, m/m)

Gylswyk, 1980), these results seem to suggest that the mainly hemicellulolytic species, such as Butyrivibrio fibrisolvens could be less sensitive to nitrite than the predominant cellulolytic species in the digest.

The change in cellulase activity during digestion is presented in Fig. 3.18. The activity of the low nitrate digest increases from an initial value of about $0,25 \mu \text{mol.mg}^{-1}.\text{min}^{-1}$ to almost $0,55 \mu \text{mol.mg}^{-1}.\text{min}^{-1}$ after 16h and then remains constant. The activity of the high nitrate digest remains virtually constant during the first 8h of digestion while nitrite is present, then slowly increases to almost $0,4 \mu \text{mol.mg}^{-1}.\text{min}^{-1}$ after 16h digestion and remains constant afterwards.

Results presented in Fig. 3.19 show the decrease in cellulolytic microbial count and cellulase activity in the presence of nitrate over the digestion period. The decrease in cellulolytic microbes is associated with a similar initial decrease in cellulase activity. However, after 8h digestion the cellulase activity in the presence of nitrite relative to that of the control remains constant. Interpretation of these results is complicated by the large standard deviation of analyses. The percentage decrease in cellulase activity was found to be smaller than the percentage decrease in the number of cellulolytic microbes, indicating no direct effect of nitrite upon cellulase synthesis.

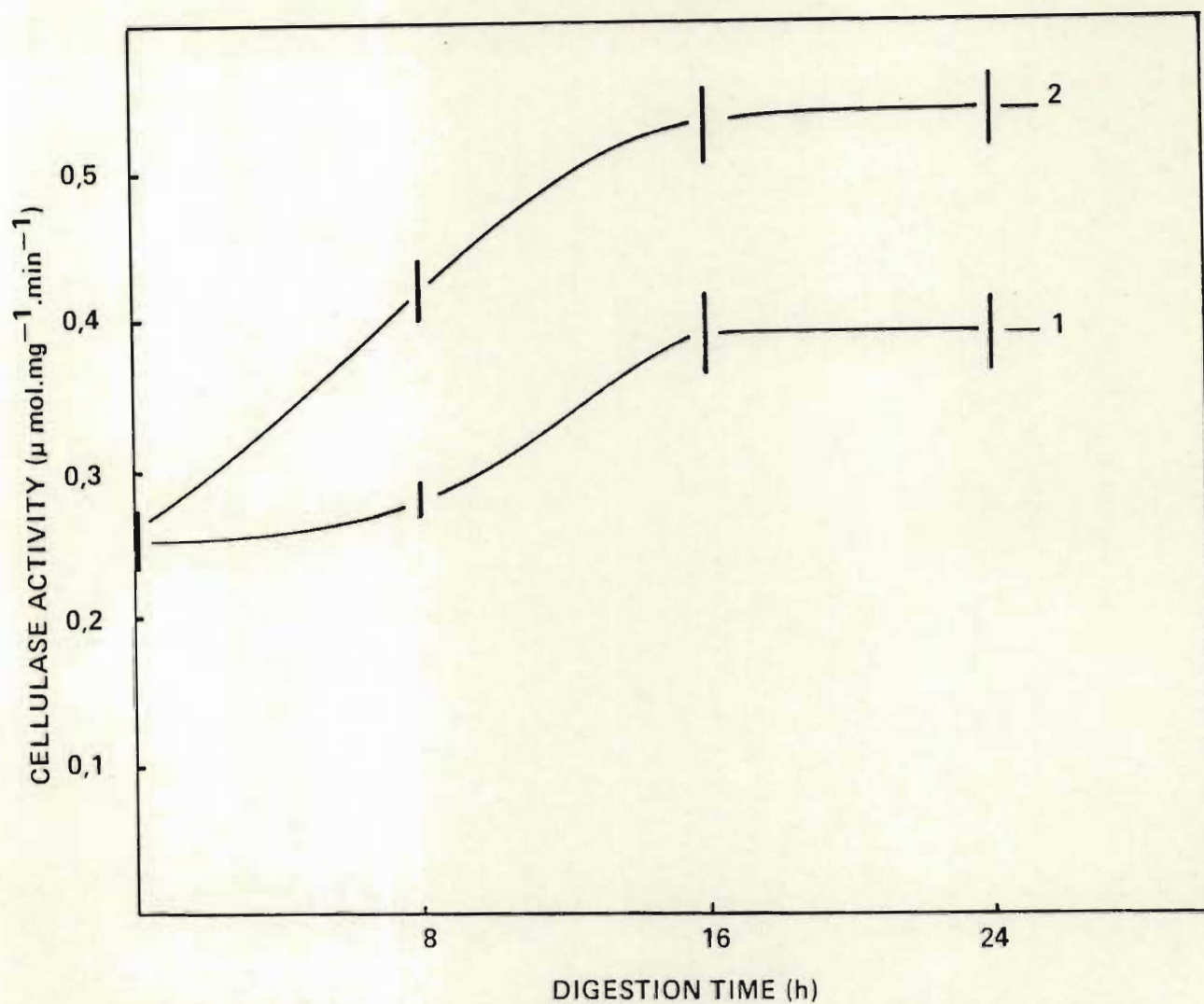


FIG. 3.18 Effect of nitrite upon the cellulase activity of the digest. (1) High nitrate kikuyu grass (600 mg⁰/o N, m/m); (2) Low nitrate kikuyu grass (100 mg⁰/o N, m/m)

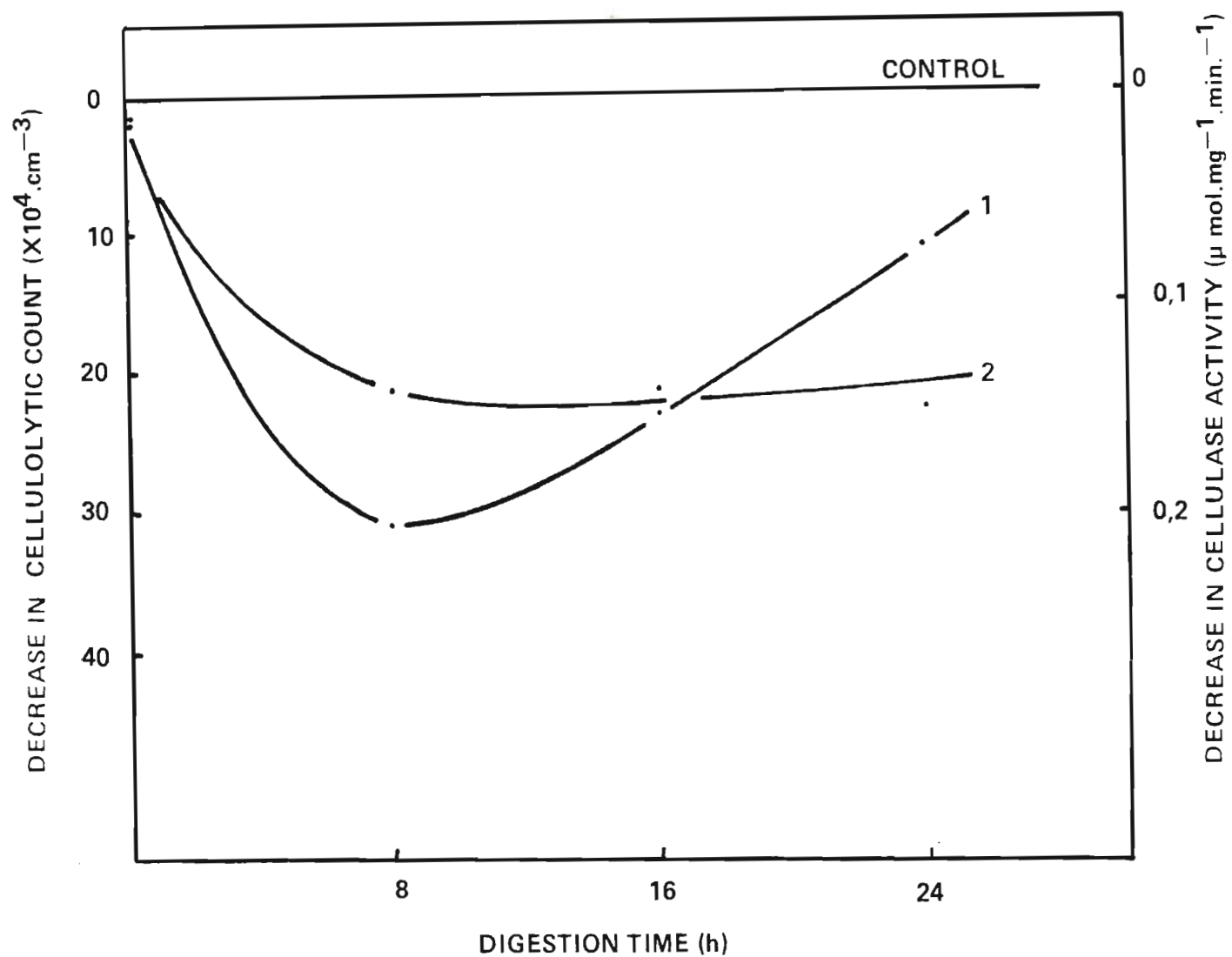


FIG. 3.19 Change in cellulolytic microbes and cellulase activity of a high nitrate kikuyu grass digest (600 mg% N, m/m) over the digestion period, relative to the low nitrate control digest (100 mg% N, m/m). (1) Cellulolytic count; (2) Cellulase activity

These results suggest that nitrite affects digestion by inhibiting the growth of the xylanolytic, cellulolytic and other microbial populations, while it seems to have no effect upon the synthesis of carbohydrases by these organisms.

Nitrite may exert its effect upon the rumen microbes in several ways. It may affect cellulolytic microbes in particular by affecting the formation of essential branched chain fatty acids, it may affect the microbial population indirectly by an effect upon the redox potential of the medium or it may have a direct bacteriostatic or bactericidal effect upon the microbes.

3.4.2 Production of essential branched chain fatty acids in the presence of nitrite

During rumen digestion, carbohydrates are converted to volatile fatty acids with acetic, propionic and butyric acids forming the main components. Branched chain fatty acids such as isobutyric and isovaleric acids are present in much lower concentrations.

Bryant & Doetsch (1954) demonstrated the absolute requirement of cellulolytic microbes for certain branched chain fatty acids. They found that Bacteroides succinogenes requires either isobutyric or 2-methylbutyric acid. Allison, Bryant & Doetsch (1958) showed that Ruminococcus flavefaciens requires isobutyric acid or isovaleric acid while Ruminococcus albus

has an absolute requirement for either isobutyric, 2-methyl butyric or 2-ketoisovaleric acid.

The presence of nitrate in the rumen causes considerable changes in the volatile fatty acid composition of the digest. Bryant (1965) demonstrated an increase in acetic acid and a decrease in propionic acid and butyric acid in the presence of nitrate in in vitro fermentation of rumen liquor, but did not comment on branched chain fatty acids. Farra & Satter (1971) obtained similar results in vivo by increasing the dietary nitrate of Jersey cows, but also did not record branched chain acids. Earlier work by Jamieson (1959) on the effect of nitrate on rumen metabolism of grazing sheep showed little change in branched chain fatty acids in the presence of nitrate.

Miura, Horiguchi, Ogimoto & Matsumoto (1983) showed that the growth rate of cellulolytic microbes in washed microbial suspensions on a cellulose urea medium roughly corresponds with the increase of branched chain fatty acids and amino acids in the digest. These workers interpret the lag in commencement of cellulose digestion as a response to the time required for the production of branched chain fatty acids.

In this laboratory cellulose digestion in vitro in the presence of nitrate was also found to be preceded by a lag period (Fig. 3.9). In an attempt to establish whether this lag is due to a lack of branched chain fatty acids in the in

vitro digest and to explain the more severe effect of nitrate upon cellulolytic microbes, an experiment was conducted to determine the volatile fatty acid composition of the digest in the presence and absence of nitrate. Kikuyu grass containing low nitrate (100 mg% N, m/m) and high nitrate (600 mg% N, m/m) levels were digested in vitro for 7h and 24h. After digestion volatile fatty acid analyses were conducted on the digests. (Table 3.9).

TABLE 3.9 Effect of nitrate upon volatile fatty acid production during digestion *in vitro*

Digestion time (h)	Volatile fatty acid	Volatile fatty acid content			
		Low nitrate kikuyu grass (100 mg % N, m/m)		High nitrate kikuyu grass (600 mg % N, m/m)	
		$\mu \text{ mol. cm}^{-3}$	molar %	$\mu \text{ mol. cm}^{-3}$	molar %
7	Acetic acid	14,31 \pm 0,19	71,23	12,65 \pm 0,07	71,11
	Propionic acid	3,90 \pm 0,04	19,41	3,44 \pm 0,03	19,34
	Isobutyric acid	0,18 \pm 0,01	0,90	0,17 \pm 0,00	0,96
	Butyric acid	1,35 \pm 0,02	6,72	1,21 \pm 0,01	6,80
	Iso valeric acid	0,19 \pm 0,01	0,95	0,18 \pm 0,00	1,01
	Valeric acid	0,16 \pm 0,01	0,80	0,14 \pm 0,01	0,79
	Total	20,09		17,79	
24	Acetic acid	22,09 \pm 0,22	68,28	19,15 \pm 0,07	71,08
	Propionic acid	7,40 \pm 0,09	22,88	5,40 \pm 0,00	20,05
	Iso butyric acid	0,22 \pm 0,01	0,68	0,22 \pm 0,01	0,82
	Butyric acid	2,17 \pm 0,02	6,71	1,71 \pm 0,04	6,35
	Iso valeric acid	0,21 \pm 0,01	0,65	0,22 \pm 0,01	0,82
	Valeric acid	0,26 \pm 0,01	0,80	0,24 \pm 0,01	0,89
	Total	32,35		26,94	

Results (Table 3.9) show a decrease in the total volatile fatty acid content of the high nitrate digest. A difference was noticeable after digestion for 7h, while after 24h the total volatile fatty acid content is about $27 \mu \text{mol.cm}^{-3}$ compared to about $32 \mu \text{mol.cm}^{-3}$ in the low nitrate digest. These results are in agreement with the observations of Bryant & Ulyatt (1965) who showed that total rumen volatile fatty acids in sheep decrease within 2h after consumption of high nitrate rye grass.

Results also show an increase in acetate production relative to propionate and butyrate. After digestion for 24h the molar percentage of acetate, propionate and butyrate in the high nitrate digest is 71%, 20% and 6% respectively compared to 68%, 23% and 7% respectively in the low nitrate digest. These results substantiate the results obtained by Bryant (1965) and Farra & Satter (1971).

Results further show an increase in molar percentage of the total branched chain fatty acids in the high nitrate digest, although, the actual concentrations of isobutyric and isovaleric acid remained constant in the presence or absence of nitrate. It therefore, seems unlikely that branched chain fatty acids could limit the growth of cellulolytic microbes in high nitrate digests in vitro.

3.4.3 Change in redox potential in the presence of nitrate

The redox potential of a redox couple is a measure of its electron transfer ability. Microbial cultures contain a multitude of different redox reactions and the redox potential measured is, therefore, the resultant of many contributory couples, some of which may be irreversible or electromotively inactive. According to Morris (1975) redox potential readings should therefore not be invested with too great a theoretical significance. However, in the study of anaerobes these readings are useful indications of the degree of anaerobiosis in the growth environment (Kistner, 1960).

It is generally recognised that the predominant rumen microbes require a low redox potential in the ambient medium for normal growth (Hungate, 1966). Huhtanen & Gall (1953) reported a redox potential of the rumen content of cattle on a roughage diet of -300 mV, while Smith & Hungate (1958) obtained values varying between -335 mV and -346 mV in the rumen of cattle used in their experiments.

Rumen methanogenic bacteria are among the most rigorous of obligate anaerobes and only thrive in locations where the redox potential of the medium is lower than -350 mV at pH 7.0 (Hungate, 1966), but most other rumen microbes seem to be able to survive at less negative potentials (-200 mV). Low redox potentials can be achieved by the inclusion into culture media for rumen microbes of reducing agents. In some

instances cysteine or sodium dithionite has been used (Hungate, 1950), which lowers the redox potential of the medium to approximately -200 mV while Bryant & Robinson (1961) proposed the use of a combination of cysteine and sodium sulphide.

Nitrite is an oxidising agent which could possibly increase the redox potential of the digest to growth inhibiting values. Experiments were therefore conducted to establish whether the redox potential is increased in the presence of nitrite and whether an increased redox potential could be a contributing factor to the observed decrease in microbial numbers. The redox potential of an in vitro digest of kikuyu grass containing low (100 mg% N, m/m) and high levels (600 mg% N, m/m) of nitrate was followed over a digestion period of 26h. Results are presented in Fig. 3.20.

These results show a positive redox potential in the digest of +70 mV at zero time. In the absence of nitrate the redox potential drops rapidly, reaching 0mV after approximately 10h and -48 mV after 26h. In the presence of nitrate the redox potential remains positive much longer and attains negative values only after digestion for about 19h.

Compared to the redox potential existing in the rumen in vivo the values recorded in the in vitro digest and especially those in the high nitrate digests are extremely high. Evidence in the literature suggests that a positive

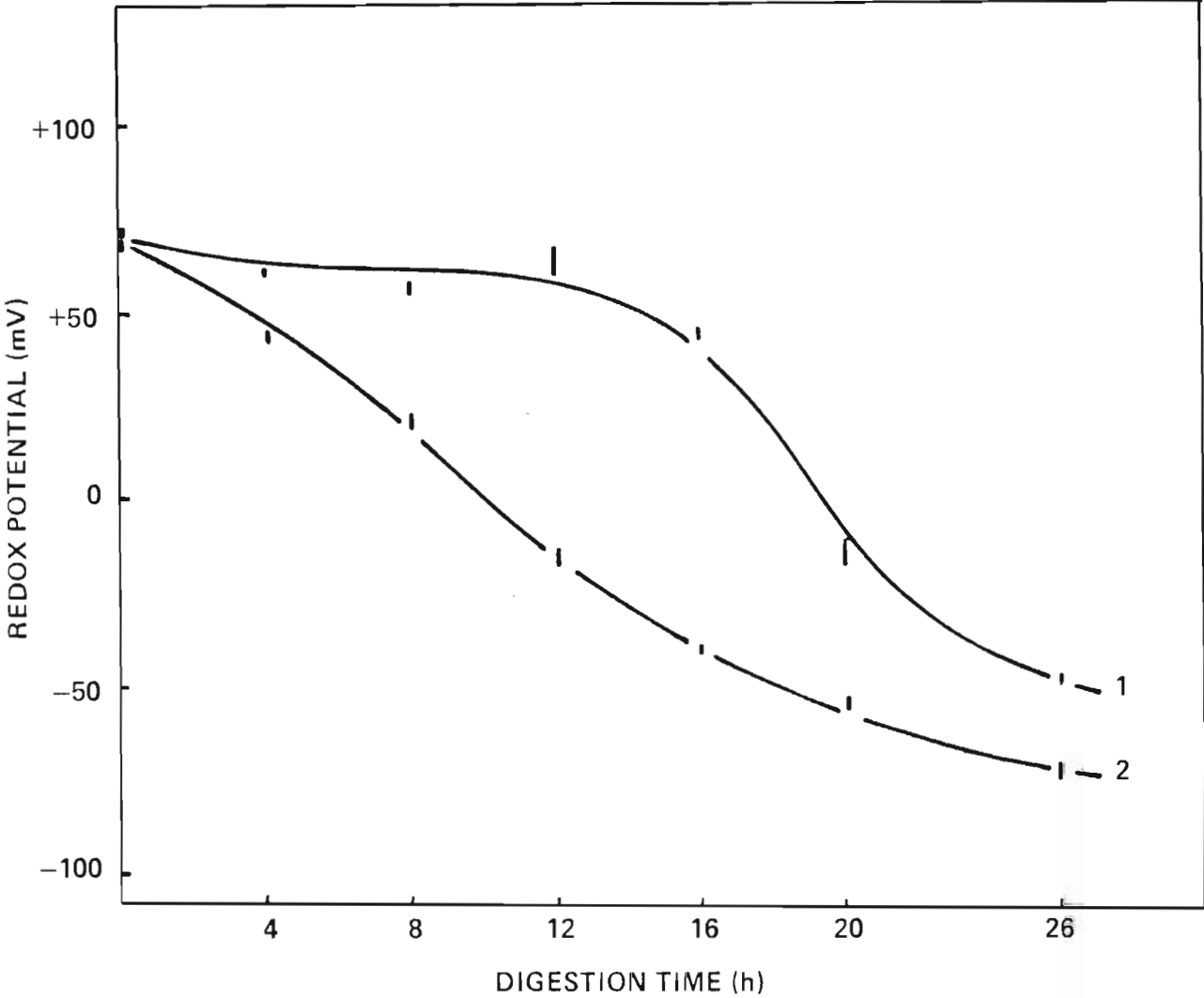


FIG. 3.20 Change over time in redox potential of a kikuyu grass digest *in vitro*. (1) High nitrate kikuyu grass (600 mg⁰/o N, m/m); (2) Low nitrate kikuyu grass (100 mg⁰/o N, m/m)

redox potential, as such, in the medium could be a growth limiting factor for anaerobic microbes. Hanke & Katz (1943) controlled the redox potential of a culture of Bacteroides vulgaris and Clostridium sporogenes by means of an electrolytic technique and showed that these organisms can grow in a continuous current of air provided the oxidation reduction potential of the media is kept sufficiently low electrolytically.

In most instances, however, oxygen itself seems to be the primary factor interfering with the growth of anaerobic microbes, a positive redox potential being only indicative of the presence of oxygen in the medium (Hentges & Maier, 1972). In a study of three anaerobic intestinal bacteria, Clostridium perfringens, Bacteroides fragilis and Peptococcus magnus grown in a medium with the redox potential adjusted to +325 mV by the addition of potassium ferricyanide, Walden & Hentges (1975) showed that despite the positive redox potential, these microbes multiply normally provided the medium is kept oxygen free.

Many workers have documented the inhibitory effect of molecular oxygen on anaerobic bacteria (Loesche, 1969; Onderdonk, Johnston, Mayhew & Gorbach, 1976). Ayers (1958) showed that oxygen has a lethal effect on Ruminococcus flavefaciens. No further development of the microbes could be observed under anaerobic conditions, following a brief exposure (15 min) of the organisms to air. Morris (1975)

reviewed the different effects of oxygen upon anaerobic microbes and showed that different species may react differently towards oxygen and several mechanisms may be operating simultaneously in limiting growth.

In order to determine whether the influence of nitrite upon digestion is due to a direct effect upon the redox potential or to an effect similar to that observed for oxygen, cysteine was added to the digest to maintain a low redox potential in the presence of nitrite. High nitrate (600 mg% N, m/m) and low nitrate (100 mg% N, m/m) kikuyu grass samples were digested in vitro in the presence and absence of cysteine (0,04%, m/v). The redox potential of the digest was followed over a period of 24h and samples were analysed for nitrite. The dry matter digested after 24h was determined. Results are presented in Fig. 3.21.

In the absence of cysteine the redox potential of the digests gives a pattern similar to that obtained in previous experiments (Fig. 3.20). In the presence of cysteine however, the redox potential is lowered and remains constant at approximately -123mV throughout the digestion period. The digestibility of the low nitrate sample in the absence of cysteine is $48,5 \pm 0,3\%$ and in the presence of cysteine, $46,3 \pm 0,5\%$. Cysteine, therefore, seems to have a slight depressing effect upon digestion.

The digestibility of the high nitrate sample in the absence

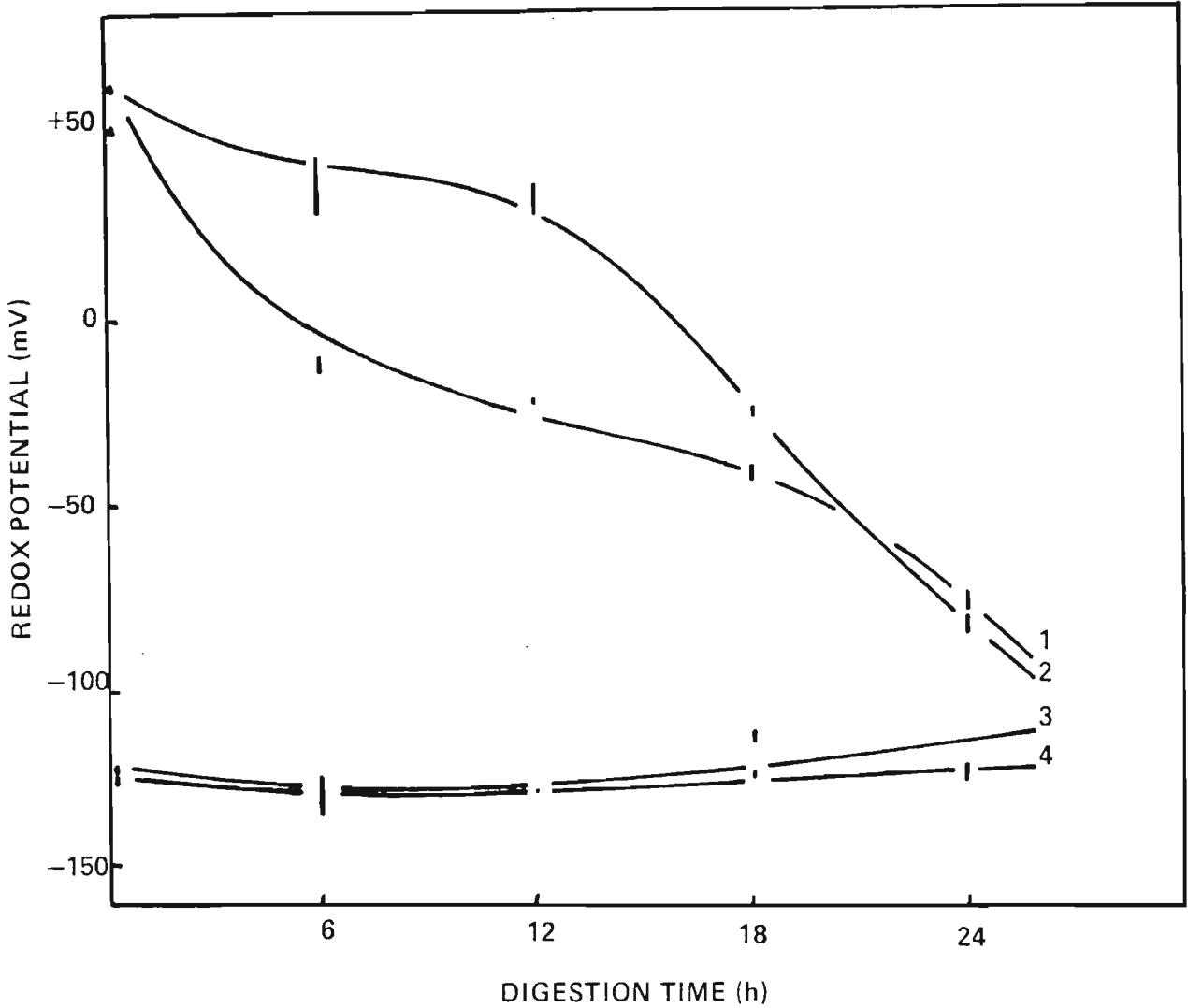


FIG. 3.21 Effect of cysteine upon the redox potential of a kikuyu grass digest *in vitro*.
 (1) Low nitrate kikuyu grass (100 mg⁰/o N, m/m), no cysteine; (2) High nitrate kikuyu grass (600 mg⁰/o N, m/m), no cysteine; (3) High nitrate kikuyu grass + cysteine (0,04 ⁰/o, m/v); (4) Low nitrate kikuyu grass + cysteine (0,04 ⁰/o, m/v)

of cysteine was $36,9 \pm 0,5\%$ and in the presence of cysteine it was increased to $39,7 \pm 0,4\%$. Cysteine, however, seems to lower the concentration of nitrite in the digest. The nitrite content of the digest in the presence and absence of cysteine after 8h digestion is $11,3 \mu\text{g.cm}^{-3}$ and $15,0 \mu\text{g.cm}^{-3}$ respectively and after 16h digestion, $1,0 \mu\text{g.cm}^{-3}$ and $2,5 \mu\text{g.cm}^{-3}$ respectively. These results are in agreement with the findings of Azoulay, Puig & Martins Rosada de Sousa (1969) who demonstrated a depression in the production of membrane bound nitrate reductase on addition of cysteine to Escherichia coli. The improvement in digestibility of the high nitrate samples could, therefore, be due to the reduction in the nitrite level of the digest. The results, however, clearly show that nitrite has a profound effect upon digestion, even at sufficiently low redox potentials, suggesting a more direct effect upon rumen microbes.

CHAPTER 4

EFFECT OF NITRITE UPON THE SPECIFIC GROWTH
RATE OF RUMEN BACTERIA

Results presented in Chapter 3 suggest a more direct effect of nitrite upon the growth rate of rumen bacteria. Little information is available regarding the effect of nitrite upon the growth and metabolism of rumen microbes. However, nitrite has long been used as an antimicrobial agent for meat curing. Despite its general use in the meat processing industry, the mechanism of inhibition of bacteria has not been satisfactorily elucidated (Hansen & Levin, 1975; O'Leary & Solberg, 1976; Yarbrough, Rake & Eagon, 1980). The inhibiting properties of nitrite appear to be quite complex and could be attributed to more than one mechanism.

In the meat-curing process, the sporostatic effect of nitrite depends largely upon heat treatment (autoclaving), suggesting the conversion of nitrite to more active substances (Incze, Farkas, Mihályi & Zukál, 1974). These workers identified nitrosothiols with definite microbial inhibitory properties which were formed from the reaction of nitrite and sulphhydryl compounds such as cysteine. Morris & Hansen (1981), studying the effect of nitrosothiols (general structure, $R-S-N=O$) on spore outgrowth in Bacillus cereus, showed that the

effectiveness of nitrosothiols as bacteriostatic agents increases with increasing electron-withdrawing capacity or polarity of the R group. Their results point towards sulphhydryl groups on the bacterial membrane or spore coat as the reactive sites. It was suggested that inhibitory action is achieved by the following reaction: $R-S-N=O + HS-X \rightarrow R-S-N(OH)-S-X$, where HS-X represents an essential sulphhydryl group on the bacterium.

Hansen & Levin (1975) showed that nitrosothiols and other heat-induced inhibitors effectively inhibit the incorporation of uracil into the ribonucleic acid of Bacillus cereus, while controls containing unheated nitrite had little effect upon uracil incorporation.

The action of heat-induced inhibitors is considered to be relatively pH independent (Perigo, Whiting & Bashford, 1967; Hansen & Levin, 1975). However, the inhibitory action of nitrite has often been shown to be pH dependent (Castellani & Niven, 1955; Buchanan & Solberg, 1972). Castellani & Niven (1955) showed that the bacteriostatic effect of nitrite upon Staphylococcus aureus increases as the pH decreases over the range pH 7 to pH 5 and found that the inhibiting power is related to the calculated amount of undissociated nitrous acid in the medium. At pH near neutrality nitrite concentrations of 140 to 1400 $\mu\text{g N.cm}^{-3}$ was found inhibitory and the bacteriostatic effect further increased ten-fold for every unit decrease in pH.

O'Leary & Solberg (1976) pointed out the extremely reactive nature of nitrous acid which is capable of interacting with a wide variety of substances including myoglobin, ascorbic acid, phenols, secondary amines and amino and thiol groups. These workers investigated the effect of nitrite in the form of nitrous acid at pH 6,3 on intracellular sulphydryl groups of Clostridium perfringens. They observed a browning of cells in the medium in the presence of nitrite ($1000 \mu\text{g.cm}^{-3}$), which seems to indicate an interaction with cell wall components. Nitrite was shown to cause a 91% decrease in the free sulphydryl content of the soluble cellular extract of inhibited Clostridium perfringens cells. Such a decrease in sulphydryl groups, could result in a serious disruption of cell function due to the inhibition of enzymes containing essential sulphydryl groups. These workers indeed observed a complete loss of glyceraldehyde-3-phosphate dehydrogenase and a 67% decrease in aldolase activity. Both enzymes are known to contain reduced sulphydryl groups essential for activity (Harris, Merriweather & Park, 1963; Rutter, Hunsley, Groves, Calder, Rajkumun & Woodfin, 1966). Furthermore, enzyme activity could be partly restored by the addition of dithiothreitol, substantiating the involvement of reduced sulphydryl groups. Sodium nitrite also reacts with primary amino groups at pH 5,5, but the reaction is not as rapid as with sulphydryl groups (Mirna & Hofmann, 1969).

Nitrous acid has also been shown to inactivate viruses,

partly by its action on the protein moiety of the virus particle (Lytle & Ginoza, 1970), but the primary mode of inactivation, it has been suggested, is the deamination of the nucleic acid bases of deoxyribonucleic acid (Lytle & Ginoza, 1969).

Stouthamer (1979) showed that the inhibitory effect of nitrite upon the bacteria, Enterobacter aerogenes, Clostridium perfringens, Paracoccus denitrificans and Propionibacterium pentosaceum is reflected in its effect upon the molar growth yield of the bacteria. The inhibition was not due to reduced growth but to a reduced efficiency of conversion of substrate to cell mass.

Bauchop & Elsdon (1960) approximated the apparent cell yield of bacteria to the value of 10,5g per mole of ATP, produced ($Y_{ATP} = 10,5$). Growth yields for many anaerobic bacteria, based upon known or suggested routes of substrate-level production of ATP were found to be considerably higher than 10,5. This observation and the fact that many anaerobic bacteria possess cytochromes, ferredoxin, flavoproteins and quinones, point to ATP synthesis coupled to electron transport phosphorylation, a mechanism previously thought to occur only in phototropic organisms and chemotropic aerobes (Decker, Jungermann & Thauer, 1970).

At present several hydrogenation reactions evidently coupled to phosphorylation are known in both facultatively and

obligately anaerobic bacteria (Thauer, Jungermann & Decker, 1977). The following hydrogenation reactions have been demonstrated in rumen bacteria:

- 1) Reduction of carbon dioxide to methane by Methanobacterium spp.
- 2) Reduction of sulphate to sulphide by Desulfovibrio spp.
- 3) Reduction of fumarate to succinate by Vibrio succinogenes.
- 4) Reduction of nitrate to nitrite by Veillonella alcalescens and Selenomonas ruminantium.

Thauer, Jungermann & Decker (1977), however, pointed out that these hydrogenation reactions need not always be linked with phosphorylation or ATP synthesis. In some anaerobic bacteria these reactions may serve only as a sink for electrons, the function of which is to drive substrate level phosphorylation.

Eagon, Hodge, Rake & Yarbrough (1979) showed that respiration and active transport in Escherichia coli and Pseudomonas aeruginosa, is partly inhibited by nitrite at a concentration of $140 \mu\text{g N.cm}^{-3}$, while 700 to $1400 \mu\text{g N.cm}^{-3}$ is required for complete inhibition. A nitrite concentration of $140 \mu\text{g N.cm}^{-3}$, however, completely inhibited ATP synthesis in aerobic bacteria and cells rapidly lose their ATP pools (Rowe, Yarbrough, Rake & Eagon, 1979). These workers suggested that nitrite exerts its effect in the cell membrane at the electron carrier level.

According to Rowe, Yarbrough, Rake & Eagon (1979) nitrate has no effect on glucose transport in organisms such as Streptococcus faecalis, which lack cytochromes and which are known to rely upon glycolysis for ATP generation (Harold & Baarda, 1968). Meijer, Van der Zwaan, Wever & Stouthamer (1979) showed that nitrite increases the proton permeability of the cytoplasmic membrane of Paracoccus denitrificans thus dissipating the proton gradient and preventing the coupling of respiration with ATP synthesis. Rake & Eagon (1980) however, found no evidence for increased membrane permeability and do not consider nitrite to act as an uncoupler of electron transport but confirmed the inhibitory effect of nitrite upon electron transport. The energy metabolism of most rumen bacteria is poorly understood. Substrates utilised and metabolic processes in these bacteria differ markedly and it can be assumed that large differences in the mode of ATP synthesis and tolerance to nitrite exist.

One of the major cellulolytic species in the rumen, Bacteroides succinogenes, also attacks crystalline cellulose (Halliwell & Bryant, 1963; Bryant 1973). It hydrolyses hemicellulose but does not utilise the hydrolysis products (Dehority, 1965; Coen & Dehority, 1970). However, glucose, dextrin, maltose and pectin are fermented (Russel, 1984) and succinate is produced as the main end product; which is rapidly metabolised to propionate by Selenomonas ruminantium in mixed culture (Scheifinger & Wolin, 1973). According to

Caldwell & Rasmussen (1983) Bacteroides succinogenes is a cytochrome containing anaerobe and Dawson, Preziosi & Caldwell (1979) showed that inhibitors of electron transport in this organism lead to a reduction in cell yield per mole of glucose catabolised, due to the loss of ATP forming mechanisms. According to these workers substrate level and electron transport-mediated processes appear to contribute approximately equally to energy formation.

Ruminococcus flavefaciens, another major cellulolytic species in the rumen, reduces fumarate to succinate as an end product which is rapidly decarboxylated to propionate by rumen microbes such as Selenomonas ruminantium (Scheifinger & Wolin, 1973). According to Thauer, Jungermann & Decker (1977) dissimilatory reduction of fumarate to succinate proceeds via an electron transport system involving specific dehydrogenases, electron carriers and fumarate reductase, but Hopgood & Walker (1967, 1969) found no evidence of ATP synthesis coupled to fumarate reduction.

Ruminococcus albus is an important cellulose digester in the rumen but can also digest xylan (Glass, Bryant & Wolin, 1977). It has the advantage over Ruminococcus flavefaciens and Bacteroides succinogenes in that it can utilise more of the pentoses produced during hydrolysis (Morris & Van Gylswyk, 1980). Ruminococcus albus has been shown to have a branched energy metabolism, each branch leading to a different ATP gain and thermodynamic efficiency of ATP

synthesis (Thauer, Jungermann & Decker, 1977). If grown in a monoculture system, it ferments glucose mainly to ethanol with smaller amounts of acetate, carbon dioxide and hydrogen and the formation of 3,3 moles of ATP per mole of glucose. If grown in the presence of hydrogen utilising bacteria such as Vibrio succinogenes, electrons are shifted away from the production of ethanol to form the more oxidised product, acetate, as a major component and smaller amounts of ethanol, formate, carbon dioxide and hydrogen. In mixed culture four moles of ATP per mole of glucose is formed (Iannotti, Kafkewitz, Wolin & Bryant, 1973). Growth yield measurements by Hungate (1963) implied that ATP formation also takes place via electron transport but at present it is generally accepted that Ruminococcus albus is able to synthesise ATP only via substrate level phosphorylation (Thauer & Kröger, 1984).

Some strains of Butyrivibrio fibrisolvens are cellulolytic but only the more amorphous types of cellulose in the rumen are attacked. (Halliwell & Bryant, 1963; Bryant, 1973). It is a predominant degrader of hemicellulose but is also able to utilise pectin, starch, cellobiose, glucose, xylose, fructose, lactose, sucrose and maltose (Hungate, 1966). According to Caldwell & Rasmussen (1983) Butyrivibrio fibrisolvens is devoid of cytochromes. However, Dawson, Preziosi & Caldwell (1979) showed that Butyrivibrio fibrisolvens obtains some energy via electron transport but substantially more from substrate-level processes.

Selenomonas ruminantium is one of the predominant non-cellulolytic rumen bacteria which is able to utilise an array of different carbon sources, but maximum growth yields are obtained on glucose and sucrose (Russel, 1984). Wallace (1978) showed that Selenomonas ruminantium produces mainly lactate from glucose if ammonia is limiting, but if glucose becomes limiting acetate and propionate are the main fermentation products. Propionic acid is formed via the succinate pathway (Hungate, 1966; Hobson & Summers, 1972). Scheifinger & Wolin (1973) showed that Selenomonas ruminantium decarboxylates succinate, formed by cellulolytic species in the rumen, to propionate. De Vries, Van Wijck-Kapteyn & Oosterhuis (1974) further demonstrated a membrane bound cytochrome-linked electron transport system in Selenomonas ruminantium capable of using fumarate as an electron acceptor and reduced pyridine nucleotide as electron donor. Their results strongly support the hypothesis that the reduction of fumarate by molecular hydrogen, reduced pyridine nucleotide or some other electron donor, may serve as an additional source of ATP. Furthermore, Hobson & Summers (1972) obtained a maximum growth yield for this organism of $Y_{\text{glucose}} = 62$ during continuous culture, which suggests the formation of ATP by electron transport. Dawson, Preziosi & Caldwell (1979), however, who investigated the effects of uncouplers and inhibitors of electron transport in Selenomonas ruminantium concluded that during growth in batch culture this organism obtains ATP solely from substrate-level

reactions.

In view of the available knowledge on nitrite as a meat preservative and the existing differences in energy metabolism of some of the major rumen bacteria, a study was made of the effect of nitrite upon the specific growth rate of pure cultures of these rumen bacteria to establish, firstly, to what extent individual species are affected by nitrite and, secondly, to determine whether growth inhibition could be correlated with the mode of energy metabolism in these bacteria.

4.1 Materials and methods

4.1.1 Determination of specific growth rate

A meaningful way of quantifying growth is by expressing it in terms of the growth parameter, specific growth rate (Pirt, 1975). Specific growth rate is defined as the quantity of dry matter synthesised per gram of dried cells per hour. Analysis of specific growth rate was based upon the turbidimetric procedure described by Therion (1981). If the growth requirements of a bacterial culture are optimal, the increase in biomass (dx) during an infinitely small time interval (dt) is proportional to the amount of biomass (x) present and to the time interval and can be expressed as:

$$dx = \mu x . dt \quad 4.1$$

The population growth rate can therefore be expressed as:

$$\mu x = dx/dt \quad 4.2$$

and the specific growth rate μ , which is the rate of growth per unit amount of biomass $dx/(x . dt)$, can be expressed as:

$$\mu = dx/(x . dt) \quad 4.3$$

and has the dimensions of reciprocal time.

Since specific growth rate is a function of the increase of biomass with time, measurement of biomass is a prerequisite for determining specific growth rate.

One of the most widely used methods for measuring biomass is based on the scattering of light by cell suspensions (Koch, 1961). With this method, biomass concentration (x) and the length of the light path through the sample (l) are related to the intensities of incident light (I_o) and transmitted light (I_t) by the equation:

$$\log (I_o/I_t) = Axl \quad 4.4$$

The value of $\log (I_o/I_t)$ is termed optical density (O D) or

extinction and the factor A is a constant at low biomass concentrations. Due to multiple scattering, at high biomass concentrations the relation with OD ceases to be linear.

During the exponential phase of growth of bacteria, the specific growth rate (μ) is constant. Integration of equation 4.2 then gives:

$$\ln x = \ln x_0 + \mu t \quad 4.5$$

where x_0 is the biomass when $t = 0$. This relation can also be expressed as:

$$\mu = (\ln x_2 - \ln x_1) / (t_2 - t_1) \quad 4.6$$

where x_1 and x_2 are the biomass concentrations of a culture in the exponential growth phase at times t_1 and t_2 respectively.

When the biomass is measured as OD in the range where OD is proportional to biomass, biomass can be substituted by OD in equation 4.6 giving the following:

$$\mu = (\ln(OD_2) - \ln(OD_1)) / (t_2 - t_1) \quad 4.7$$

where OD_1 and OD_2 are optical density reading at times t_1 and t_2 respectively.

In order to improve the accuracy of the method multiple readings were taken and μ was calculated as the slope of the least-squares linear regression plot of $\ln(\text{OD})$ versus time.

Organisms

The organisms, Bacteroides succinogenes strain S85 and Ruminococcus flavefaciens strain FDI came from the culture collection of Dr. Marvin P. Bryant and Ruminococcus albus strain 22.08.6A, Butyrivibrio fibrisolvens strain Ce 51 and Selenomonas ruminantium strain ATCC 19205 are from the culture collection of the Anaerobic Microbiology Division, CSIR Laboratory for Molecular and Cell biology.

Reagents

Potassium nitrite solutions. Potassium nitrite (3,8856g) was dissolved in distilled water (1 dm^3). An aliquot ($0,5 \text{ cm}^3$) of this solution added to growth medium ($9,5 \text{ cm}^3$) gave a final nitrite-N concentration of $32 \mu\text{g} \cdot \text{cm}^{-3}$. Further dilutions were made to obtain final nitrite concentrations of $16 \mu\text{g}$, $8,0 \mu\text{g}$, $4,0 \mu\text{g}$, $2,0 \mu\text{g}$, $1,0 \mu\text{g}$ and $0,5 \mu\text{g} \cdot \text{cm}^{-3}$.

Mineral solution I. Dipotassium hydrogen phosphate (11,84g) was dissolved in distilled water (1 dm^3).

Mineral solution II. Potassium dihydrogen phosphate (7,08g), ammonium sulphate (17,97g), sodium chloride (1,78g),

magnesium sulphate (3,75g) and calcium chloride (2,41g) were dissolved in distilled water and made up to 1 dm³.

Haemin solution. Haemin (0,1g) was dissolved in a solution consisting of ethanol (500 cm³) and 0,05 mol.dm⁻³ sodium hydroxide (500 cm³).

Volatile fatty acid solution. Acetic acid (170 cm³), propionic acid (60 cm³), butyric acid (40 cm³), isobutyric acid (10 cm³), isovaleric acid (10 cm³) and DL- α -methylbutyric acid (10cm³) were mixed, chilled in an ice bath and the pH adjusted to 7,7 with 10 mol.dm⁻³ sodium hydroxide. The solution was made up to 1 dm³ with distilled water.

Cysteine-sodium sulphide solution. Cysteine hydrochloride (1,25g) was dissolved in 0,4 mol.dm⁻³ sodium hydroxide solution (50 cm³). Sodium sulphide (1,25g) was dissolved in the cysteine solution and made up to 100 cm³. The solution was purged with 95% nitrogen containing 5% hydrogen and the pH adjusted to 11,08 with hydrochloric acid if too high. Aliquots (10 cm³) of the solution were dispensed into nitrogen purged 28 cm³ McCartney bottles and the bottles were autoclaved (120°C, 20 min).

Preparation of medium

Culture vessel. The medium was prepared in an anaerobic

culture vessel (3 dm^3). The vessel, illustrated by Therion (1981), was fitted with a vibratory mixer ('Vibromixer' type El, Chamap, Mannendorf, Switzerland), a combination pH electrode, a thermistor temperature sensor and a quartz-sheathed, 50 watt miniature immersion heater.

Procedure. The medium was based on medium 10 of Caldwell & Bryant (1966). Cellobiose (3,0g) (or glucose, 3,0g, in medium for Selenomonas ruminantium), trypticase (Merck)(2,0g), yeast extract (Difco Laboratories, Detroit, USA)(0,5g), mineral solution I (25 cm^3), mineral solution II (25 cm^3), haemin solution (10 cm^3) and volatile fatty acid solution (10 cm^3) were dissolved in deoxygenated distilled water (862 cm^3). The medium, kept at $38,5^\circ\text{C}$, was purged with an oxygen-free gas mixture consisting of 68% nitrogen, 30% carbon dioxide and 2% hydrogen. A constant gas pressure of 588 N.m^{-2} was maintained in the system by a pressure sensor actuating a solenoid valve in the gas line. The pH was adjusted to either 6,0 or 6,8 by the addition of solid sodium bicarbonate.

Samples ($9,3 \text{ cm}^3$) of medium were dispensed by means of an automatic syringe fitted with a sterile 25mm Millipore micro-syringe filter holder with prefilter and $0,2 \mu\text{m}$ porosity membrane. The medium was injected into sterile 20 cm^3 serum bottles closed with a butyl rubber stopper and a crimped metal seal. The bottles had previously been flushed with an oxygen-free gas mixture, also used for purging the culture medium. Cysteine-sodium sulphide solution ($0,2 \text{ cm}^3$)

and the appropriate concentration of potassium nitrite solution ($0,5 \text{ cm}^3$) were dispensed under anaerobic conditions, through sterile Millipore $0,2 \mu\text{m}$ porosity bacterial filters, into the serum bottles.

Preparation of inocula

An agar slope was inoculated with the water of syneresis ($0,2 \text{ cm}^3$) from a stock culture, using the procedure described by Roche, Albertyn & Kistner (1973). The agar slope was incubated at $38,5^\circ\text{C}$ until growth was visible on the agar surface. The cells were washed off the surface of the agar and the resulting suspension used to inoculate a portion (10 cm^3) of the growth medium. As soon as the culture attained an optical density of approximately 0,6 it was used to inoculate the media containing different levels of nitrite.

Measurement of growth rate

Apparatus. The growth rate of the bacteria was monitored by measuring the change in turbidity of the cultures at 578 nm in a mercury vapour lamp filter photometer (Model 6120, Eppendorf Gerätebau, Hamburg, W. Germany). The cuvette holder of the photometer was modified to accommodate the serum bottles. To allow for imperfections in the glass and small variations in the thickness of the bottles, reference marks on the bottles were aligned with an index line on the edge of the sample well to ensure that all measurements were taken

with the bottles in the same position.

The serum bottles were incubated in a thermostatic water bath at $38,5 \pm 0,1^{\circ}\text{C}$.

Procedure. The bottles containing medium and nitrite of different concentrations (done in triplicate) were pre-warmed in the water bath and their blank optical density values read against a similar bottle containing membrane filtered distilled water. Blank values were subtracted from all subsequent readings on the same bottles. Inoculum ($0,2 \text{ cm}^3$) was injected into each bottle of medium and the turbidity measured immediately after inoculation and then at hourly intervals. Readings were taken until growth stopped or until the turbidity values increased beyond the range of a satisfactory linear relationship between biomass and optical density.

Experimental data was processed with the aid of a microcomputer (Model P6060, Ing. C. Olivetti & C., Ivrea, Italy). The optical density data were corrected for the blank readings and converted to their natural logarithm values. These values were plotted against incubation times by the integral thermal printer of the microcomputer. From these plots, the onset and end of the exponential growth phase could be determined. By entering these figures into the computer, the specific growth rate at a particular nitrite level and pH value, could be calculated.

4.2 Results and discussion

The work of Dawson, Preziosi & Caldwell (1979) showed that Bacteroides succinogenes obtains approximately 50% of its ATP via electron transport mediated reactions. If nitrite acts by inhibiting electron transport in rumen bacteria, in a similar manner as was found in Escherichia coli and Pseudomonas aeruginosa (Rowe, Yarbrough, Rake & Eagon, 1979), it could be expected that nitrite would severely affect the growth rate of this organism.

Results presented in Fig. 4.1 indeed shows a considerable decrease in the growth rate of Bacteroides succinogenes, strain S85 in the presence of nitrite. At pH 6,8 a nitrite-N concentration of $2 \mu\text{g.cm}^{-3}$ causes an approximately 60% decrease in the specific growth rate, while a nitrite concentration in the growth medium of $4 \mu\text{g.cm}^{-3}$ totally inhibits growth.

Fig. 4.1 further shows the effect of pH upon growth in Bacteroides succinogenes strain S85. Growth at pH 6,0 is markedly retarded even in the absence of nitrite as this pH is close to the lower limit for growth in this organism (Holdeman & Moore, 1975). Since the slopes of the specific growth rate curves are practically identical, it is inferred that the nitrite effect is not pH dependent. Inhibition by

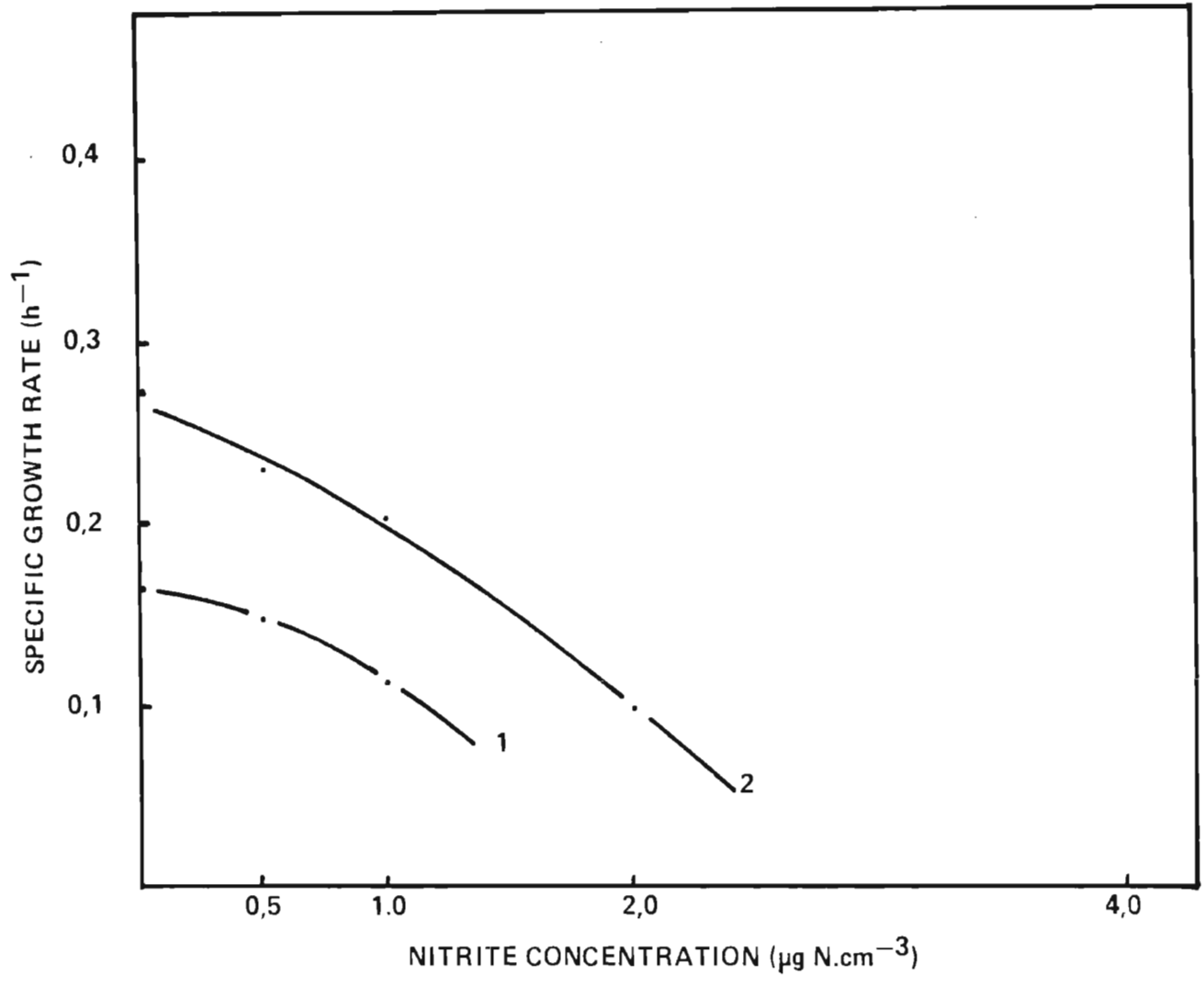


FIG. 4.1 Effect of nitrite upon the specific growth rate of *Bacteroides succinogenes* S85.
(1) pH of growth medium 6,0; (2) pH of growth medium 6,8

nitrite at the concentrations used in this experiment, therefore does not seem to depend upon the amount of undissociated nitrous acid formed in the medium as Castellani & Niven (1955) observed in Staphylococcus aureus.

Fig. 4.2 shows that, in comparison with Bacteroides succinogenes strain S85, the growth of Ruminococcus albus strain 22.08.6A, is only very slightly depressed by nitrite at a concentration of $4,0 \mu\text{g N.cm}^{-3}$. In subsequent experiments the nitrite concentration of the medium was increased to $32 \mu\text{g.cm}^{-3}$, resulting in a decrease in specific growth rate of approximately 50% at pH 6,8. If nitrite acts by inhibiting electron transport, Ruminococcus albus would be expected not to obtain much energy via these reactions. Thauer & Kröger (1984) indeed showed that ATP is formed in this organism only via substrate level phosphorylation. The inhibition of growth at high nitrite concentrations could be due to a general effect of nitrite upon essential sulphhydryl groups.

Results presented in Fig. 4.2 further show that the specific growth rate of Ruminococcus albus strain 22.08.6A, is decreased by approximately 50% at pH 6,0, even in the absence of nitrite. As in the case of Bacteroides succinogenes strain S85, results do not show an increased inhibiting effect of nitrite at pH 6,0.

Although an electron transport system has been demonstrated in Ruminococcus flavefaciens (Thauer, Jungermann & Decker, 1977), evidence for ATP synthesis via electron transport

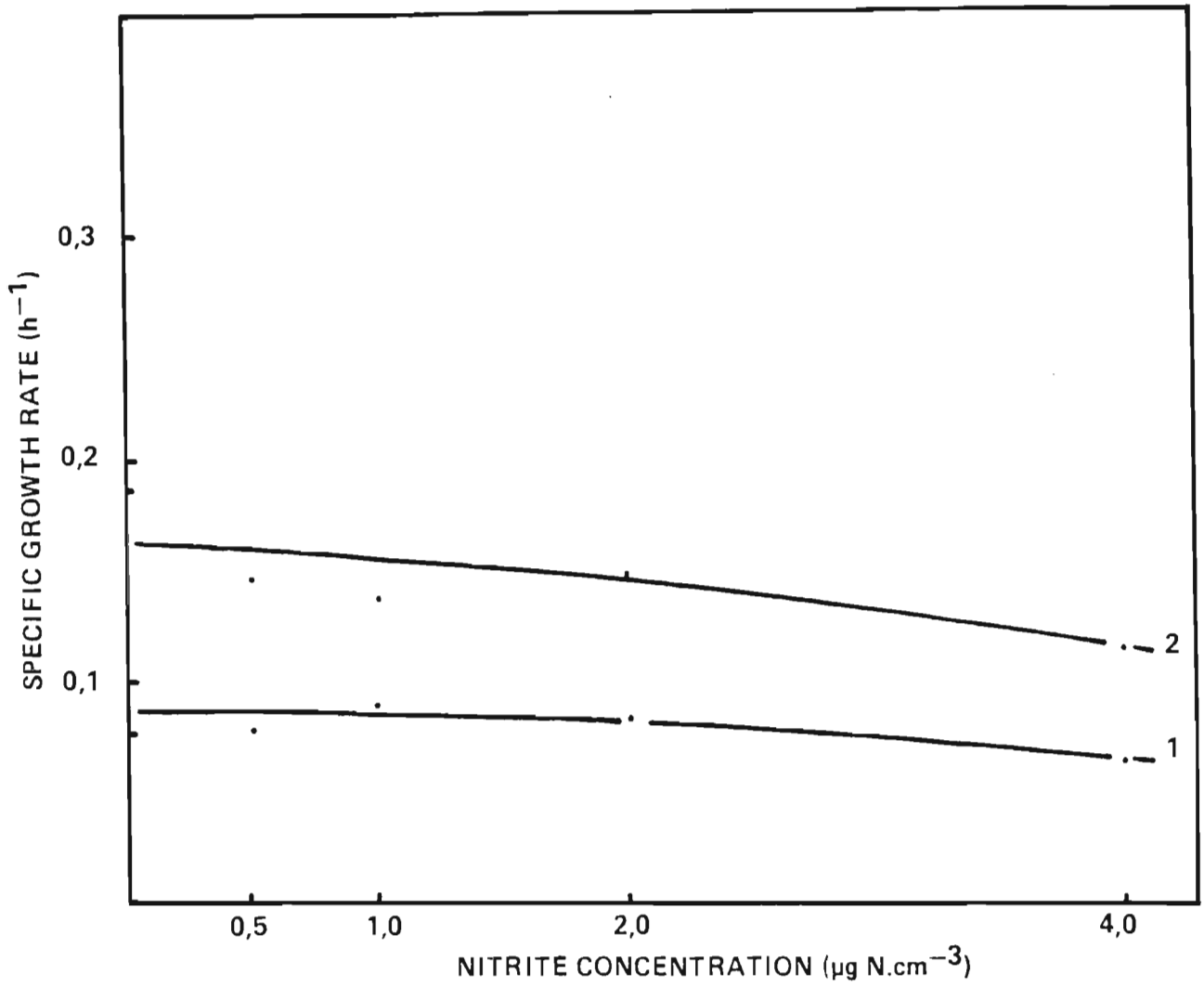


FIG. 4.2 Effect of nitrite upon the specific growth rate of *Ruminococcus albus* 22.08.6A.
(1) pH of growth medium 6,0; (2) pH of growth medium 6,8

processes is lacking (Hopgood & Walker, 1967; 1969). Results presented in Fig. 4.3 show a decline in the specific growth rate of Ruminococcus flavefaciens strain FDI, in the presence of nitrite, which could indicate the inhibition of electron transport mediated ATP synthesis. The decline in specific growth rate of Ruminococcus flavefaciens strain FDI, is much less severe than of Bacteroides succinogenes strain S85. A nitrite-N concentration in the medium of $4 \mu\text{g}.\text{cm}^{-3}$ causes a 60% decrease in specific growth rate at pH 6,8. Ruminococcus flavefaciens strain FDI, however, is much more sensitive to nitrite than Ruminococcus albus strain 22.08.6A, suggesting marked differences in ATP producing processes in different Ruminococcus species. Differences are also evident from reports showing metabolic pathways leading to different fermentation products in Ruminococcus flavefaciens and R.albus (Scheifinger & Wolin, 1973; Thauer, Jungermann & Decker, 1977).

Ruminococcus flavefaciens strain FDI, did not show any growth in either the presence or the absence of nitrite at pH 6,0.

Fig. 4.3 also shows the effect of nitrite upon the specific growth rate of Butyrivibrio fibrisolvens strain Ce51. A nitrite-N concentration of $2,0 \mu\text{g}.\text{cm}^{-3}$ in the growth medium causes a 16% decrease in the specific growth rate while $4,0 \mu\text{g}.\text{cm}^{-3}$ suppresses the growth rate by 80%. Butyrivibrio fibrisolvens strain Ce51, is therefore less sensitive to nitrite than Bacteroides succinogenes strain S85, which seems

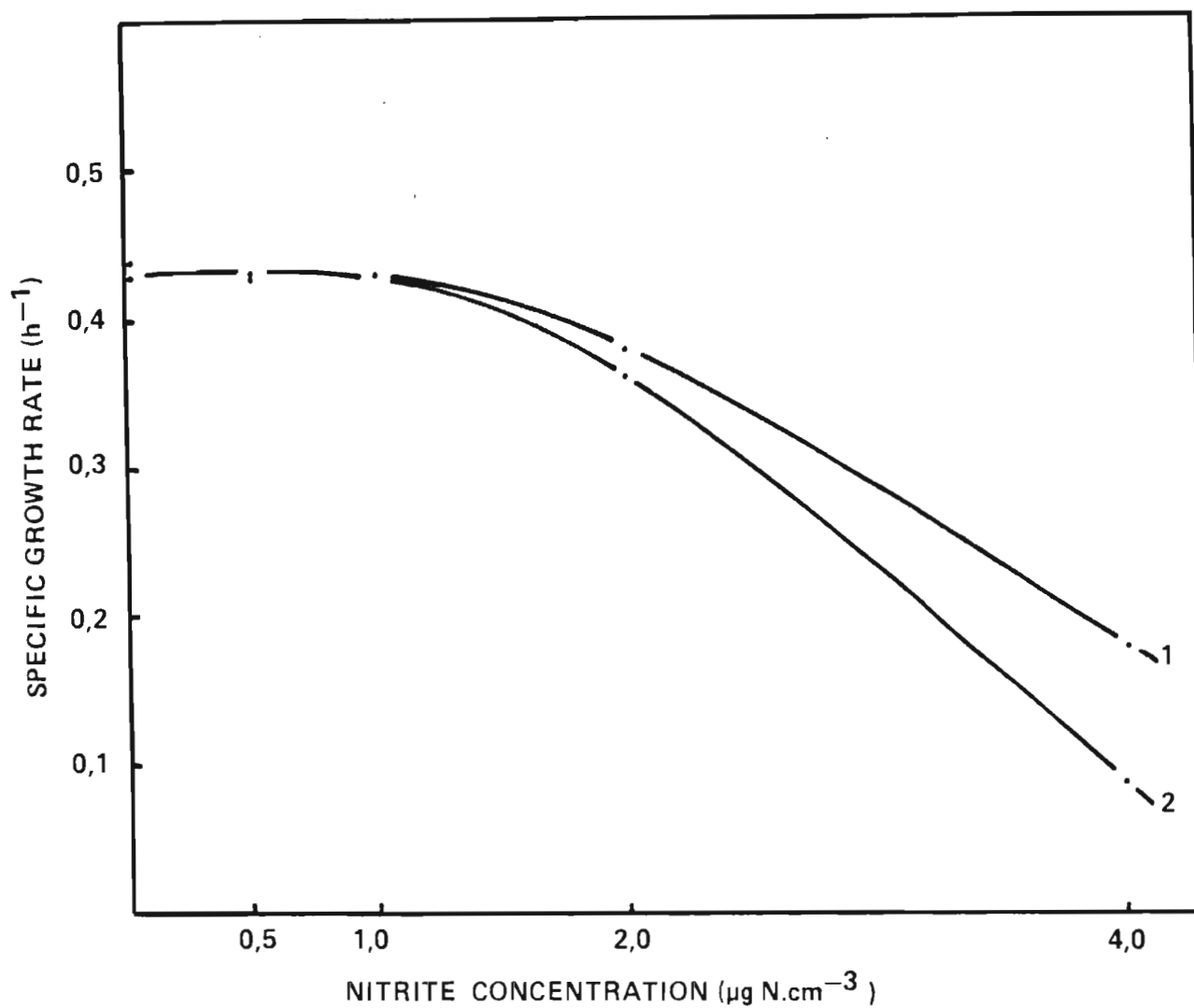


FIG. 4.3 Effect of nitrite upon the specific growth rate of (1) *Ruminococcus flavefaciens* FD1 and (2) *Butyrivibrio fibrisolvens* Ce 51 at pH 6,8

to suggest a greater involvement of substrate-level processes in ATP production in Butyrivibrio fibrisolvens strain Ce51, than in Bacteroides succinogenes strain S85. These results are in agreement with the findings of Dawson, Preziosi & Caldwell (1979) who suggested that substrate-level and electron transport-mediated processes contribute equally to energy production in Bacteroides succinogenes but that a substantially smaller proportion of energy is obtained from electron transport processes in Butyrivibrio fibrisolvens.

Butyrivibrio fibrisolvens strain Ce 51, also showed no growth at pH 6,0, even in the absence of nitrite.

The growth of the only non cellulolytic rumen bacterium investigated, Selenomonas ruminantium strain ATCC 19205, is hardly affected at all by the presence of nitrite ($4 \mu\text{g N.cm}^{-3}$) in the growth medium at pH 6,8 (Fig. 4.4). Selenomonas ruminantium strain GFA, was shown to reduce nitrate to nitrite (De Vries, Van Wyck-Kapteyn & Oosterhuis, 1974). The nitrite accumulated in the medium suggesting the absence of nitrite reductase. It is not known whether Selenomonas ruminantium strain ATCC 19205 could reduce nitrite, thus eliminating it from the medium. For this reason the nitrite content of the medium was checked at the end of the specific growth rate determination. No loss of nitrite could be detected, indicating an insensitivity of this organism to nitrite at pH 6,8. These results, therefore, suggest the absence of an electron transport mediated ATP

generating mechanism in this organism. This is in agreement with the findings of Dawson, Preziosi & Caldwell (1979) who demonstrated by means of inhibitors and uncouplers of electron transport that Selenomonas ruminantium, obtains ATP solely from substrate-level processes.

Results presented in Fig. 4.4 further shows that a reduction in pH from 6,8 to 6,0 in the absence of nitrite causes a decrease in specific growth rate. At low levels (0,5 and 1,0 $\mu\text{g N.cm}^{-3}$) the bacteriostatic effect of nitrite seems to be unaffected by pH. Above a level of 1,0 $\mu\text{g N.cm}^{-3}$ nitrite in the medium, the pH seems to have a marked effect upon the growth of Selenomonas ruminantium strain ATCC 19205, and all growth ceases at a nitrite content in the medium of 2,0 $\mu\text{g N.cm}^{-3}$. In this organism inhibition could be due to the formation (under mild acidic conditions) of nitrous acid reacting with essential sulphydryl groups, as proposed by O'Leary & Solberg (1976) for Clostridium perfringens.

Growth inhibition of the rumen bacteria tested seems to depend largely upon the extent to which these microbes derive their energy from electron transport-mediated processes. In most studies reported in the literature, on nitrate inhibition of bacteria, nitrite concentrations of the order of 140 to 1400 $\mu\text{g N.cm}^{-3}$ were used. The present investigation seems to point to a much greater sensitivity of electron transport-mediated ATP synthesis to nitrite in certain rumen microbes, but the exact nature of this inhibition is not

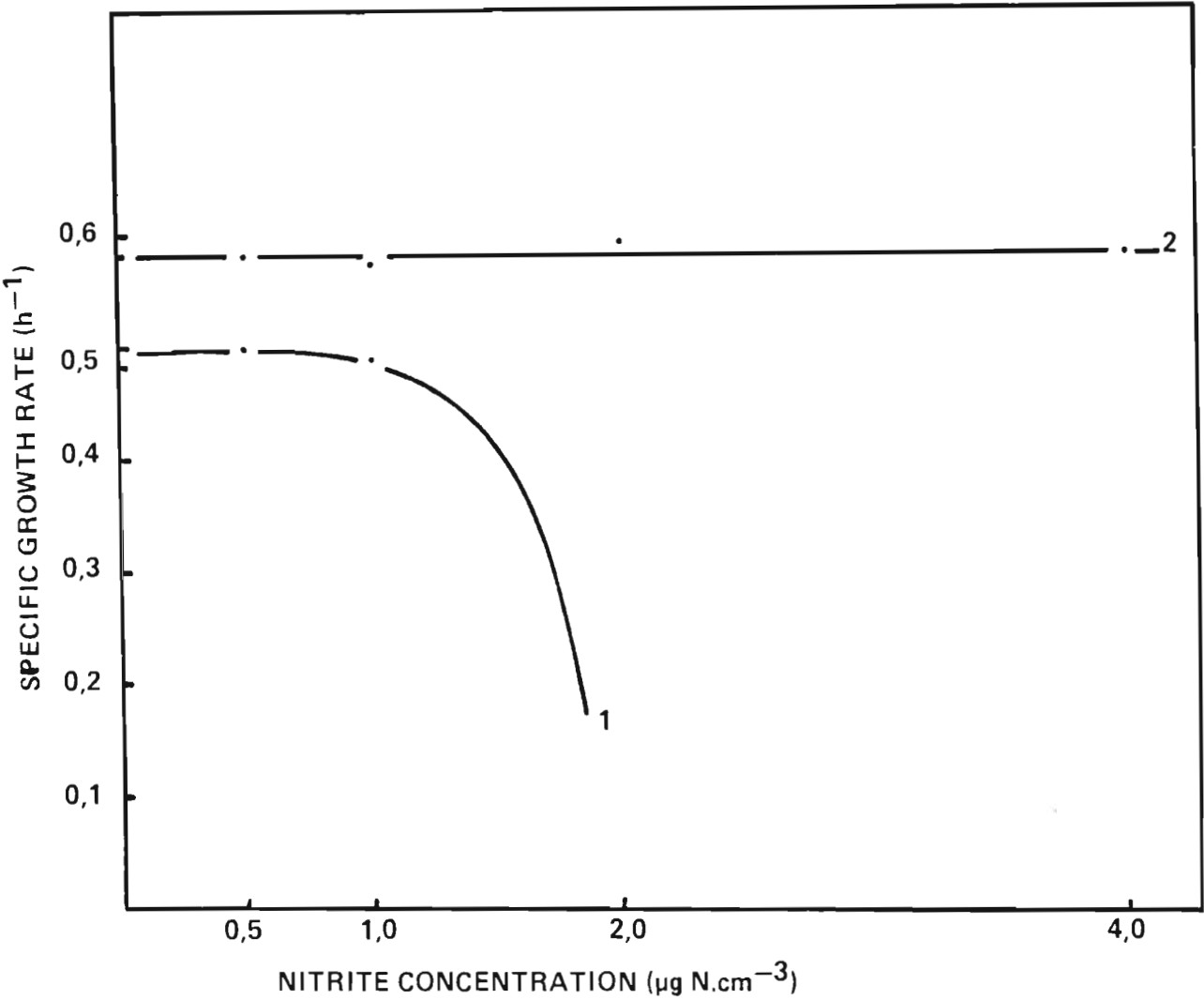


FIG. 4.4 Effect of nitrite upon the specific growth rate of *Selenomonas ruminantium* ATCC19205. (1) pH of growth medium 6,0; (2) pH of growth medium 6,8

known. In addition to this effect, nitrite seems to inhibit bacterial growth by its general affinity for essential sulphhydryl groups.

CHAPTER 5

GENERAL DISCUSSION

The studies reported in this thesis were undertaken to obtain a better understanding of the factors controlling the nitrate level in kikuyu pastures and to determine the effect of nitrate upon digestion in the ruminant.

In recent years a number of observations have led to concern about the potential risk to animal and human health resulting from the presence of nitrate and nitrite in drinking water and fodders consumed by the grazing animal and in human food products. Despite the growing awareness of the potential hazards associated with nitrates and nitrites, the possible depressing effect of these substances on animal performance due to their interference with rumen digestion, has received scant attention.

The nitrate content of a plant is controlled by the interaction of absorption and assimilation processes. Many internal and environmental factors are known to regulate or affect absorption and assimilation and these factors are bound to vary from one situation to another. In the kikuyu grass pastures under investigation, the nitrogen fertilizer level, the potassium level in the plant tissue, the morphological development of the plant and environmental

factors such as rainfall, light and temperature have been shown to be involved.

The nitrogen status of the soil seems to be one of the more important of these factors. An accurate assessment of the nitrogen status of a grazed perennial pasture such as kikuyu grass is complicated by the re-cycling of nitrogen from decaying plant matter and from faeces and urine. In this investigation no attempt was made to quantitate the contribution from these sources, but results obtained clearly suggest that this contribution is substantial. The interconversion of inorganic and organic forms of nitrogen in perennial pastures is a much neglected field of research and should receive more attention.

This investigation further focusses attention upon the high potassium levels in high nitrate kikuyu grass pastures and its role in the accumulation of nitrate in the plant tissue. Potassium is essential for normal vigorous plant growth and is readily absorbed in large quantities. Quantitatively, however, potassium plays a much less important role in animal tissue. Under normal circumstances the potassium in pastures is far in excess of the requirements of the grazing animal and is excreted in the faeces, urine, saliva and by the skin. In the grazing situation much of this potassium is returned to the pasture, where excessive amounts accumulate in the soil. It has been found that as much as 95% of the potassium of high potassium pastures is returned almost immediately to

the soil via the urine of the grazing animal (Voisin, 1963). It is therefore of prime importance that fertilizer programmes allow for the amount of potassium returned to the soil. Failure to do this may result in an ever-increasing excess of potassium in grazed pastures. The present study substantiated, for kikuyu grass pastures, the enhancing effect of potassium on nitrate absorption and accumulation observed by Blevins, Hiatt, Lowe & Leggett (1978), Frost, Blevins & Barnett (1978) and Rufty, Jackson & Raper (1981) for maize, barley and wheat seedlings.

Nitrate accumulates mainly in stem tissue. In most pasture grasses stem tissue remains out of reach of the grazing animal for a large part of the growing season and elongates only when flowering is initiated. Animals grazing kikuyu grass pastures, however, are exposed to potentially high levels of nitrate to a greater extent, due to the continuous elongation of stem internodes throughout the growing season.

A study of the effect of nitrate upon digestion in the ruminant constitutes the main thrust of this thesis. A study of this nature in the intact animal is complicated by the fact that nitrate also causes methaemoglobinaemia with its additional adverse influence upon animal health and performance. This effect could partly mask any reduction in digestion derived from the interference of nitrate with rumen microbial metabolism. The in vitro digestion technique employed in this study successfully eliminates this problem

and facilitates elucidation of the chemical changes taking place during nitrate metabolism by the rumen microbial population. A disadvantage is that it does not give a quantitative measure of the effect of nitrate and nitrite in the in vivo situation.

A quantitative comparison of the effect of high and low levels of nitrate in plant material upon rumen digestion is complicated by the fact that digestion is also influenced by the presence of other components in the plant material such as carbohydrates, proteins and fibrous substances. However, the concentrations of these substances are, in turn, influenced by the nitrogen or nitrate status of the plant. Unless stated otherwise, experimental samples with high and low levels of nitrate, but which were identical with respect to all other components, were obtained by using low nitrate kikuyu material and adjusting the nitrate level with potassium nitrate. However, in interpreting these results, it should be kept in mind that added nitrate would become available to the rumen microbes at a faster rate than the nitrate within cell structures.

By employing these techniques, it was clearly shown that the digestibility of kikuyu grass is reduced by high levels of nitrate. However, nitrate levels below 100 mg% N (m/m) in the sample actually stimulated digestion. At this concentration the rumen microbes seem capable of utilising the nitrate as a nitrogen source.

High nitrate levels in kikuyu grass is associated with elevated protein and increased levels of other non-protein nitrogenous substances of unknown composition. The present investigation did not completely eliminate the possibility that these non-protein nitrogenous substances may also affect digestion. The presence of proteins and readily metabolisable carbohydrates seem to ameliorate the effect of nitrate upon digestion. Since kikuyu grass contains a low level of readily available carbohydrate and protein compared to temperate grasses and some sub-tropical grasses, the digestibility of kikuyu grass is more severely depressed than that of these grasses.

In vitro studies showed that nitrate is rapidly reduced to nitrite and then to ammonia. The nitrite concentration of the digest peaked after a digestion period of 8 to 16h. Nitrite causes the observed reduction in digestion, while nitrate and ammonia appear to have no effect upon digestion. The concentration of nitrite, and the period of time it is present in the rumen, which are determined by the relative rates of nitrate and nitrite reduction, therefore, seem of crucial importance in determining its adverse effect upon digestion. Results have shown that animals on high nitrate kikuyu grass diets can adapt to metabolise both nitrate and nitrite more readily. The successful adaptation of animals on rotational grazing systems could be hampered by the fact that adjacent pastures or even parts of the same pasture often

vary markedly in nitrate content. Rotation between high and low nitrate pastures could lead to continuously changing, unstable rumen populations.

Adaptation is attributed to a substantial increase in the number of nitrate and nitrite reducing organisms in the rumen, but the rumen nevertheless seems to maintain a larger capacity to reduce nitrate than to reduce nitrite. The nitrite reducing capacity, therefore, seems to be the limiting factor in nitrate metabolism. Nitrite reduction could be improved by the introduction into the rumen of readily metabolisable carbohydrates, which tend to lower the rumen pH to within the optimum range of nitrite reductase activity.

The rate of formation of nitrite in the rumen can be reduced by dosing the animal with sodium tungstate, an inhibitor of nitrate reductase. However, Geurink, Malestein, Kemp, Korzeniowski & Van't Klooster (1982) pointed out that tungstate should be used with caution, as its effect upon animal health has not been fully assessed.

Since the rumen seems to have a higher capacity to reduce nitrate, care should be taken not to introduce nitrate at a rate in excess of the nitrite reducing capacity of the rumen microbes. The high moisture content of fresh kikuyu grass limits its intake. Nevertheless, results showed that the

intake and release of nitrate from fresh high nitrate (500 mg% N) kikuyu grass is sufficiently high to cause the accumulation of nitrite in unadapted sheep. In adapted sheep, however, the release was not sufficient to cause the accumulation of nitrite. The further introduction into the rumen of nitrate in a freely available form, readily exceeded the nitrite reducing capacity and led to nitrite accumulation and methaemoglobinaemia.

In contrast to fresh grass, the cell membranes of preserved roughage such as silage or hay are completely permeable and the nitrate in this material would readily diffuse into the rumen fluid, leading to higher rumen nitrite levels.

Different ruminants behave differently to nitrite. In the present investigation sheep were used as experimental animals, but cattle apparently produce much more methaemoglobin than sheep on equivalent nitrate intakes and seem to be more susceptible to poisoning (Bradley, Eppson & Beath, 1939; Setchell & Williams, 1962).

A large part of this investigation was devoted to a study of the mode of action of nitrite in rumen digestion. A reduction in digestibility involves a decrease in the solubilisation of the structural carbohydrate components of the grass. During digestion solubilisation is effected by enzymes, produced mainly by the cellulolytic and xylanolytic rumen microbes. Attempts to isolate these enzymes by means of gel filtration,

PAGE and isoelectric focusing on Bio Gel P-150, in order to study the effect of nitrite upon their production and activity, largely failed due to the affinity of the enzymes for the cellulose substrate. Adsorption of cellulases onto the substrate, providing sugars in close proximity to the cellulolytic microbes, which are also associated with the substrate, must be of considerable advantage to these microbes in the rumen environment where many other microbes also compete for liberated sugars. The advantage of adsorption to the substrate is further increased by the fact that the soluble fraction of the digest has a faster flow rate than solid components.

The adsorptive properties of the enzymes could be exploited by considering the digested grass as a crude enzyme extract. This made possible the semi-quantitative assessment of enzyme activities during digestion, in the presence and absence of nitrate. Using this technique it could be shown that nitrate, nitrite and ammonia do not directly affect cellulase activity and a chromatographic study of the end products showed no accumulation of hydrolysis products and ruled out the possibility of end product inhibition of cellulase activity in digests in vitro.

Nitrite was shown to retard the growth of cellulolytic, xylanolytic and total rumen microbes. The cellulolytic bacteria were affected most and they recovered only relatively slowly from exposure to nitrite. Low cellulolytic

and xylanolytic microbial counts were associated with similarly low levels of carbohydrases produced by these microbes in the digests, suggesting a direct inhibiting effect of nitrite upon bacterial growth rather than an effect only upon carbohydrase synthesis.

The retarded growth of cellulolytic bacteria observed in the presence of nitrite was shown not to be due to a lack of branched chain fatty acids known to be essential for the growth of these bacteria. Furthermore, measurement of the redox potential of digests in vitro revealed conditions of high and often positive redox potentials prevailing during the initial stages of digestion. These conditions are aggravated by the presence of nitrite due to its oxidising properties. However, retarded growth of the rumen microbes could not be ascribed to the unfavourable redox potential of the high nitrate digests, as lowering the redox potential by adding cysteine did not eliminate the inhibiting effect of nitrite. The rapid growth of the different groups of bacteria under conditions of elevated redox potential seems to point to the ability of these organisms to create micro environments suitable for their survival.

The study of the effect of nitrite upon pure cultures of rumen bacteria revealed the extreme sensitivity of Ruminococcus flavefaciens strain FDI, Butyrivibrio fibrisolvens strain Ce 51 and Bacteroides succinogenes strain S85, to nitrite (2 to 4 $\mu\text{g N.cm}^{-3}$) in the medium. These

organisms are known to obtain a large part of their energy from electron transport mediated reactions and the inhibition by nitrite of some vital part of this process is envisaged. The exact mechanism of inhibition is as yet unresolved, but the extreme sensitivity of certain rumen bacteria, and the lack of a pH effect on inhibition in most of the bacteria investigated, may suggest a different mechanism than that observed in Staphylococcus aureus and Clostridium perfringens, which are sensitive to nitrite at concentrations of 140 to 1400 $\mu\text{g N.cm}^{-3}$ (Castellani & Niven, 1955; O'Leary & Solberg, 1976). However, it may also point to a limited contribution of electron transport to the overall energy production of Staphylococcus aureus and Clostridium perfringens.

The growth of Ruminococcus albus strain 22.08.6A and Selenomonas ruminantium strain ATCC 19205, which obtain their energy from substrate level processes only, is not affected by nitrite at low concentrations. Higher nitrite concentrations ($32 \mu\text{g N.cm}^{-3}$) have been found to be partly inhibitory to Ruminococcus albus strain 22.08.6A. This is not unexpected since nitrite has been shown to react with many cell substances, especially sulphydryl groups. The effect of nitrite is sometimes aggravated at low pH values (as was observed in Selenomonas ruminantium) due to its conversion to more reactive nitrous acid. However, in the rumen this effect may be ameliorated by the more rapid reduction of nitrite to ammonia at low pH conditions.

The growth rate is an important factor in determining the success of an individual bacterial species within the rumen. The dilution rate of the rumen liquid phase is usually lower than the maximum growth rate of rumen bacteria, but a decrease in growth rate of individual bacteria could lead to the washing out of these microbes, especially those moving in the liquid phase of the rumen digest. Small differences in the growth rate of bacteria could, therefore cause considerable changes in the composition of the microflora of the rumen in the long run. Fortunately many rumen microbes and especially the cellulolytic bacteria are particle bound and move more slowly than the rumen fluid. The organisms attached to these particles have a better chance of survival despite a reduction in growth rate in the presence of nitrite. Growth inhibition of three of the four major cellulolytic microbes in the rumen readily explains the adverse effect of nitrite upon digestion in vitro. In addition, it may also have serious long term effects in vivo due to the washing out of slower growing microbes.

In the presence of nitrite, Ruminococcus albus has an advantage over the other major cellulolytic microbes and is likely to increase in numbers. However, it is not known if, or to what extent, Ruminococcus albus and perhaps other less important nitrite tolerating cellulolytic organisms, can replace the normal cellulolytic populations and restore cellulose digestion in the presence of nitrite. This

therefore constitutes a promising area for further study.

CHAPTER 6

SUMMARY

In the high rainfall areas of Natal milk producers rely heavily upon kikuyu grass pastures during the summer months. The nutritive value of kikuyu grass is often lower than is expected. As high levels of nitrate are often present in this grass, attempts were made to establish the cause of these high nitrate levels and to study the effect of nitrate upon digestion in the ruminant.

The nitrate level of kikuyu grass was shown to be affected by the nitrogen level of the soil, the potassium level of the plant, the developmental stage of the plant and by climatic factors such as temperature, light and rainfall. The nitrogen and potassium levels of the soil seem to be major factors controlling nitrite levels in kikuyu grass. Furthermore, the morphology of the kikuyu plant allows constant exposure of high nitrate containing tissue to the grazing animal.

The presence of high levels of nitrate in kikuyu grass was shown to reduce digestion in vitro. The degree of reduction depends upon the level of nitrate in the plant, but is further modified by the presence of readily available carbohydrates and protein in the digest.

It was shown, by means of nitrate reductase inhibiting studies using tungstate, that the adverse effect of nitrate upon digestion, is due to the formation of nitrite by certain rumen microbes during the assimilatory reduction of nitrate to ammonia. Studies in vivo showed that the rumen microbial population of sheep can, to a certain degree, adapt by reducing nitrate and nitrite more rapidly, thus preventing nitrite from accumulating in the rumen under ad lib. feeding of fresh high nitrate (550 mg% N) kikuyu grass. However, if the capacity of the nitrite reducers is exceeded by the introduction into the rumen of readily available forms of nitrate, nitrite accumulates. The increased level of nitrite has a deleterious effect upon the animal, both by suppressing digestion and by elevating levels of methaemoglobin in the blood.

Examination revealed that although nitrite reduces the digestion of cellulose, it has no direct effect upon the activity of rumen cellulases. The carbohydrases of the rumen digest are particle bound. Unsuccessful attempts were made to isolate these carbohydrases by means of gel filtration, PAGE and isoelectric focusing. Furthermore, the possibility of end product inhibition of cellulases was discounted as a possible cause of the observed decreased cellulose digestion in vitro.

Nitrite was shown to have a direct effect upon the rumen microbial population. In the presence of nitrite, cellulolytic, xylanolytic and total microbial numbers

decrease with a concomitant decrease in cellulase and xylanase activity of the digest. The decrease in microbial numbers could not be attributed to an increase in the redox potential of the digest due to the oxidising nature of nitrite. Cellulolytic microbes are most sensitive to nitrite, but although the presence of nitrite changes the volatile fatty acid composition of the digest, the reduction in cellulolytic microbes was shown not to be the result of a lack of essential branched chain fatty acids required for cellulolytic microbial growth.

A study of the effect of nitrite upon pure cultures of rumen bacteria revealed the extreme sensitivity of some of the major cellulolytic microbes to nitrite. The growth of Ruminococcus flavefaciens strain FDI, Butyrivibrio fibrisolvens strain Ce51 and Bacteroides succinogenes strain S85, was severely inhibited in the range of 2 to 4 $\mu\text{g N.cm}^{-3}$. In turn, Ruminococcus albus strain 22.08.6A and Selenomonas ruminantium strain ATCC 19205, a non-cellulolytic species, were only slightly affected by these nitrite levels. Ruminococcus albus and Selenomonas ruminantium obtain their energy from substrate-level processes only, while Ruminococcus flavefaciens, Butyrivibrio fibrisolvens and Bacteroides succinogenes in addition obtain energy from electron transport mediated processes. Low levels of nitrite, therefore, seem to inhibit growth in these bacteria by affecting some vital part of the electron transport energy producing system. Higher concentrations of nitrite seem to

have a further general inhibiting effect upon rumen microbes, possibly by reacting with essential sulphydryl groups.

Nitrate affects digestion of kikuyu grass primarily through the action of nitrite upon energy producing electron transport processes in some key cellulolytic bacteria in the rumen. The extent of the effect upon digestion and animal production, however, depends upon the degree to which nitrite accumulates in the rumen and on the ability of the rumen microbial population to change towards a population containing an increased proportion of species capable of tolerating nitrite, especially such species which can also digest cellulose.

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