PATTERN OF VFA. PRODUCTION FROM ROUGHAGES AND CONCENTRATES COMPARED TO THOSE FROM PURE NUTRIENTS IN 'IN VITRO' FERMENTATION OF GOAT'S RUMEN CONTENT.

---x---

Thesis

submitted to the faculty of Veterinary Science and Animal Husbandry, Magadh University, in partial fulfilment of the requirements for the Degree of MASTER OF SCIENCE in Animal Husbandry.

...

BIHAR VETERINARY COLLEGE
PATNA
Acc. No. 2993
Date 22.1.'65

AN/128

BY:

SURYANARAYANA RAO,
B.V.Sc., & A.H.,
Bihar Veterinary College, Patna.
(October, 1963).

oOo

I certify that this Thesis has been prepared under my supervision by Sri Suryanarayana Rao, a candidate for the M.Sc. (A.H.) with Animal Nutrition as major subject, and that it incorporates the results of his independent study.

D. B. Mukherjee
16.10.63

(D.B.MUKHERJEE)
M.Sc., Ph.D.,
Professor of Animal Nutrition,
Bihar Veterinary College, PATNA.

C O N T E N T S.

		Page No.
INTRODUCTION.	... ...	1
CHAPTER (I):	Review of Literature. ...	12
CHAPTER (II).	Materials & Methods. ...	34
CHAPTER (III).	Estimation of errors in various experimental procedures.	52
CHAPTER (IV).	R e s u l t s. ...	57
CHAPTER (V).	D i s c u s s i o n. ...	88
CHAPTER (VI).	S u m m a r y. ...	122
	References ...	(i) to (vii)

A C K N O W L E D G M E N T S.

I like to express my sincere gratitude to my revered teacher, Dr. D.B.Mukherjee, M.Sc., Ph.D. (Aberdeen), Professor of Animal Nutrition, for suggesting the problem, continuous guidance, valuable suggestions and constant encouragement throughout the work.

I also wish to express my grateful feelings to:

(1) Sri S.A.Ahmad, B.Sc., M.R.C.V.S., Principal, Bihar Veterinary College, Patna, for extending generous facilities for the work.

(2) Sri J.N.Prasad, M.Sc., Research Officer, Statistics, Live Stock Research Station, Bihar, Patna, for advice on the methods of statistical analysis performed and on arriving at inferences therefrom.

... SURYANARAYANA RAO.

CHAPTER (I)

INTRODUCTION.

I N T R O D U C T I O N.

The study of the ruminant nutrition in many respects is the study of a remarkable symbiosis between the animal and the vast population of bacteria and infusoria which inhabit its digestive tract. This salient feature of ruminant metabolism makes it well-nigh impossible to apply results of nutritional investigations made with the common laboratory animals to ruminants.

The rumen has long been filled with mystery. Heard (1906) stated that the rumen of a cow was the darkest place on earth. Thanks to the shining light of science, many of these mysteries are being resolved. For the last twenty years, there has been a growing recognition of the importance of the rumen to the overall nutrition of the ruminant.

A note-worthy peculiarity of digestion in ruminants is their ability to break down cellulose and other complex polysaccharides of fiber. Ruminants do not secrete any enzyme in their digestive tract which can digest cellulose and other complex polysaccharides. It is the host of microorganisms which inhabit the rumen, both bacteria and protozoa, bring about its digestion. This microbial activity enables a much wider variety of carbohydrate materials in the diet of these herbivores to be digested.

Rumen microbes, irrespective of the type of the carbohydrate supplied ferment them to VFA, Co_2 and methane. The chief VFA produced are Acetic, Propionic and Butyric acids. Apart from these small amounts of other higher fatty acids are also produced. Phillipson (1947) pointed out that cellulose is broken down to cellubiose, glucose, pyruvate, lactate and ultimately to VFA. The relative proportions of the VFA produced varies with the nature of the acid and possibly with the species and individuality of the animal. The proportions of the VFA in the rumen liquor of the goats on common diets are: Acetic 50 - 70%, Propionic 20 - 40%, n-butyric 10 - 20% and other fatty acids together constitute less than 1% of the total.

Rumen microflora apart from playing a major role in the utilization of the energy-yielding constituents; they also attack the proteins of the foodstuff. Sym (1938) found rumen contents to be strontly proteolytic and Pearson and Smith (1943) got clear evidence of proteolytic activity with soluble proteins. The intermediates of this protein breakdown are the Amino Acids which are partly synthesized into microbial proteins and partly metabolized to Ammonia and the short-chain fatty acids, mostly branched chain isomers of C_4 and C_5 fatty acids (El-Shazly, 1952). The ammonia so produced is partially absorbed into the

blood stream through the rumen wall (McDonald 1948) which is later metabolized in the liver to urea and partly utilised by the bacteria to form their own body protein.

As the microorganisms pass down the digestive tract, their body proteins are utilized by the host like food proteins. The nature of the microbial proteins are quite different from the food protein. The microbial proteins, however, have a high Biological value and help the host to be largely independent of the Amino Acid composition of the diets. The experiments of Gray (1953) on hay fed sheep suggest that about 50% of the Nitrogen of the rumen contents is microbial protein nitrogen and that this degree of conversion of food Nitrogen to microbial Nitrogen continues in the rumen.

Rumen microorganisms have got the ability to convert NPN compounds into microbial protein that contains all the essential Amino acids. These findings are of great economic importance, since a part of the crude protein in roughages are NPN. Ruminants no longer need to compete with monogastric animals for protein concentrates because urea (NPN) is being produced in ever increasing quantities. It has been found that a liberal supply of soluble carbohydrates, particularly starch, is needed for effective utilisation of urea.

The most efficient use of NPN is obtained only when urea is added to rations containing low levels of protein. Rumen bacteria are also able to convert inorganic Sulphur to the sulphur containing Aminio Acids.

Microorganisms during their existence in the rumen also synthesize all the components of Vitamin B group and Vitamin K, before the food enters the true digestive and absorptive tracts, so that especially favourable conditions exist for the utilization of the products.

For bringing about various degradative and synthetic processes mentioned above, microorganisms of the rumen require a suitable environment. Constant passage of food in the rumen, secretions of the salivary glands, the process of rumination, passage of digesta into the small intestine absorption of the end products of microbial activity through rumen wall, constant temperature of 39° C, pH buffered neutral and anaerobiosis through the production of Co_2 and other gases by fermentation of the food stuffs, help in keeping the 'fermentation vat' working.

All these peculiarities would seem best considered as by-products or consequences of its peculiar microbiological method of utilizing nature's most abundant living source of energy, Cellulose. This method is a perfect

example of obligate symbiosis for the micro-fauna and microflora of the rumen cannot persist long without the rumen host. The host, likewise soon becomes very sick if its rumen microbiology is extensively disturbed.

As the end products of microbial digestion in the rumen are short chain fatty acids, one is naturally inclined to know the rates at which these different fatty acids are produced. This cannot be determined directly because of the following factors which stand on the way of their estimation 'in vivo'. Firstly, fatty acids which are produced are being diluted by constant passage of saliva, in take of food and water. Secondly, because of the appreciable interconversion of butyrate into acetate (Mukherjee, 1960). Thirdly, due to the metabolism of the acids, particularly butyric by the rumen wall itself (Pennington, 1952). Fourthly, due to absorption of the fatty acids through the rumen wall (McAnally & Phillipson, 1942; Barcroft etal. 1944a).

Rates of production can, however, be estimated by fermenting the foodstuffs 'in vitro', which can only be approximate, as it imposes lot of unnatural conditions on the microbiota and the limitations of which will be dealt later. Mukherjee (1960) who for the first time gave a solution for this long awaited problem by the

application of Isotope dilution technique 'in vivo' rates of production of the acids are as follows: Acetic 4.79, Propionic 1.10 and Butyric 0.6 mil eq/hr/kg of rumen content respectively.

It has been shown by various workers that absorption of lower VFA's occurs from the rumen itself (Barcroft, McAnally & Phillipson, 1944b). Danelli et. al. (1945) have studied the mechanism of absorption from the rumen as it affects the lower fatty acids, acetic propionic and butyric. Absorption of the lower fatty acids from the rumen is influenced by pH of the rumen content. It has been seen that maximum absorption occurs at pH 5.8. The determination of the rate of absorption of the different fatty acids from the rumen is also difficult process because of the difficult surgical procedures involved. Mukherjee (1960) intelligently estimated for the first time accurately the rates of absorption of the three acids in the rumen from their 'in vivo' production rates by the application of Isotope dilution technique in fasting sheep which are as under: Acetic 38.8, Propionic 9.66, Butyric 4.22 mil mole/hr/kg. rumen contents respectively.

Probably the most outstanding rumen research has been concerned with the end products of carbohydrate digestion. It is well recognised that the end products of mocrobial fermentation of the carbohydrates being short

chain fatty acids, mainly acetic, propionic and butyric as against glucose in simple stomached animals, naturally one is inquisitive to know the fate of these acids.

Propionic acid is a precursor of glycogen (Eckstein, 1933; Deuel et·al·1935). The administration of propionic acid to Phloridizinised dogs results in the formation of equivalent amounts of glucose in the urine (Ringer, 1912). Pennington and Sutherland (1956) have shown that it enters the Tricarboxylic acid cycle by Carboxylation to Succinate.

Butyrate is more controversial. It is generally acknowledged to be Ketogenic. Pennington (1952) showed that it is metabolised by rumen wall resulting in the production of Ketone bodies. There is some evidence that it can cause a rise in the level of blood glucose, lactate and pyruvate in the sheep (Potter 1952).

Acetic acid is quantitatively the most important acid produced in the rumen. It is an important metabolic intermediate and a precursor of wide variety of compounds. Acetic acid is utilized by liver (Leloir & Munoz, 1939) and by brain tissue (Reid, 1950). It is also used in the synthesis of milk fat (Popjak et·al·1951) and Lactose (Cowie et·al·1951). The carbon moeity of Acetic acid has been traced also to Cholesterol (Bloch et, al, 1946) and Milk Casein (Kheiber et, al, 1952).

The short chain fatty acids are quantitatively much more important than glucose as a source of energy to the ruminant Carroll and Hungate (1954) have calculated

that in cattle some 6000 - 12000 Cal of energy are available from the acids produced by fermentation in the rumen. This was found to meet 70% of the maintenance energy requirement in steers. With sheep, Phillipson & Cuthbertson (1956) have calculated from the results of Schambye (1951a, b, 1955) that atleast 600 - 1200 Cal of energy in the form of steam VFA's could be absorbed every 24 hours. Maintenance requirement of sheep was calculated by Armstrong and Blaxter (1956) to be 1200 Cal/day on this basis about 85% of the maintenance energy requirement of the sheep will be met with from VFA. Mukherjee (1960) calculated that even in fasting sheep where production rate is minimal about 25% of the maintenance need is met from VFA.

Among other things, ruminant animal has by virtue of the activity of the microflora of its digestive tract the ability to digest cellulose and other plant constituents not attacked by the digestive secretions of Vertebrates, nevertheless, this distinction and ability is not without certain disadvantages. Apart from the fact that the steam VFA which are the major end products of bacterial digestion of cellulose are used less efficiently than is hexose the bacterial dissimilation in itself is a wasteful process.

Nutritionists all the time were striving hard to provide a measure of the ability of foods to meet particular energy requirement. Forbes et.al.(1931) stated that the nutritive value of an individual foodstuff was not constant, because they noted a threefold increase in heat increment with steers fed an all-corn meal diet as compared to a diet of corn meal plus alfalfa hay.

Recently Armstrong et.al.(1957) have found that acetic acid has the highest heat increment of 41%, propionic 14% and butyric 16%.

In recent years, considerable interest has been focussed on the rumen fatty acids, their importance as a source of energy for the ruminant and their possible implications in bovine Ketosis. Since the various acids present have different energy values, information on factors affecting their relative concentration in the rumen is of considerable importance.

In order to predict the ability of a food to serve as an energy source, it is necessary to know not only how much of it is apparently digested, it is also necessary to know the chemical nature of the end products of digestion which are finally absorbed into the body.

If the major metabolites arising from bacterial dissimilation of Carbobydrate which reach the tissues of the ruminant are the SVFA's (Phillipson and Cuthbertson, 1956), then foods must have different nutritive values in terms of energy when they are used above and below maintenance datum. Above maintenance a unit of food will be less efficiently used than below maintenance. This has long been realised. Results from several laboratories show that with normal rations the net availability of the metabolizable energy of food for maintenance is about 85% and that for fattening about 55% (Blaxter, 1950-1). What is implied is that besides there being a general reduction of efficiency above maintenance, the reduction might be greater with some feeds than with others.

Combining the above two ideas i.e. that both food-stuffs and individual fatty acids vary in their nutritive values, because food stuffs differ in their nutritive value and also in the fatty acids produced from them. It is logical to presume that different foodstuffs which differ in their nutritive value must also differ in their production of the proportion of VFA's.

The present study was designed to evaluate the 'in vitro' production of VFA by a mixed cell suspension from goat, fermenting various substrates, consisting of feeds both similar and dissimilar to the ration consumed by the donor animal.

An effort has also been made to find out the pattern of VFA's produced by different pure nutrients 'in vitro'; with the idea of correlating the fatty acids structure of different foodstuffs with their nutritive value.

Mukherjee (1960) in an effort to find out the distribution of activity on incubation of rumen content with labelled acetate, propionate and butyrate, surprisingly enough found that with butyrate 35% of the initial activity could not be recovered in the fatty acids.

An effort has also been made here to investigate, if the unaccounted butyrate activity observed by Mukherjee (1960) can be traced to the formation of Ketone bodies, one of the logical end products of butyric acid, similar to that occuring with rumen epithelium (Pennington, 1952).

--ooOoo--

CHAPTER (II)

REVIEW OF LITERATURE .

Chapter I.

REVIEW OF LITERATURE.

Artificial Rumen:-

The advent in recent years of the rumen fistula and the artificial rumen technique has provided important research tools which have contributed greatly to our knowledge of rumen function. The artificial rumen has been very useful in exploring the untapped resources held with the dark recesses of the rumen. In this technique an attempt is made to impose upon the withdrawn rumen sample conditions which duplicate those within those within the animal, so far as these are known as the cellulolytic bacteria (Bungate, 1950) and Protozoa (Bungate, 1942) of rumen are very sensitive to change in its environment.

Elsden, Johns and other co-workers first adopted the procedure of incubation of rumen content of mixed microbial population to study the rumen fermentation which opened a new chapter in this field. They used 'washed suspension' of rumen microorganisms and since then various improvements have been achieved in this field. Generally, the artificial rumen technique developed which is now adopted universally to conduct 'in vitro' studies.

The early attempts at developing an 'in vitro' system for studying rumen microbial activity consisted of primarily incubating strained rumen fluid with various substrates and measuring microbial activity in several ways.

Wegner et.al.(1940) used whole rumen contents. These workers criticised the artificial rumen in that there was no movement and no diffusion. Quin (1943) incubated various carbohydrates with rumen fluid in gas tight flasks and measured gas production manometrically. Although the system was crude, he concluded candidly that in view of the close relation between ruminant digestion and bacterial activity, the nutrition of ruminants is vitally linked to various products derived from bacterial metabolism - hence the necessity of further investigation. Pearson and Smith (1943) and Smith and Baker (1944) used samples of rumen ingesta strained through muslin. Since their experiments were short term, no elaborate attempt was made to duplicate exactly the rumen contents. Marston (1948) improved the same by attempting to regulate the pH with the addition of suitable buffer and maintained anaerobic condition.

Burroughs et.al.(1950) made further improvement by providing flow of artificial saliva at definite rate. Louw et.al.(1949) used semipermeable vessel which dialized the fermentation product by diffusion resembling close to 'in vivo' condition. Warner (1956) used 'Cellophane Sac' containing rumen liquor and substrate - it is dialysed against a mineral solution similar in composition to rumen liquor and got predictable results even upto 8 hours of incubation.

The use of various types of artificial rumen has also made possible the screening of many compounds in the laboratory to study their effect on digestion and synthesis by rumen microbes.

Though many valuable improvements have been made with 'in vitro' systems, the fact that the live ruminant is the animal without which we are finally concerned must be remembered. Rumen maintains an extremely complex and interdependent sequence of microbial reactions, which is inevitably grossly disorganized when removed from its natural environment. Data obtained with 'in vitro' technique must, ofcourse, be interpreted with caution.

Volatile Fatty Acids in Rumen:- Tappeiner (1883) first demonstrated that the fermentation of cellulose in the rumen of ox resulted in the production of large amounts of volatile fatty acids. He ascribed little nutritional value to the fatty acids. This idea was overwhelmed by the work of Kellner (1900) in which cellulose was found to be of the same energy value as starch when fed to steers. Woodman (1930) postulated that glucose was a normal intermediary in cellulose fermentation, glucose was the product which was absorbed.

Woodman and Evans (1938) admitted that lower volatile fatty acids were among the principal end products of fermentation of cellulose. Marston and his colleagues had long suspected their importance. It was, however, the development of new Chromatographic technique of Condsen et.al.(1944) that led Elsden (1946) and later Hoyle et.al.(1948) to design reliable methods for characterizing the VFA which arise from fermentation in the rumen. Phillipson and McAnally (1942) observed that hexoses were rapidly fermented to lactic acid in the sheep rumen and this metabolite was consequently converted to lower VFA's mainly propionic acid. Elsden (1945-46) for the first time separated VFA's and stated that under natural conditions acetic, propionic

and butyric are the main acids present and acetic acid predominates in concentration. Marston (1948) showed that there is little doubt that the fatty acids arising from the dissimilation of Carbohydrates in the paunch provide for the ruminant its main source of energy.

Gray et.al.(1951) incubated wheaten hay 'in vitro' for varying lengths of time, found that in earlier stages there was remarkable production of acetic acid almost free from other fatty acids. In later stages, larger amounts of the higher acids and particularly propionic acid, were formed.

Gray et.al.(1952) found that a large amounts of higher fatty acids are produced when wheaten hay was incubated 'in vitro' for longer periods of incubation. They showed with labelled propionic and acetic acid that considerable part of the higher fatty acids in the rumen fluid were produced from synthesis by condensation of the lower ones with two carbon compounds in equilibrium with acetic acid.

Annison (1954) stated that small amounts of formic acid as well as some branched chain acids are occasionally present. At present the major fatty acids such as acetic, propionic and butyric acids are considered as energy sources and key anabolic intermediates rather than waste products of digestion.

Rate of production of VFA in Rumen:- Determination of rate of production of VFA 'in vivo' is a complex one. Gray and Pilgrim (1951) have tried to assess the rates of production of the individual VFA's 'in vivo' after feeding by following changes in the relative proportions of the acids in the rumen. Later Gray and Pilgrim (1952) have fermented powdered wheaten hay 'in vitro' by rumen liquor from a sheep-fed on the same diet and obtained a rate of 8.1 mmoles between 2 - 4½ hours and 8.7 m moles between 4½ to 7½ hours. Thereafter, the rate fell off sharply, possibly due to lack of substrate. Carroll and Hungate (1954) found that fermentation of rumen content for 4 hours from a hay fed steer, drawn 16 hours after feeding produced acids at a rate of 8.3 m moles per hour per lit. of rumen liquor, while the 13 hours sample gave a rate of 12.5 m moles. Apparently the rate tended to fall off between 13 and 16 hrs. Mukherjee (1960) for the first time by the application of Isotope dilution technique determined the rate of production in sheep normally maintained on oats and hay, ~~in~~ ~~in~~ 'in vivo' to be 7.18 m moles per kg/hr of VFA in fasting sheep. These results cannot be compared with any similar data as no previous work has been done on that line. An alternative approach to the problem of measuring ruminal VFA production is based on the incubation of whole rumen contents for short periods under conditions which simulate those of the rumen

(Carrol and Hungate 1954; Halse and Velle 1956). The VFA production 'in vitro' was compared with the changes in the concentration of VFA's in the rumen during the same time interval. An assumption implicit in this work is that VFA production 'in vitro' proceeds at a rate similar to that which occurs in the rumen which is far from correct.

Pattern of VFA production on various Dietary Regimes:

A number of investigations have shown that the relative concentrations and proportions of individual VFA production are dependent on the nature of diet, species of animal and even with individuals of the same species (Phillipson, 1947). Until recently there was very little data on the pattern of VFA production in the rumen on different diets, because of the lack of better techniques for separation of fatty acids. The identity of the acids present in the rumen remained in doubt until Elsden (1945-46), developed a partition Chromatogram which separated quantitatively mixtures of fatty acids. With the introduction of Elsden's Silica gel partition chromatogram introduced a new chapter in the ruminant nutrition. Some of the findings of the various workers have been shown in table (1).

Table (1)

Table showing the products of fermentations 'in vitro' on various substrates.

SUBSTRATE.		Animal.	A.	P.	B.	Reference.
Cellulose.	...	Sheep.	42.10	54.50	3.40	Elsden (1945-46)
Glucose	...	Sheep.	23.20	51.20	25.60	
Dried grass	...	Sheep	52.50	34.50	13.00	
Wheaten hay	...	Sheep	46.00	41.00	13.00	Gray et.al. (1951)
Leucern hay	...	Sheep	59.00	27.00	14.00	
Hay.	...	Steer	60.30	21.10	18.60	Carroll and Hungate (1954)
Grain.	...	Steer	63.40	22.60	14.00	
Hay and concentrate.	...	Fasting sheep	75.00	16.00	12.00	Mukherjee (1960)
Hay and concentrate	...	Non-fasting sheep.	51.20	24.40	24.40	
Baggage	...	Goat.	58.80	37.00	4.20	Maitra (1962)

Balch et.al.(1955) found that when rations that are high in concentrate and low in roughages are fed to cattle the ratio of acetic to propionic acid in the rumen has been found to be lower than the ratio obtained when rations containing moderate amounts of concentrates are fed. This effect of high concentrater rations is enhanced if a cooked concentrate such as broad is used (Shaw et.al.1959) or if the concentrate is heated before being fed (Eusebio et.al.1959).

Eusebio et.al.(1959) showed that feeding of such rations altered the microbial metabolism of the rumen probably by bringing about a change in the relative proportions of the microbial species present in the rumen. It is not clear just how this change is brought about by heating the feed. They also noted that changing from roughage and concentrate diet to all concentrate diet resulted in a large decrease and increase in the molar proportions of acetate and propionate respectively.

Stewart and Schultz (1955) observed that corn meal additions to rumen liquor caused increased propionate production.

Hershberger et.al. (1956) found that when starch was fermented, VFA mixtures were produced with lower acetate propionate rations than when cellulose was fermented.

Emery et. al. (1956) found that on hay more acetic and propionic and less butyric acid was formed than on concentrates.

Belasco (1956) using 'in vitro' methods reported that the total production and relative proportions of the VFA's are dependent on the type and amount of Carbohydrate used.

Waldo and Schultz (1956) found that Butyric acid showed an increase in concentration as well as an increase in percentage of total VFA's with an increase in grain feeding, propionic acid showed an increase in concentration with grain feeding but showed less increase than butyric acid as a percentage of total VFA's. Acetic acid showed similar concentration on all levels of grain feeding with a decrease as a percentage of the total VFA's with grain feeding.

Waldo and Schultz (1956) also found that roughage rations produced marked changes in the VFA's. Butyric acid had the lowest concentration on grass hay. Propionic acid concentrations were lowest for the grass hay ration.

Barnett and Reid (1957) incubated fresh grass with rumen liquor and found that 25% of the total was butyric and higher fatty acids.

Reid (1957) observed relationship between high dietary intakes of wheat, wheat starch or crushed maize and high proportions of propionate in the rumen fluid.

Balch and Rowland (1957) also observed low levels of acetate and high levels of propionate and higher acids in the rumen fluid of cows fed predominently of flaked maize presumably due to the heating of the corn during the flaking process. They observed that mean molar percentage of these acids varied with the different diets as follows:- Acetic from 40.6 to 73.7; Propionic from 16.5. to 39.1; Butyric from 6.6 to 13.9 and higher acids from 2.5 to 12.7. In general, the percentage of butyric and higher acids increased with increase of protein in the diet. The values for acetic and propionic acids varied inversely. The ratio of acetic to propionic acid decreased with decrease in the ratio of fibrous to starchy concentrates. Replacing long hay, in a diet of high flaked maize content, by finely ground hay also decreased the ratio of acetic to propionic acid. In that particular case, they also observed a marked increase in the range of concentration of TVA of 21.3 m eq/100 ml ofrumen liquor, when finely ground hay was fed which was the highest.

Shaw et.al.(1957) noted that cooked diets made up primarily of bread or of cooked rice, cooked potato meal, bread and molasses resulted in marked decrease of molar proportions of the rumen acetate as well as increases in

the molar proportions of propionate and in the fractions made up of valerate and higher acids. These changes were far greater than those observed with diets made up predominently of uncooked concentrates.

Stewart and Schultz (1958) incubated 'in vitro' various feeds using steers rumen content and found that fresh hand-clipped legume mixed grass caused marked depression of propionic acid formation, compared to hay. Molasses consistently decreased acetic acid production. Beet pulp significantly increased acetic acid production and corn meal increased propionic acid production.

Elliot and Loosli (1959) also noted that as concentrates made up an increasing proportions of the ration the relative proportion of acetic acid decreased with concomitant increases in propionic and butyric acids.

Gray and Pilgrim (1952) have shown that part of the butyric acid is synthesized from acetic acid.

Mukherjee (1960) for the first time studied quantitatively the extent of interconversion of the three ruminal acids both in 'in vivo' and in 'in vitro' and found to be not more than 3% excepting in the case of butyrate to acetate.

In 'in vitro' studies of all percentage conversion from butyrate to acetate in 3 hours were 12.8 and 29.3 for non-fasting and fasting animals respectively. Whereas in 'in vivo' condition, it was in the order of 88.9 and 73.0%, the last one being the data for fasting sheep.

Some of the important findings on VFA levels in the rumen is shown in table 1.1. (See page 26).

It is seen from the table 1 and 1.1 that the proportions of the three acids both in 'in vitro' and 'in vivo' are widely different depending upon the condition of the experiment.

The products of fermentation of both Carbohydrates and protein together give rise to various straight and brahched chain fatty acids and all these are utilised by host. Butyric acid and some branched chain fatty acids are utilized by the rumen wall itself. (Annison and Pennington, 1954; Pennington, 1954; Pennington and Sutherland, 1956).

Table 1.1

Table showing VFA levels in the rumen under various dietary regimes.

DIET.	Animal.	Rumen VFA's (Molar proportions). Acetic.	Propionic.	Butyric.	Reference.
Flaked maize.	Sheep.	60.0	35.0	5.0	Phillipson (1952)
Conc. (plus) 50 Gm. Casein.	Sheep	56.9	23.7	15.3?	El-Shazly (1952)
Frozen grass.	Sheep	54.0	22.6	18.2?	
Dried grass.	Sheep	57.7	22.2	11.4?	
Silage.	Sheep	53.2	25.4	12.9?	
Wheaten hay.	Sheep	65.4	22.3	12.3	Gray et. al. (1952)
Leucerne hay.	Sheep	66.9	22.5	10.6	
Groundnut cake.	Sheep	60.0	26.0	14.0	Annisen (1954)
Maize.	Sheep	46.0	32.0	22.0	
Casein	Sheep	60.0	22.0	16.0?	
Hay.	Sheep	81.0	13.0	6.0	
Hay and concentrate.	Cow Sheep	40.6	36.5	23.0?	Balch and Rowland (1957)
Hay and concentrate.	Sheep	75.50	15.30	9.0?	Mukherjee (1960)

CHROMATOGRAPHY OF FATTY ACIDS.

The lower fatty acids with the exception of formic acid, are, so similar in their chemical properties that their quantitative separation and estimation by purely chemical methods have not proved possible. Therefore, the methods available for the estimation of these compounds in mixtures should be physical rather than chemical.

In recent years, the importance of Chromatography as a preoperative and analytical methods has increased greatly.

For the estimation of ruminal VFA, Smith (1942) using silica gel column, has been able to resolve chloroform plus 1% Butanol solution for formic, acetic, propionic, n-butyric and n-valeric acid, into their component compounds. Although the butanal-chloroform-water system gave satisfactory separation of C_1 - C_4 acids, it is not effective for acids having long chains (Elsden, 1946) and (Ramsey et.al. 1945). Elsden (1946) who for the first time devised a liquid-gel partition chromatogram which permitted accurate analysis of mixtures of acetic, propionic and butyric acids. Moyle et al (1948) used buffered silica-gel tube, such a system permitted separation of C_2 - C_8 acids. Peterson and Johnson (1948) were able to isolate the C_2 - C_{10} acids by employing Benzene-aqueous-sulphuric acid in Celite packed tubes.

Dueding and Yalo (1951) used Celite for separating VFA's by which it was possible to separate quantitatively and reproducible from each other volatile fatty acids containing from 2 - 6 Carbon atoms. A modified procedure of the same was used by Mukherjee (1960).

A major advance in the analysis of VFA's was the gas-liquid partition Chromatographic method of James & Martin (1952). This method made possible for the separation of not only the higher and lower acids and also the branched chain Isomers of butyric and valeric acids as well as the esters of both saturated and unsaturated fatty acids.

Paper Chromatography gives satisfactory results for quantitative estimation by measurements of the spot areas was achieved by Reid and Lederer (1951). Henon et.al. (1962) used a new indicator which is very sensitive but cannot be used for quantitative estimations. Pant et.al. (1962) qualitatively separated the fatty acids by paper Chromatography as their anions using mixture of Ammonium salts of fatty acids.

However, the use of paper Chromatography seems limited by their relative inaccuracy when compared with columns.

KETOGENIC NATURE OF BUTYRIC ACID.

Schultz et.al.(1949) reported that the administration of butyric acid to goats proved Ketogenic.

~~Smith and~~ Schultz and Smith (1951) have shown that blood Ketones can be increased in ruminants by the administration of certain fatty acids, particularly butyric and Caproic acids.

Pennington (1952) clearly demonstrated the Ketogenic nature of butyric acid. He also demonstrated that the epithelium of rumen, reticulum and omasum as well as liver tissue are sites for the conversion of butyric acid to Ketone bodies. He postulated that propionate in the intact animal facilitate the complete oxidation of other fatty acids and thus prevent the excessive formation of Ketone bodies.

Jarret and Potter (1950) found that administration of acetate to sheep increased the level of Ketone bodies in the blood. But simultaneous administration of acetate and propionate produced little rise in Ketone bodies.

In view of both the above findings of Jarret and Potter (1950) and Pennington (1952), it appears that oxidation of acetate and butyrate is closely associated with the metabolism of propionate.

El-shazly (1952) found that high protein diets produce more of butyric and higher VFA's. He speculated that the high protein diets may be responsible for abnormally high levels of blood Ketone bodies.

HEAT INCREMENT AND CALORIMETRIC CONSIDERATION OF VFA.

Grouven (1864) found the very high heat increment of Carbohydrate in ruminants, he suggested that it may be due to the metabolic stimulus of the acids produced in the rumen fermentation.

Armsby (1917) in experiments with maize given above maintenance, found the net availability of its metabolizable energy to be 63.5%.

Kellner (1920) ignored this hypothesis and attributed the heat increment to the work of digestion.

Forbes et al (1931, 1933) made the significant observation that the nutritive value of an individual foodstuff was not constant, but the efficiency of the utilization of the energy of a feeding stuffs by a fattening ruminant can be altered by the ration combination in which it is fed.

Ritzman and Benedict (1938) in a long series of experiments found that their cows required slightly more metabolizable energy to maintain than when the food was hay (32% crude fiber) than when it was maize (3% crude fiber).

Marston (1948) explained the high heat increments of ruminants to be due to the transfer of atleast some of the absorbed fatty acids into the cycles of intermediary metabolism involving considerable thermodynamic losses.

McClymont (1962) suggested that very rapid oxidation in non-lipogenic tissue was the main reason for the high heat increment and that two carbon units arising from acetic acid or butyric acid undergo a continuous partition between oxidation and lipogenesis the former being thermodynamically, a wasteful process.

Important papers of Armstrong and Blaxter gave a radical solution to the confusing problem of heat increment of VFA.

Armstrong and Blaxter (1957 a) found the heat increment of individual steam VFA in fasting sheep to be acetic 41 Cal, propionic 13 Cal, and butyric 16 Cal/100 Cal of metabolizable energy respectively.

Armstrong et.al.(1957 a) demonstrated with the help of indirect calorimetry that mixtures of acetic, propionic and butyric acids when used as the sole source of energy for fasting sheep, were utilised quite efficiently by the animals for their basal activities, as only about 15% of the total metabolizable energy appeared as heat increment when 1117 Cal/24 hours of metabolizable energy was utilized by the animals which was far below from that of the anticipated results.

Later Armstrong and Blaxter (1957 b) have also determined the heat increment of these acids above maintenance for fattening sheep to be acetic 67 Cal, propionic 44 Cal and n-butyric acid 38 Cal/100 Cal of metabolizable energy.

The above results suggest that the utilization of the steam VFA above the maintenance datum thus differs very markedly from their metabolism below maintenance. Not only are heat increments higher above maintenance than below, but above maintenance the composition of the mixture of acids has a marked effect on the magnitude of the increment of heat, whereas below maintenance mixture of widely different composition are used with equal efficiency (Armstrong et.al, 1957b).

Armstrong et.al.(1958) estimated the heat increment of two different mixtures of VFA above maintenance, one high acetic mixture 75% acetic acid and another low acetic acid mixture containing 25% acetic acid to be 68 Cal and 42 Cal respectively. It appears, therefore, that the heat increments of acids mixtures above maintenance are those to be expected on the basis of direct proportionality of response to the individual acids.

It appears, therefore, that the energy of the steam VFA appears to be more efficiently utilized oxidatively in meeting maintenance needs than it is for lipogenesis. Differences in the relative nutritive value of concentrates and for roughages for maintenance and for lipogenesis can possibly be explained in this way.

-xlr-

CHAPTER (II)

MATERIALS AND METHODS

Chapter (II)

MATERIALS AND METHODS.

Analysis of feed:- The analysis was done only for the ground-nut shell.

The sample was collected from different centres and the material was pooled and samples from different parts were taken to the laboratory for routine analysis.

For moisture the material was subjected to 102° C. for 10 hours. Protein was estimated by Kjeldahl's method. For crude fiber analysis Weende's method was employed. Total ash was determined by igniting the sample in muffle furnace at 575° C. for 4 hours. For ether extract Seshan's method (1936) was employed i.e. extraction of the material in Soxhlet by petrolium ether for 60 hours without any consideration of number of extraction per hour and uniform extraction.

Rumen liquor used in incubation:- The rumen content used for incubation throughout this experiment was brought from slaughtered goats from the butcher's shop very near to the College immediately after the slaughter. The rumen wall was slited by a sharp knife and about a pound of rumen content was taken in a wide-mouth bottle which was previously

gassed with carbondioxide to drive out the air within it, from the laboratory. The bottle was taken to the laboratory and carbondioxide was again passed for a few minutes and the bottle was immersed in warm water at about 40° c.

The rumen content was strained through a double folded fine muslin cloth and rumen liquor was pipetted into incubation flasks.

'In vitro' incubations:- Two different methods of incubations were used during the course of this experiment.

In the first method, incubation was carried out in 100 ml conical flask (Pyrex), fitted with rubber cork No.4, provided with both inlet and outlet device in it. The inlet tube being well below the outlet one in the incubating flasks. The foodstuffs used in incubation were all finely powdered and 1 gram of each was added in different experimental flasks and kept ready.

The foodstuffs tried were maize, Guar meal, Berseem hay, raw Berseem green, and spear grass hay. The pure nutrients used were glucose, lactose, starch and cellulose.

To each of the flasks 20 ml. of the rumen liquor was pipetted and 2 ml. of 2 M phosphate buffer of pH 6.6 was added into the flasks. The flasks were immersed in

a water bath maintained at 39° - 40° C. and were connected in series, and the corks were fixed firmly to the flask with the help of a drop or two of water, so as to make it air-tight. The incubation experiments were always run in duplicate. Two other flasks treated similarly, without the foodstuffs were always used as controls in each incubation.

All the flasks were gassed with Carbondioxide, which is passed along the inlet tube from carbondioxide cylinder at the same time for 10 minutes to maintain anaerobic condition which was tested by passing the outlet gas in Barium Chloride solution, and also by a lighted taper. Once when it was sure that carbondioxide was coming at the outlet, both the inlet and outlets were closed. The rumen content was thus incubated under anaerobic conditions. The flasks were gently shaken every half an hour, throughout the incubation period.

Leakage in the flasks was tested by (1) placing a drop of water at the junction of flask and cork; (2) it was also further tested by connecting the outlet tube to the mercury Manometer.

As the incubations were of longer duration, it was presumed that Methane, H_2S and Co_2 which was produced during incubation, if accumulated would prevent the normal thrive of the bacteria, so outlets were opened at every hourly interval and fresh carbondioxide was passed in all the flasks.

After the specified incubation time, all the flasks were acidified with 2 ml. of 10N H_2SO_4.

The whole process of starting an incubation experiment did not take more than 15 minutes from the time the sample was brought to the laboratory.

In a rough check on the effect of exposure of rumen content to air, it was noticed that neither the pH nor the fermentative activity was changed perceptibly on exposure to air for 15 minutes. It is, therefore, safe to infer that no deleterious effect takes place during exposure in air of rumen content for atleast 15 minutes.

At each incubation immediately after straining the rumen liquor, 20 ml. of it was pipetted in a separate beaker and to it 2 ml. of 2 M. Phosphate buffer at pH 6.6 was added. It was acidified with 2 ml. of 10N H_2SO_4. This is for the initial concentration of volatile fatty acids.

Second method of incubation:- (Pant et.al.1962).

The second method of incubation was practically similar in design to that accepted by Barnet and Reid (1957 a).

This was carried out in 250 ml. conical flask fitted with No.10 rubber cork. In this method, mineral mixture (Burroughs et al.1950) of the following composition was used:-

Mineral mixture.

Sodium Phosphate (Monobasic).		...	52.50	Gm.
Sodium Bicarbonate.	...	...	52.50	"
Ammonium Sulphate.	...	...	37.50	"
Potassium Chloride.	...	...	77.50	"
Sodium Chloride.	...	...	7.50	"
Magnesium Sulfate.	...	...	2.25	"
Calcium Chloride.	...	...	0.75	"
Ferrous Sulphate.	...	...	0.15	"
Manganous Sulfate.	...	...	0.08	"
Zinc sulfate.	...	...	0.08	"
Copper sulfate.	...	...	0.04	"
Cobalt chloride.	...	...	0.02	"
Distilled water (ml.)	...	...	2000	

Each glass artificial rumen was charged with 25 ml. of the above mineral mixture. To it 110 ml. of water was added. The flasks so treated were allowed to reach a temperature of 39° C. by keeping them in an incubator for overnight.

Next day in the morning prior to the collection of rumen liquor, carbon-dioxide was bubbled into the flasks at a rapid rate for about 10 minutes to ensure anaerobic condition. Next 25 ml. of the strained rumen liquor was added to all the flasks and 1 gm. of foodstuff to be incubated was added to the experimental flasks only. In this no phosphate buffer was added. Rest of the treatment was similar to that in the previous method.

In this procedure, pH of the contents was checked every 6 hours and was brought to the original pH by adding 0.6 ml. of Na_2Co_3 solution (106 gm./liter) for every 0.1 deviation in the pH.

This procedure was used only for digestion of pure cellulose and ground-nut shells; since incubation was carried for much longer period of 24 hours in this case.

Estimation of VFA in rumen liquor:-

The total VFA in rumen liquor was estimated by the method of Elsden et.al.(1946).

Before distillation each sample was treated as under: The acidified content of each of the flasks was transferred to a measuring cylinder. The flasks were washed 2 - 3 times with 4N H_2So_4 saturated with Magnesium sulphate and poured to the cylinder and the volume was made upto 40 ml. The addition of Magnesium sulphate served not only to

Photo showing chromatographic columns of Celite and Bottles containing CB_0, CB_5 and CB_{20} used in the experiment.

Photo showing chromatographic columns of Celite and Bottles containing CB_0, CB_5 and CB_{20} used in the experiment.

ly in

increase the rate of distillation of the volatile fatty acids, but also to hold back HCl which otherwise came over in appreciable quantities.

A 10 ml. aliquot of this mixture was taken in "Markham still" and was steam distilled. About 150 ml. distillate was collected and was titrated against N/50 NaOH solution using 0.02% Bromo-thymol blue as indicator. Further 25 ml. of the distillate was also collected and titrated as a precautionary measure against complete distillation. The second 25 ml. of the distillate invariably did not contain any acid.

Sufficient care was taken to see that the steam distillation was not too fast to make the tip of the condensor hot.

CHROMATOGRAPHIC SEPARATION OF THE VFA.

The Chromatographic procedure followed was a modified procedure of Bueding and Yale (1951). The whole process consists of the following steps which are described individually below:-

(1) Preparation of the VFA sample for Chromatography:

The first 150 ml. of Markham distillate was made distinctly alkaline after titration by the addition of an extra drop of N. NaOH and evaporated down to a volume of about 20 ml. over an even flame. The small volume of distillate was then transferred to a round-bottomed 100 ml. flask and dried down completely in an oven.

The VFA were extracted according to the method of Elsden (1946 a) into Chloroform solution which is necessary for Chromatography. The dry powder was dissolved in 0.2 ml. water and sufficient anhydrous $KHSO_4$ powder was added to form a dry mass which did not stick to the sides of the flask. The powder was then extracted 6 times with small volumes of 5% butanol in chloroform dried over Na_2So_4 and then made upto volume.

(2) Preparation of the Chromatographic column:

The Celite-Johns-Manville No.535 (Celite 535) used in the Chromatogram was prepared for this purpose by boiling it vigorously in concentrated Hydrochloric acid for 30 minutes, to remove most of the Iron in it. The supernatent HCl was decanted off and the celite was repeatedly washed with about 3 liter portions of tap water till nearly free from HCl, when it was washed twice with the same volume of distilled water till completely free from HCl. During the washing, a fair amount of the finer particles of celite that failed to settle down within 15 minutes was also decanted off. The celite was then dried in an oven at 110° C. to a constant weight. The dried celite was then washed with ether by pouring it over the Celite in a Buchner's funnel and drawing off the solvent by suction. The ether washed Celite was finally dried in a desiccator under continuous evacuation.

6 Gm. of this processed Celite was thoroughly ground in a mortar with 3.6 ml. of 2 M. Phosphate buffer at pH 6.5, so that the buffer was uniformly distributed in the Celite. Sufficient chloroform equilibrated with the buffer was added to the Celite until a slurry was obtained.

The end of the column, of about 12 mm. internal diameter, was plugged with glass wool to act as a support for the Celite. A few ml. of chloroform was run through the glass wool which was tampered again to remove the air bubbles in it. About 0.5 gm. of the Celite slurry was introduced into the column with the help of a funnel and sufficient Chloroform was poured to make a suspension of it. The suspension was stirred by a slender rod and then tamped down by another rod which had a flattened end almost as large in diameter as the column. The rod was given a twisting motion as it was lowered and an even downward pressure was exerted on the Celite, while packing it. The column was rotated slowly and the edge of the pack was tampered round with the slender rod. The next charge of another 0.5 Gm. of Celite was then introduced and all the abovementioned steps were carefully gone through. After the last addition of the Celite, all floating particles were tamped down so that the column surface was flat and even. Particular care was taken to follow this packing procedure in every detail so as to avoid the formation of channels and consequent poor resolution.

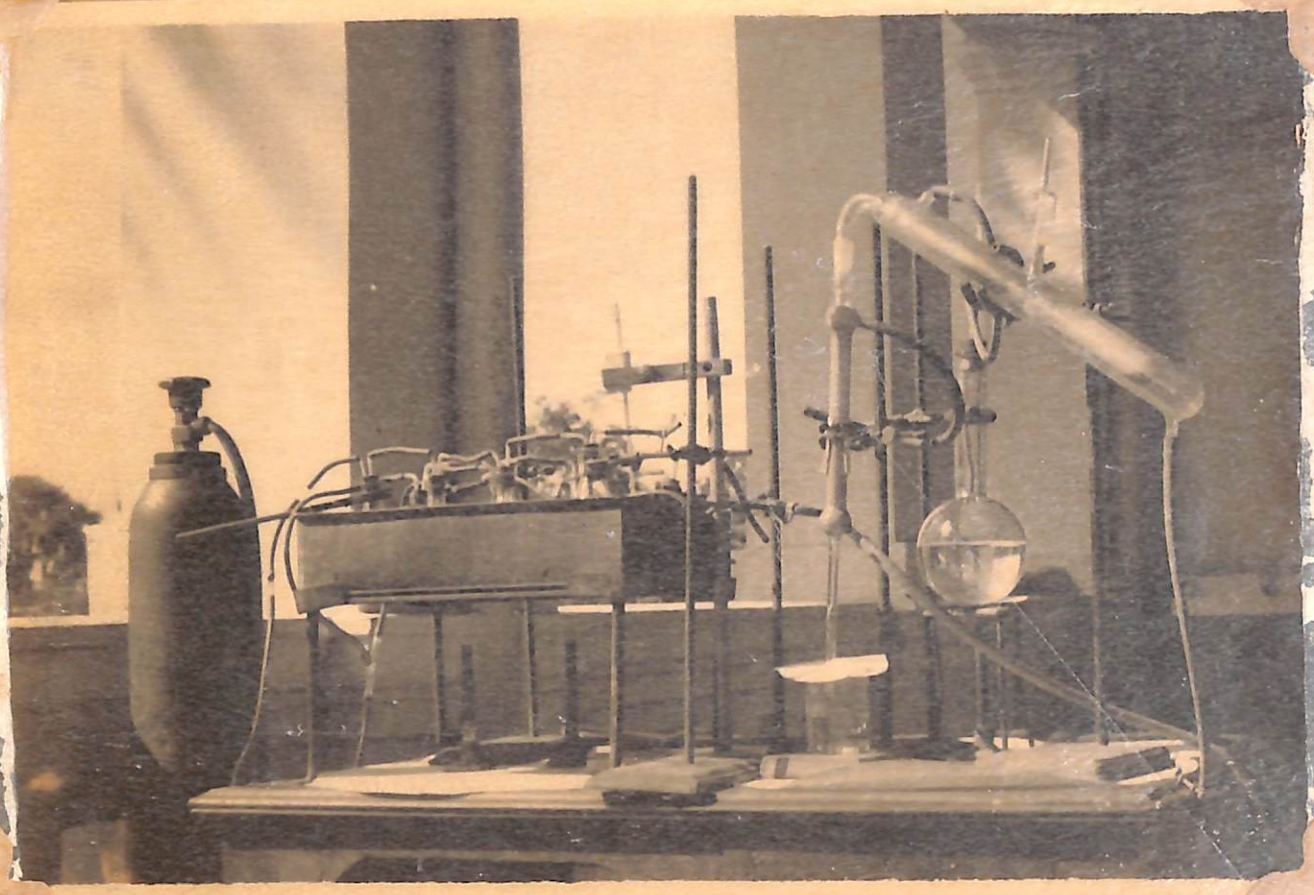


Photo showing a set of incubation and distillation apparatus used in the experiment.

(3) Separation of the acids on the Chromatogram and their titrations:-

The small volume of the solvent always remaining above the Celite column was allowed to flow through until its level just reached the surface of the Celite. From 3 - 5 ml. of the solution of the acids in the solvent containing 100 - 150 micromoles of total acids was allowed to flow into the Celite. The sides of the column were washed twice with 3 ml. portions of pure Chloroform (CB_0). The column was then filled with CB_0 and 5 or 10 ml. samples were collected for titration. Very often the first fraction of the elute was collected in bulk of 50 ml. to save time and error in the titration.

When the titration reading of the last sample of elute dropped down to blank value, the receiver was changed and after the remaining volume of CB_0 passed through the column, it was filled up with the next solvent 5% butanol in Chloroform (CB_5) and the same procedure as described above, was followed till the titration reading again dropped down to the blank value when the last solven 20% butanol in Chloroform (CB_{20}) was used.

The titration of the elute was carried out with N/200, methanolic NaOH, using Bromothymol blue as the indicator. Before the titration of the CB_0 fractions 10 drops of methanol was added to it so as to avoid the false end point ~~that is often to avoid the false end point~~

that is often noticed when the amount of butyric acid is less than 5 micromoles. This step was found to be essential to avoid confusion in the titration of the CB_0 fractions as pointed out by Peterson and Johnson (1948).

The natural flow of the solvent through the column was sometimes found to be too slow. The efficiency of separation of the acids was found to be unaffected by variation in the rate of flow of the solvents.

RECOVERY OF THE SOLVENTS FOR REPEATED USE.

The used solvents were pooled and vigorously shaken with about 5 ml. of strong methanolic NaOH to neutralize the free acids if any. The pooled sample was then washed with running tap water till the washing did not show any sign of alkalinity. The solvent was separated from the water with the separating funnel and was shaken with $CaCl_2$ granules for further drying. The dry solvent was distilled in a glass distillation apparatus. The distillate consisted of undetermined mixture of Chloroform and butanol which was used to prepare fresh CB_5 and CB_{20} by adding calculated amounts of pure Chloroform or pure butanol to it based on specific gravity measurements with the hydrometer. The CB_0 was, however, always prepared from fresh Chloroform. The same pooled solvent was used indefinitely without any apparent deleterious effect.

REAGENTS USED.

1. 2 M. Phosphate buffer pH. 6.5 - 100 Gm. KH_2Po_4 and 190 Gm. KH_2PO_4/liter.
2. Butanol - n-butanol, reagent quality.
3. Chloroform - Reagent quality, washed with distilled water for 2 - 3 hours to remove the alcohol added to it for stabilisation.
4. Methanol - reagent quality.
5. Solvents - pure Chloroform (CB_0), 5% butanol in Chloroform (CB_5) and 20% butanol in Chloroform (CB_{20}).

All solvents were equilibrated with the same buffer which is used as the stationary phase of the column. 2-3 ml. of the buffer/100 ml. of solvent was thoroughly shaken in a separating funnel. After separation of the two layers, the solvent was filtered in order to remove any water remaining in suspension.

ESTIMATION OF KETONE BODIES IN RUMEN LIQUOR.

The total Ketone bodies in rumen liquor was estimated by the method of Greenberg and Lester (1944) with slight modifications.

This method of estimation of total Ketone bodies is based upon the conversion of acetoacetic acid to acetone by acid hydrolysis and the oxidation of B-hydroxybutyric acid to acetone with acid dichromate; followed by reaction of acetone with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone, the separation of the hydrazone

REAGENTS USED.

1. 2 M. Phosphate buffer pH. 6.5 - 100 Gm. KH_2Po_4 and 190 Gm. KH_2PO_4/liter.
2. Butanol - n-butanol, reagent quality.
3. Chloroform - Reagent quality, washed with distilled water for 2 - 3 hours to remove the alcohol added to it for stabilisation.
4. Methanol - reagent quality.
5. Solvents - pure Chloroform (CB_0), 5% butanol in Chloroform (CB_5) and 20% butanol in Chloroform (CB_{20}).

All solvents were equilibrated with the same buffer which is used as the stationary phase of the column. 2-3 ml. of the buffer/100 ml. of solvent was thoroughly shaken in a separating funnel. After separation of the two layers, the solvent was filtered in order to remove any water remaining in suspension.

ESTIMATION OF KETONE BODIES IN RUMEN LIQUOR.

The total Ketone bodies in rumen liquor was estimated by the method of Greenberg and Lester (1944) with slight modifications.

This method of estimation of total Ketone bodies is based upon the conversion of acetoacetic acid to acetone by acid hydrolysis and the oxidation of B-hydroxybutyric acid to acetone with acid dichromate; followed by reaction of acetone with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone, the separation of the hydrazone

by extraction with carbontetrachloride and its colourimetric determination in the carbontetrachloride layer.

The whole process consists of the following steps which are described individually.

1. Deprotoinization:- 2 ml. of rumen liquor which has been made up to a known volume after removing coarser particles by centrifuging for 15 minutes, was taken in a glass centrifuge tube. To it 1 ml. of 0.15N $Ba(OH)_2$ solution is added. The contents of the tube are mixed and 1 ml. of 2.5% zinc sulfate solution added. The contents of the tube are again mixed and the tube stoppered and centrifuged for 2 - 3 minutes.

2. Oxidation of Acetoacetic acid and B-hydroxybutyric acid to Acetone:- Whole of supernatent fluid, after centrifuging are then delivered to the bottom of the clean dry 25 ml. pyrex tube containing several glass beads, 0.6 ml. of acid dichromate solution is added and the tube tightly stoppered with the cold finger. Then ice cold water is passed through the cold finger. The contents of the tube are heated by means of a micro Bunsen burner and gentle boiling is continued for 10 minutes. At the end of this time, the burner is removed and when boiling has ceased, the cold finger is lifted slightly and 0.5 ml. of 10% dichromate solution is introduced. This solution is

allowed to run down the cold finger. The tube is then closed tightly and boiling continued for an additional 10 minutes. Throughout the heating process, sufficient care was taken to keep the upper half of the reflux tube cold. Then the cold finger is removed and the contents of the reflux tube are ready for the determination of acetone.

3. Development of colour:- To the fluid in the reflux tube, 1 ml. of 15% sodium sulphite solution is added to destroy the dichromate. To it is added 2 ml. of 2,4-dinitrophenylhydrazone solution and 7 ml. of Carbon tetrachloride. The contents of the tube are shaken for 10 minutes. The supernatent acid solution is then thrown off, the tube is again filled twice with distilled water which is also drawn off each time. Then 3 ml. of 0.5N NaOH solution are added and is again shaken for 3 minutes.

4. Colourimetric determination:- The contents of the tube are then poured into a Klett - Summerson colourimeter tube and read against a similar tube containing carbon tetrachloride, a Klett filter 42 which transmits light of wave length 420 mμ being used. From this reading is substracted that of a blank determination in which distilled water is used instead of the solution for analysis; this

blank results from impurities in the reagents and the retention of a small amount of hydrazine in the carbontetrachloride. It remains constant for any one preparation of the reagents.

The blank B, which must be substracted from the reading obtained, is determined as follows:- To 3 ml. of distilled water are added 0.6 ml. of acid dichromate solution and 0.5 ml. of the 10% dichromate solution. This mixture is then carried through the same procedures as the filterate after it has been refluxed.

Preparation of standard curve:- A standard solution of acetone is prepared containing 1 mg/ml. This solution is diluted 100 times just before use so that each ml. of the diluted solution contains 10 µg. This solution is taken in increasing amounts i.e. containing 10 µg, 20 µg, 30 µg and 40 µg of acetone and colour was developed as described above and colourimetric readings were noted. Figure (1) represents standard curve for acetone. It will be seen that Beer's law is strictly adhered to over range noted.

REAGENTS USED.

1. Acid 2,4-dinitrophenylhydrazine solution,
   0.1% 2,4-dinitrophenylhydrazine in 2.0 N/ HCl.
2. Carbontetrachloride reagent.
3. NaOH solution 0.5N.
4. Zinc sulphate solution approximately 2.5%.
5. Barium hydroxide solution approximately 0.15N.
6. Acid dichromate solution 0.46 % Potassium dichromate in 15.6N H_2SO_4.
7. 10% Potassium dichromate solution.
8. 0.15% Anhydrous Sodium sulphite solution.

STATISTICAL METHODS.

1. Analysis of variance:- This analysis was done for all the fatty acids produced from foodstuffs separately. The idea of this analysis was to know whether the variations in the production of fatty acids between the foodstuffs were significantly different or not. The following is the set up of the analysis:-

Sources of variation.	d. f.	Total S.S.	Mean Square	F.
Total. ...	N - 1	X	-	
Between foodstuffs.	Y - 1	P	$\frac{P}{Y-1}$	$\frac{\frac{P}{Y-1}}{\frac{Z}{N-Y}}$
Within foodstuffs or error. ...	N - Y	Z	$\frac{Z}{N-Y}$	

t ... table value with error d.f.

s^2 ... error mean square.

r_1 and r_2 ... No. of replicates.

The critical difference value was then compared with the average of the percentage of fatty acid produced from each foodstuff with the other.

Correlation coefficient:- Correlation is a measure of association between the variables. The degree of association is measured mathematically by calculating the correlation coefficient. The index of correlation is symbolized by 'r' and is obtained by the formula:

$$r = \sqrt{\frac{\text{Covariance of XY.}}{\text{Variance of X and variance of Y.}}}$$

X ... independent variable i.e. crude fiber content or TDN content of foodstuff.

Y ... dependent variable, fatty acid.

Correlation coefficient between crude fiber and TDN content of foodstuffs and fatty acids produced were calculated by the above formula and tested for significance from correlation coefficient table 7.61 of Snedecor (1956).

--oOo--

where,

N ... total number of observations.

Y ... total number of food-stuffs.

X ... total corrected sum of squares.

P ... between foodstuff corrected sum of square.

Z ... error sum of squares.

The analysis of variance table provides a ready means of testing the significance of difference between class means. A comparison of the mean square between foods with the error mean square provides a test of significance of differences arising from foodstuffs. The comparison is done by finding the ratio of the mean square concerned to the error mean square. This ratio is the variance ratio and is denoted by 'F'. This was seen in the F table at that particular degree of freedom for significance.

Critical difference:- By the differences between individual foodstuffs were tested for significance only after the overall significant differences between foodstuffs are indicated by F test in the analysis of variance.

Critical difference was calculated both at 5% and 1% level by the following formula:-

$$\text{Critical difference} = t \text{ (with error d. f.)} \sqrt{s^2 \left(\frac{1}{r_1} + \frac{1}{r_2}\right)}$$

CHAPTER (III)

ESTIMATION OF ERRORS IN VARIOUS EXPERIMENTAL PROCEDURES.

Chapter (III)

ESTIMATION OF ERRORS IN VARIOUS EXPERIMENTAL PROCEDURES.

(I) Efficiency of Markham distillations:- The efficiency of the recovery in this method was tested by adding known amounts of standard Acetic acid solution to the rumen liquor, whose VFA content has been estimated. Portions of rumen liquor which contain known amounts of VFA's was distilled. 150 ml. of the distillate was collected which was titrated against a standard alkali. The close agreement of duplicate recovery from rumen liquor which was invariably obtained also testify the accuracy of the full process.

The result of the recovery is shown in table 3.1.

Table 3.1.

TABLE SHOWING THE EFFICIENCY OF DISTILLATION OF VFA IN 'MARKHAM STILL'

Material.	No. of Obs.	Amount of acid used (ml. $\frac{N}{10}$ NaOH)	Amount of acid recovered (ml. $\frac{N}{10}$ NaOH)	Percentage recovery.
Rumen liquor with 2.5 ml. of N/10 acetic acid ...	4	7.6	7.55	99.6 ±0.5
Rumen liquor with 5 ml. of N/10 acetic acid ...	4	10.1	10.2	100.0 ±0.4
Rumen liquor with 10 ml. of N/10 acetic acid ...	4	15.1	15.05	99.6 ± 0.3

It is seen from the table 3.1 that recovery from the distillation process adopted in this work invariably recovered nearly 100% of the VFA. The very small standard indicates the high degree of reproducibility of the process. It is also seen that the next 25 ml. of the distillate after the first 150 ml. as a rule did not contain any trace of acidity. The efficiency of distillation both too slow and too fast rate produced errors. A period of 20 - 25 minutes was found to be optimum and was regularly followed.

(II) Efficiency of separation and recovery of acetic, propionic and butyric acids from mixtures by Chromatography on Celite.

A standard solution of acetic, propionic and butyric acids were prepared in CB_5 and a mixture of the three solutions were prepared in the ratio of 4:2:1 so that the concentration of 5 ml. of the mixture contained about 150 micromoles of VFA which is guarded as a safe load for the column.

5 ml. of this mixture containing known amount of acid was used in Chromatogram and recovery of the each of the acids was tested by the usual way. The result is shown in table 3.2. (See page 54).

It is seen from the table 3.2 that although butyric, propionic and acetic acids were taken in the ratio of 1:2:4 respectively, the recovery of all the three acids was of the same order, being 97.2 for butyric, 98.6 for propionic and 100.8 for acetic acids.

Table 3.2

Table showing the percentage recovery of VFA in Chromatography.

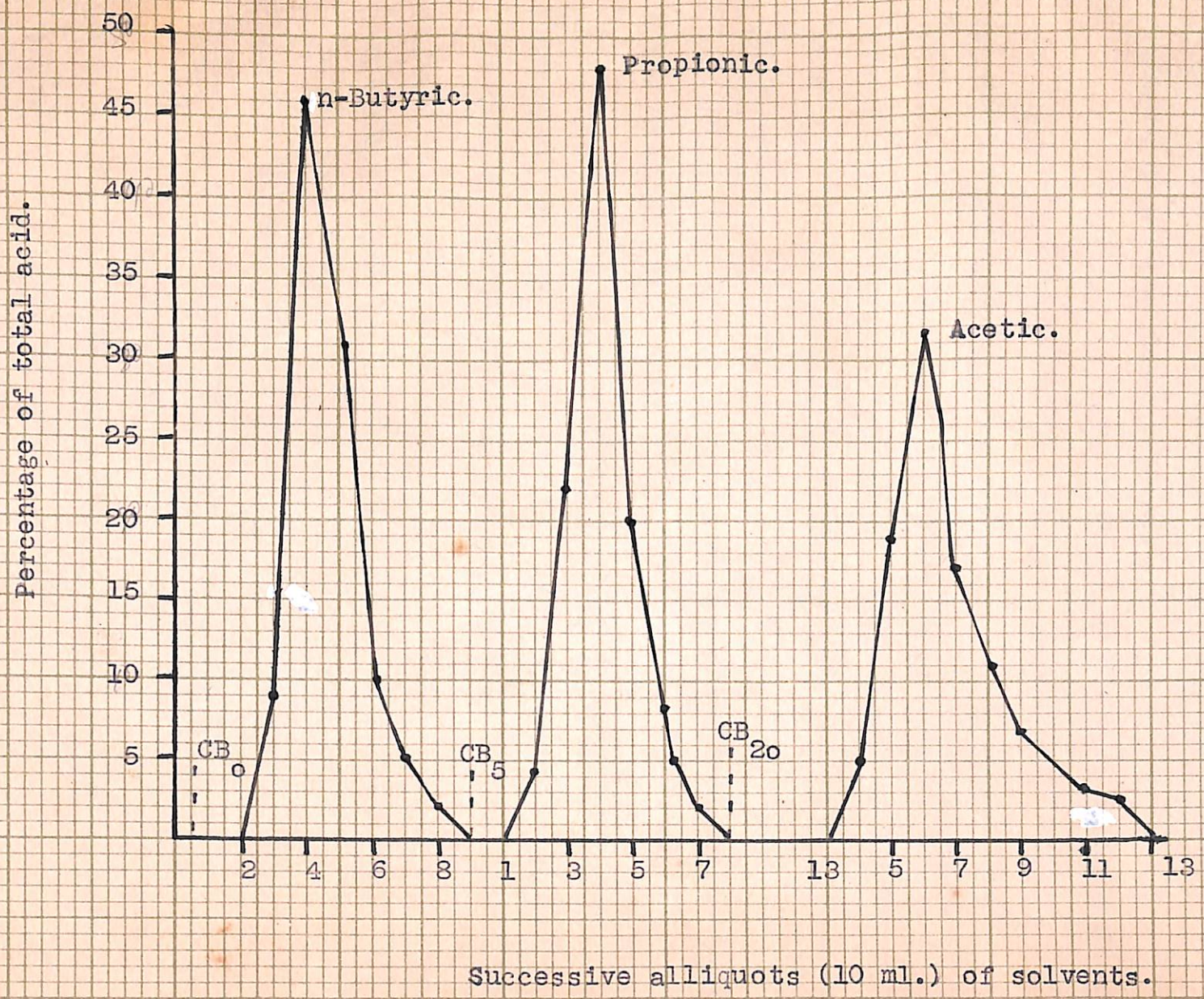
No. of columns used.	No. of observations.	Eluents.	As N/200 NaOH. Amount of acid used.	Amount of acid recovered.	Percentage recovery.
3	6	CB_0	3.6	3.5	97.2 ±1.0
3	6	CB_5	7.3	7.2	98.6 ±1.5
3	6	CB_{20}	15.2	15.3	100.6 ±0.4

In a preliminary trial run, the volume of eluent needed to elute completely the related acids, successive 10 ml. of the elute was titrated with alkali. The process was continued till a particular 10 ml. elute showed no trace of acid in it.

The elution curve for acetic, propionic and butyric acids in mixtures based on the mean values of a number of runs in the same as well as in different columns is represented in figure (1) in which abscissa represents successive aliquots of elute and the ordinate the percentage of total acid in each.

Fig. 1.

ELUTION CURVES OF N-BUTYRIC, PROPIONIC AND ACETIC ACIDS.



It is seen from figure 1 that an an average 80 ml. of CB_0, 70 ml. CB_5 and 120 ml. CB_{20} are required to completely elute butyric, propionic and acetic acids respectively. The interval between the complete elution of butyric and the appearance of propionic is uslally 10 ml. of the eluent and acetic acid is 20 ml.

For accurate reproducibility of the Chromatographic readings, it is essential that the Chromatograms should behave identically between different runs as also between different columns. This point was critically tested by running a standard VFA solution. (1) Successively in the same column and (2) in different columns, for Chromatography. The results shown in table 2.1 was obtained by taking into consideration both the abovementioned factors. The general conclusions drawn from the very large number of observations with butyric, propionic and acetic acids in mixtures are that their Chromatographic separation by the procedure described in the chapter on 'Methods' is invariably quantitative, extremely accurate and highly reproducible between columns and between runs of the same columns. It was also observed that the speed of elution did not affect the accuracy of the process, atleast upto a speed of 1 ml. per minute which was the usual speed used.

In experimental run, the process was shortened by collecting bulk samples of 50 ml. in case of CB_0 and CB_5 and 80 ml. in case of CB_{20}; and subsequent 10 ml. samples till the reading dropped down to blank value.

(III) <u>To test butyric acid against the presence of acetone in it:-</u>

It has been shown in the chapter on 'Results' that addition of butyric acid to rumen liquor and incubation for 4 hours caused production of Ketone bodies. In order to ascertain if the butyric acid used contains any Acetone bodies or not, known amounts of butyric acid is added to rumen liquor and ketone bodies were estimated without incubation, and also same rumen content without addition of butyric acid served as control, as described in chapter on 'Methods'. The result is shown in table 3.3.

<u>Table 3.3.</u>

Table showing testing of Butyric acid against presence of Acetone.

Material.	No. of observations.	Reading of Colourimeter. (Scale reading).
Rumen liquor. ...	3	63
Rumen liquor with 10 mg. butyric acid. ...	3	63
Rumen liquor with 20 mg. butyric acid. ...	3	63

It is seen from the above table that addition of butyric acid to rumen content did not cause any increase in the Colourimeter reading which is indicative of the absence of Acetone bodies in butyric acid.

-x-x-x-

CHAPTER (IV)

RESULTS.

Chapter (IV)

R E S U L T S .

FERMENTATION OF DIFFERENT FOODSTUFFS IN VITRO' FOR 4 HOURS.

The rumen samples were collected from slaughtered goats as described under the chapter on 'Materials & Methods'. First method of incubation mentioned therein was followed. The foodstuffs tried were Maize, Guar meal, Berseem hay, Berseem green and Spear grass hay all finely powdered. Total volatile fatty acids produced from each source and their rate of production were determined. The result is shown in table 4.1.

Table 4.1.

Result of incubation of different foodstuffs for 4 hrs.
(m moles of VFA/1000 ml. of rumen liquor)

SOURCES.	No. of obs.	Total VFA produced. Average with S.E.	Total VFA produced. Range.	Rate per hour. Average with S.E.	Rate per hour. Range.
Rumen liquor.	3	12.9 ± 5.8	5 - 24.2	3.2 ± 1.4	1.25 - 6.05
Maize. ...	3	42.4 ± 2.3	38 - 43.2	10.6 ± 0.6	9.5 - 11.5
Guar meal...	3	82.7 ± 0.7	81.4 - 83.6	20.6 ± 0.1	20.3 - 20.9
Berseem hay.	3	105.5 ± 6.3	98.9 - 118.2	26.4 ± 1.6	24.7 - 29.5
Berseem green	3	34.8 ± 7.1	22.2 - 46.8	8.7 ± 2.0	5.5 - 11.7
Spear grass hay.	3	35.2 ± 2.9	30.0 - 40.0	8.8 ± 0.7	7.5 - 10.0

The table 4.1 indicates that all the foodstuffs which were incubated were easily fermentable.

The rumen liquor alone on incubation for 4 hours produced 12.9 m moles of VFA/1000 ml. of rumen liquor, whereas VFA produced on fermentation of Berseem hay, Guar meal, Maize, Spear grass hay and Berseem green were in the descending order of 105.5, 82.7, 42.4, 35.2, and 34.8 m moles/1000 ml. of rumen liquor respectively. The high VFA production from these foodstuffs clearly suggests that the organic nutrients in them were all fermentable. Berseem hay produced maximum amount of VFA of 105.5 m moles/1000 ml. of rumen liquor, although the same quantity of foodstuff was used in each case.

Rate of fermentation:- The rate of production of VFA is fairly high in all cases. It is noteworthy that the rate of fermentation in Berseem which was highest was calculated to be 26.37 m moles per hour/1000 ml. of rumen liquor. Whereas the rate of VFA production in Guar meal, Maize, Spear grass hay and Berseem green was in the descending order 20.65, 10.6, 8.8 and 8.7 m moles/hr/1000 ml. respectively.

Berseem green used in incubation was but coarse. The lower production of TVFA and its rate may be attributed to its coarseness.

The range of the rate of production of VFA per hour per 1000 ml. of rumen liquor has also been indicated in table 4.1. The rate of production of VFA varied considerably between samples. The range in rate for rumen liquor was found to be 1.25 - 6.05 m moles per hour. Wide fluctuations were also noted for other foodstuffs.

'IN VITRO' INCUBATION OF GROUNDNUT SHELLS.

Analysis of groundnut shells:- A representative sample of groundnut shells used in connexion with the work was analysed. The average composition of the material is shown in table 4.2.

Table 4.2.

Table showing the average composition of groundnut shells expressed in percentages.

Material.	Moisture.	Ash.	C.F.	E.E.	N.F.E.	Protein.	Organic matter.
Groundnut shells...	10.73	9.31	55.77	3.41	14.21	6.57	79.96

Incubation by first method:- This part of the investigation was undertaken to determine the extent of fermentation of groundnut shells in 'in vitro' with the idea of exploring of its use for maintenance for goats.

Finely powdered groundnut shells was incubated by the first method with rumen liquor for varying lengths of time to determine the total VFA produced. The period of incubation was 3 hours, 6 hours, 12 hrs. and 24 hrs. The result is shown in table 4.3.

Table 4.3.

VFA produced on fermentation of groundnut shells at different length of incubation.
(m moles acid/1000 ml. of rumen liquor)

Period of incubation.	No. of obs.	TVFA produced. Average with SE.	TVFA produced. Range.	Rate per hour. Average with S.E.	Rate per hour. Range.
3 hours ...	4	14.0 ± 0.4	12.8-14.8	4.7±0.1	4.3 - 4.9
6 " ...	4	13.8±0.5	12.4-14.8	2.3±0.08	2.1 - 2.5
12 " ...	4	12.8±0.5	12.4-13.6	1.1±0.04	0.9 - 1.1
24 " ...	4	13.2±0.3	12.4-14.8	0.5±0.01	0.5 - 0.6

The above table indicates that 14.0 m moles of VFA are produced upto 3 hrs., thereafter there was no fermentation of groundnut shells.

Origin of VFA produced from groundnut shells:- To see whether the slight increase in VFA at the end of 3 hours was due to actual fermentation or due to absorption of VFA already present in the shells, following experiments were conducted. In this, 1 and 5 gms. of powdered groundnut shells was dissolved in water and kept for varying periods and total VFA was determined. The result is

shown in table 4.4.

Table 4.4.

Table showing VFA released from groundnut shells on soaking in water and kept for different periods. (Expressed as ml acid/1000 ml.)

Groundnut shell.	1 Gram.				5 Gms.
Period kept.	Zero hour.	3 hrs.	6 hrs.	12 hrs.	Zero hour.
No. of obs.	3	3	3	3	3
VFA produced with S.E.	14.6 ± 0.5	15.2 ± 0.2	14.9 ± 0.3	15.9 ± 0.6	76.8 ± 0.9

The above table indicates that the slight increase in VFA's at the end of 3 hours observed in table 4.3 to be due to release of VFA already existing in the groundnut shells. It was further confirmed by the fact that by increasing the amount of groundnut shells to 5 Gm. there was correspondingly definite increase in total VFA.

Fermentation of groundnut shells with added glucose:-

As the groundnut shells contain only 14% of NFE., it was thought that addition of soluble sugar may bring about fermentation of the substance, as glucose provides an easily available source of energy for the cellulolytic organisms initially. The effect of added eglucose to rumen liquor served as control. The result is shown in table 4.5.

Table 4.5.

Result of incubation of groundnut shells for 4 hours with added glucose.

(m moles of VFA/1000 ml. of rumen liquor)

Material.	No. of obs.	Total VFA produced.	Rate per hour.
Rumen liquor with 250 mg. glucose.	3	62.4 ± 1.2	15.6 ± 0.3
Rumen liquor with 250 mg. glucose with 1 gm. groundnut shells.	3	64.6 ± 1.3	16.1 ± 0.3

It is seen from the above table that addition of glucose did not bring about any fermentation of groundnut shells as VFA concentration in the control and experimental flasks were similar.

Fermentation of groundnut shells by '2nd method':- In an effort to bring about fermentation of groundnut shells, second method of incubation was adopted. Incubation was carried out for 24 hours. The result is shown in table 4.6. (See page 63).

The table 4.6 (results) indicate that groundnut shells could not be fermented by neither of the two methods, though it is clear from the table 4.11 that 'in vitro' conditions were suitable for the cellulolytic organisms to thrive by the second method. The cause of its not being fermented was not investigated.

Table 4.6.

Result of incubation of groundnut shell for 24 hours by '2nd method'. (m N acid/1000 ml. of rumen liquor)

Source.	No. of obs.	TVFA produced.		Rate per hour.	
		Average with S.E.	Range.	Average with S.E.	Range.
Rumen liquor ...	3	23.2 ± 0.8	21.6 - 24.0	0.96 ± 0.04	0.9 - 1.0
Groundnut shells ...	3	21.3 ± 1.6	18.4 - 24.0	0.90 ± 0.5	0.8 - 1.0

EFFECT OF PHYSICAL NATURE OF THE FOODSTUFF ON FERMENTATION.

Effect of physical nature of the foodstuff on fermentation was studied. In this, Berseem hay and Jowar leaves were incubated for 12 hours both in the finely powdered form and also ~~xxx~~ chopped to about half-an-inch in length. At the end of 12 hours incubation, total VFA's were determined. The result is presented in table 4.7.

Table 4.7.

Result of incubation of Berseem hay and Jowar leaves both powdered and chopped for 12 hours. (Expressed as m moles of VFA/1000 ml.)

Food-stuff.	Berseem hay.		Jowar leaves.	
	Powdered.	Chopped.	Powdered.	Chopped.
Total VFA.	136.2 ± 2.4	Nil.	64.4 ± 1.7	Nil.

The table 4.7 indicates that in 12 hours, sufficient of the VFA's are produced from powdered foodstuffs, while the same foodstuffs in the chopped form could not be fermented even in 12 hours. This finding is interesting, in view of its practical importance.

FERMENTATION OF PURE NUTRIENTS 'IN VITRO'.

A series of pure nutrients viz. glucose, lactose, starch (soluble) and powdered cellulose (Chromatographic) were fermented for 4 hours by the first method of incubation. TVFA produced from each source and their rate were determined. pH of the contents of each flasks were recorded at the end of incubation. The result is shown in table 4.8 and is an average of three incubations.

Table 4.8.

Result of incubation of pure nutrients for 4 hrs.
(m moles acid/1000 ml. of rumen liquor)

SOURCE.	pH at the end of 4 hrs.	TVFA produced. Average with S.E.	TVFA produced. Range.	Rate per hour. Average with S.E.	Rate per hour. Range.
Rumen liquor.	6.6	27.6 ± 2.4	24.4 - 32.2	6.9 ± 0.6	6.1 - 8.1
Glucose ...	5.5	76.2 ± 1.6	73.6 - 79.2	18.9 ± 0.4	18.4 - 19.3
Lactose ...	5.7	54.7 ± 1.9	51.2 - 60.0	13.68 ± 0.7	12.8 - 15.0
Starch ...	6.3	9.6 ± 0.3	8.1 - 11.1	2.4 ± 0.2	2.1 - 2.7
Cellulose..	6.6	----------	Nil	----------	

The table 4.8 indicates that there is a wide difference in the TVFA produced by different pure nutrients in 4 hours. The rumen liquor itself produced 27.6 m moles/1000 ml. of rumen liquor, whereas glucose, lactose and starch produced 76.2, 54.7 and 9.6 m moles/1000 ml. of rumen liquor respectively.

It is evident from the table 4.7 that cellulose could not be digested in 4 hours. Absence of cellulose digestion can also be inferred from its pH at the end of 4 hours incubation which was same as in rumen liquor of 6.6.

It is interesting to note that starch which is easily fermentable, the rate of VFA production was 2.4 m moles as when compared to glucose and lactose of 18.9 and 13.68 m moles/hr/1000 ml. of rumen liquor.

pH:-

The pH fluctuated inversely with the concentration of VFA in spite of the solution being buffered at pH 6.8 with 2 ml. of 2 M phosphate buffer. This is what is to be expected from the limited buffering capacity on the buffer used as is evident from the table 4.13. Glucose produced maximum amount of VFA which had the lowest pH of 5.5. It is interesting to note that the pH on fermentation of starch was lower than that to be expected from its content of VFA, while only 9.6 m moles of VFA was produced from fermentation, the pH dropped down to 6.3 from 6.6.

The significance of these fi

the chapter on 'Discussion'.

tern incubation of starch:-

re produced in 4 hours in

effect of longer period

was expected that starc

utilised in the rum

after 4 hours. Th

12 hours is given

Table 4.2

entation

00 ml.

d method':-

fermentation of

ntrolled condition

tried. Several ex

he fermentation of ru

owder for 24 hours we

in table 4.11.

f cellulose for

d method'.

of rumen liquor)

cod.	Rate per hour.	
Range.	Average with S.E.	Range
	20.4 - 23.2	0.9 ± 0.04	0.8 - 0
8	153.6 - 201.2	7.2 ± 0.62	6.4 - 8

It is seen from the above table that by this method

cubation there was appreciable digestion of cellulose

h produced 171.9 n moles/1000 ml. as against 1 6 n moles/

0 ml. in 24 hours of incubation by the 'first method'

ble 4.10).

The significance of these findings are discussed in the chapter on 'Discussion'.

Long term incubation of starch:- As very little of VFA's are produced in 4 hours incubation in case of starch, effect of longer period of incubation was studied since it was expected that starch which is known to be appreciably utilised in the rumen, may be fermented sufficiently after 4 hours. The result of incubation of starch for 12 hours is given in table 4.9.

Table 4.9.

Result of fermentation of starch for 12 hrs.
(m moles/1000 ml. of rumen liquor)

SOURCE.	No. of obs.	TVFA produced.		Rate per hour.	
		Average with S.E.	Range.	Average with S.E.	Range.
Rumen liquor.	3	45.2 ± 1.3	43.4 - 47.3	3.7 ± 0.09	3.6 - 3.9
Starch ...	3	96.2 ± 2.9	92.6 -102.0	8.0 ± 0.2	7.7 - 8.5

It is seen from the above table that in 12 hours fairly large amounts of VFA are produced i.e. 96.2 m moles/1000 ml. The rate of production of TVFA was 8.0 m moles/hr/1000 ml. of rumen liquor.

Fermentation of cellulose by 'second method':-

In an effort to bring about fermentation of cellulose under more favourable controlled conditions. 'Second method' of incubation was tried. Several experiments were undertaken in which the fermentation of rumen liquor alone, and of cellulose powder for 24 hours were studied. The result is shown in table 4.11.

Table 4.11.

Result of fermentation of cellulose for 24 hours by '2nd method'.

(in m moles/1000 ml. of rumen liquor)

SOURCE.	No. of obs.	TVFA produced.		Rate per hour.	
		Average with S.E.	Range.	Average with S.E.	Range.
Rumen liquor.	3	21.3 ± 0.9	20.4 - 23.2	0.9 ± 0.04	0.8 - 0.9
Cellulose.	3	171.9 ± 14.8	153.6 - 201.2	7.2 ± 0.62	6.4 - 8.4

It is seen from the above table that by this method of incubation there was appreciable digestion of cellulose which produced 171.9 m moles/1000 ml. as against 1 6 m moles/1000 ml. in 24 hours of incubation by the 'first method' (Table 4.10).

The rate of VFA production from cellulose by this method is 7.2 m moles/hr/1000 ml. which is fairly high when compared to that produced by first method of 0.66 m moles/hr/1000 ml. of rumen liquor. (Table 4.10).

The pH changes in the above incubations were recordred. The pH changes recorded at 6 hourly intervals gave sufficient indication of the extent of fermentation of cellulose, with time, without actually estimating the VFA produced. The pH changes are shown in table 4.12.

Table 4.12.

Result of pH changes during incubation of cellulose by '2nd method'

DATE.	Time.	Rumen liquor.		Rumen liquor with cellulose.	
		pH observed.	Na_2CO_3 soln. added (ml.)	pH observed.	Na_2CO_3 soln. added (ml.)
04.5.63	4 p.m.	7.1	-	7.1	-
04.5.63	10 p.m.	6.7	2.4	6.7	2.4
05.5.63	4 a.m.	6.9	1.2	6.7	2.4
05.5.63	10 a.m.	7.0	0.6	6.8	1.8
05.5.63	4 p.m.	7.1	-	6.8	1.8

From the changes in the pH during incubation from the above table, it is seen that in the first 6 hours of incubation, the deviation in pH in both the flasks was 0.4 from the original pH of 7.1. From the next 6 hours onwards there was increasingly greater reduction in pH in

both the flasks was 0.4 from the original pH of 7.1. From the next 6 hours onwards, there was increasingly greater reduction in pH in the cellulose flask than in the flask which contains rumen liquor only. It appears from this that digestion of cellulose started only after first 6 hours and thereafter it increased gradually.

To test the efficiency of 2 M Phosphate buffer used in 'first Method' of incubation.

2 ml. of Phosphate buffer of pH 6.6 was routinely used in incubation flasks as in chapter (I) on 'Methods' as a substitute for natural buffers in saliva.

To test the buffering capacity to 2 ml. of Phosphate buffer at pH 6.6 was added to 20 ml. rumen liquor to it, known amount of M/10 acetic acid was added and change in the pH was recorded by means of Beckman pH meter. The result of its buffering activity is shown below.

Table 4.13.

Table showing efficiency of Phosphate buffer.

Contents.	No. of obs.	pH. recorded.	Reduction in pH.
1. Rumen liquor with 2 ml. Buffer ...	3	6.5	--
2. Rumen liquor with 2 ml. Buffer plus 5 ml. of M/10 acetic acid. ...	3	6.1	0.4
3. Rumen liquor with 2 ml. Buffer with 10 ml. M/10 Acetic acid ...	3	5.7	0.4

It is seen from the table 4.13 that 2 M Phosphate buffer was very effective for resisting against change in pH upto addition of 10 ml of M/10 acetic acid. This amount of acid is rarely produced in incubations. By adding 10 ml of M/10 acetic acid, pH has gone down to 5.7 which is within the physiological range of rumen pH.

PATTERN OF VFA PRODUCED ON FERMENTATION.

Foodstuffs:- The individual fatty acids produced on fermentation of foodstuffs were analysed by Chromatographic method described in chapter on 'Materials and Methods'. The percentage composition of the three acids viz. acetic, propionic and butyric acids, present at zero hour, on hours incubation of rumen liquor and rumen liquor with different foodstuffs have been shown in table 4.14. (See page 71).

The table 4.14 indicates that the proportions of the three acids did not change much within the samples and between the first two treatments viz. incubated and unincubated rumen liquor.

On the other hand, there was wide variation in the composition of fatty acids produced by the addition of different foodstuffs to rumen liquor.

Table 4.14.

Individual fatty acids obtained under different conditions with foodstuffs.
(Expressed as percentages)

MATERIAL.	No. of obs.	BUTYRIC. Average with S.E.	BUTYRIC. Range.	PROPIONIC. Average with S.E.	PROPIONIC. Range.	ACETIC. Average with S.E.	ACETIC. Range.
Unincubated rumen liquor.	3	20.50 ±0.32	20.07 - 21.11	19.54 ±0.14	19.26 - 19.73	59.94 ±0.16	59.63 - 60.20
Incubated rumen liquor.	3	18.89 ±0.20	18.60 - 19.28	20.57 ±0.14	20.30 - 20.72	60.46 ±0.32	60.00 - 61.30
Rumen liquor with Maize.	3	22.50 ±0.68	21.38 - 23.72	25.08 ±0.31	24.46 - 25.52	52.41 ±0.37	51.87 - 53.10
Rumen liquor with Guar meal.	3	24.53 ±0.39	23.84 - 25.19	27.76 ±0.20	27.40 - 28.02	47.69 ±0.54	46.94 - 48.75
Rumen liquor with Berseem hay.	3	18.66 ±0.86	16.95 - 19.70	25.34 ±0.66	24.08 - 26.30	55.99 ±0.51	55.01 - 56.73
Rumen liquor with Berseem green.	3	25.58 ±0.32	24.82 - 26.13	22.90 ±0.49	22.18 - 23.86	51.54 ±0.73	50.10 - 52.51
Rumen liquor with Spear grass hay.	3	20.40 ±0.76	18.95 - 21.51	20.09 ±0.56	19.35 - 21.19	59.50 ±0.20	59.14 - 59.85

It is interesting to find that the percentage composition of VFA in the slaughtered animal did not materially differ from that of the acids produced from its 'in vitro' fermentation.

A probable reason for this agreement may be found in the fact that both the 'in vitro' and 'in vivo' fermentation pattern could be similar to a great extent.

The differences in the composition of the fatty acids produced with different added substrates were analysed statistically and the result is presented in table 4.15.

Table 4.15.

Analysis of variance of relative percentages of fatty acids.

Source of variation.		d. f.	MEAN SQUARES. Butyric.	Propionic.	Acetic.
Total.	...	17	4.0	3.7	7.2
Between.	...	5	12.3**	11.4**	23.2**
Error.	...	12	0.55	0.5	0.47

N.B. (**) indicates significance at 1% level.

The above table indicates that the differences in the percentage of each fatty acids produced by different added substrates to rumen liquor are statistically highly significant at 1% level ($P < 0.01$).

The rate of production of individual fatty acids from different foodstuffs was calculated. The result is shown in table 4.16. (See page 73).

Table 4.16.

Rate of production of individual fatty acids on fermentation of different foodstuffs.

(m moles of VFA/1000 ml. of rumen liquor)

SOURCE.	BUTYRIC.		PROPIONIC.		ACETIC.	
	Average with S.E.	Range.	Average with S.E.	Range.	Average with S.E.	Range.
Rumen liquor.	0.41 ± 0.19	0.12 - 0.78	0.74 ± 0.28	0.35 - 1.29	2.07 ± 0.97	0.77 - 3.97
Maize.	2.90 ± 0.02	2.81 - 3.06	4.70 ± 0.58	3.90 - 6.36	3.22 ± 1.13	1.63 - 5.39
Guar meal.	5.96 ± 0.32	5.40 - 3.06	7.01 ± 0.58	6.25 - 8.15	7.80 ± 0.71	6.40 - 8.69
Berseem hay.	5.13 ± 0.38	4.45 - 5.76	7.41 ± 0.36	6.97 - 8.13	13.97 ± 0.94	12.38 - 15.65
Berseem green.	3.35 ± 0.79	1.80 - 4.39	2.33 ± 0.50	1.40 - 3.13	2.97 ± 0.88	1.87 - 4.71
Spear grass hay.	2.12 ± 0.23	1.70 - 2.47	1.69 ± 0.23	1.24 - 1.95	4.99 ± 0.63	3.79 - 5.91

The rate of production of different fatty acids by different foodstuffs was subjected to statistical analysis and the result is presented in table 4.17.

Table 4.17.

Analysis of variance of rate of VFA production from foodstuffs.

Source of variation.	d.f.	MEAN SQUARES.		
		Butyric.	Propionic.	Acetic.
Total. ...	17	3.99	7.57	19.97
Between. ...	5	12.14**	23.94**	59.96**
Error. ...	12	0.48	0.75	2.38

N.B. (**) indicates significant at 1% level.

It is seen from the above table that the rate of production of acetic, propionic and butyric acids by different foodstuffs are statistically highly significant at 1% level. ($P < 0.01$).

The amount and the percentage composition of the three acids freshly produced from fermentation of rumen liquor as also from different foodstuffs were calculated from the data in table 4.14 and the data is represented in table 4.17 (See page 75). The proportions of fatty acids produced from foodstuffs are presented in the form of Histogram in figure 2.

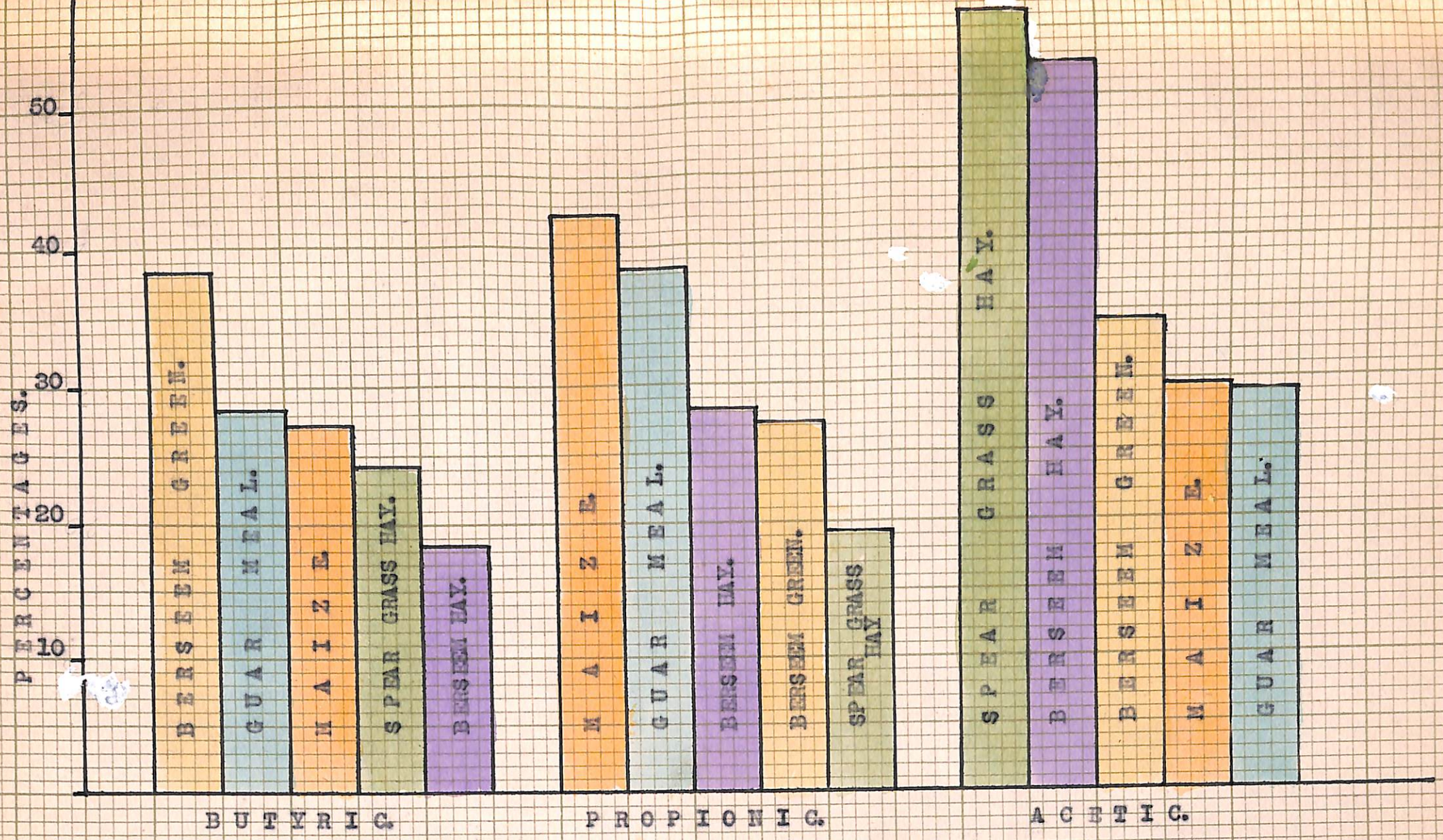
BIHAR VETERINARY COLLEGE PATNA LIBRARY

Table 4.18.

Composition of VFA produced on incubation of foodstuffs.

SOURCE.	Amount of acid (µ moles) per 1000 ml.			Percentage composition.		
	Butyric.	Propionic.	Acetic.	Butyric.	Propionic.	Acetic.
Incubated rumen liquor.	1.65 ± 0.78	2.97 ± 1.1	8.31 ± 3.8	12.20 ± 1.2	24.66 ± 2.0	63.14 ± 1.3
From Maize.	11.63 ± 0.31	17.97 ± 3.9	12.80 ± 4.2	27.54 ± 1.2	42.72 ± 9.2	29.74 ± 9.1
From Guar meal.	23.84 ± 1.3	28.04 ± 2.2	31.20 ± 2.8	28.64 ± 1.2	38.36 ± 7.1	29.60 ± 6.9
From Berseem hay.	20.00 ± 2.0	29.64 ± 1.4	55.89 ± 3.8	18.93 ± 1.4	28.10 ± 0.37	52.97 ± 1.6
From Berseem green.	13.41 ± 3.1	9.62 ± 2.0	11.90 ± 3.5	38.18 ± 3.6	27.34 ± 1.3	34.48 ± 6.8
From Spear grass hay.	8.48 ± 0.7	6.79 ± 0.9	20.00 ± 2.5	24.60 ± 4.2	19.23 ± 1.4	56.27 ± 2.8

??



(Figure 2)

Graph showing percentage composition of fatty acids produced from different foodstuffs.

It is seen from table 4.18 that in general, the butyric acid percentage in the fresh VFA formed from different foodstuffs were generally higher than that formed in the incubated rumen liquor; but showed no correlation with protein content of foodstuffs. It needs to be mentioned here that the butyric acid fraction in our Chromatographic separation always contained the higher fatty acids as well.

Percentage of propionic acid production increased with the increase in content of soluble Carbohydrate in the foodstuffs. Maize which is known to contain maximum amount of soluble Carbohydrates produced 42.72% of butyric acid which was highest.

Percentage of acetic acid increased with the increase in fiber content in the foodstuff. Spear grass hay which is known to have highest crude fiber content among the foodstuffs studied here, produced 56.27% of acetic acid which was highest.

Those results indicate the normal trends of the processes involved.

The detailed discussion of the significance of the findings are discussed in the chapter on 'Discussion'.

The differences in the amounts of individual fatty acids produced by different foodstuffs were statistically analyzed and the results are shown in table 4.19. (See page 77).

Table 4.19.

Analysis of variance of amounts of fatty acids produced by foodstuffs.

Sources of variation.		d.f.	MEAN SQUARES.		
			Butyric.	Propionic.	Acetic.
Total.	...	17	62.1	121.3	309.2
Between.	...	5	190.5**	377.9**	960.4**
Error.	...	12	8.6	14.5	37.8

??

N.B. (**) indicates significance at 1% level.

It is seen from the above table that the amounts of individual fatty acids produced by different foodstuffs are statistically highly significant at 1% level ($P < 0.01$).

Pure nutrients:-

Individual fatty acids present on fermentation of glucose, lactose, starch and cellulose were analysed and the percentage composition of the three acids viz. Acetic, Propionic and Butyric acids present are shown in table 4.20. (See page 78).

The rate of production of individual fatty acids from glucose, lactose, starch and cellulose were calculated from the data in table 4.19 and the result is presented in table 4.21. (See page 79).

Table 4.20.

Individual fatty acids obtained under different conditions with pure nutrients.

(Expressed as percentages)

MATERIAL.		Period of incubation.	No. of obs.	BUTYRIC ACID. Average with S.E.	BUTYRIC ACID. Range.	PROPIONIC ACID. Average with S.E.	PROPIONIC ACID. Range.	ACETIC ACID. Average with SE	ACETIC ACID. Range.
Unincubated rumen liquor.	...	-	-	CHROMATOGRAPHY WAS NOT DONE.					
Rumen liquor incubated.	...	4	3	23.56 ±0.91	21.74 - 25.56	16.39 ±0.69	15.09 - 17.08	59.81 ±0.64	58.53 - 60.57
Rumen liquor with glucose.	...	4	3	13.8 ±0.51	12.91 - 14.68	49.91 ±0.91	48.60 - 51.66	36.26 ±0.42	35.42 - 36.71
Rumen liquor with lactose.	...	4	3	15.15 ±0.92	13.45 - 16.63	36.16 ±0.69	34.90 - 37.29	48.68 ±0.97	46.08 - 51.64
Rumen liquor incubated.	...	12	3	13.94 ±0.86	12.90 - 15.15	20.56 ±0.75	19.12 - 21.66	65.50 ±0.12	65.33 - 65.73
Rumen liquor with starch.	...	12	3	17.03 ±0.62	15.88 - 18.04	23.15 ±0.87	21.42 - 24.26	59.79 ±0.60	58.59 - 60.52
Rumen liquor incubated.	...	24	3	17.08 ±0.37	16.44 - 17.73	19.60 ±0.31	18.98 - 20.00	63.31 ±0.13	63.09 - 63.55
Rumen liquor with cellulose.	...	24	3	7.49 ±0.55	6.46 - 8.33	51.59 ±0.55	51.03 - 53.70	42.24 ±1.80	38.96 - 45.26

		0.11	0.35 - 0.72	16.77 ± 0.41	15.96 - 17.32	1.86 - ±0.57	0.72 - 2.54
Lactose.	...	0.23 ± 0.11	0.11 - 0.45	9.19 ± 0.37	8.54 - 9.84	4.25 - ±0.71	2.96 - 5.68
Starch.	...	1.70 ± 0.05	1.61 - 1.80	2.13 ± 0.15	1.88 - 2.39	4.19 ±0.10	3.98 - 4.30
Cellulose	...	0.23 ± 0.07	0.11 - 0.37	4.74 ± 0.49	4.14 - 5.73	2.18 ±0.04	2.13 - 2.27

BBBB

Table 4.21.

Rate of production of individual fatty acids on fermentation of pure nutrients.

(m moles of VFA/1000 ml. of rumen liquor)

SOURCE.	BUTYRIC. Average with S.E.	BUTYRIC. Range.	PROPIONIC. Average with S.E.	PROPIONIC. Range.	ACETIC. Average with S.E.	ACETIC. Range.
Glucose. ...	0.52 ± 0.11	0.35 - 0.72	16.77 ± 0.41	15.96 - 17.32	1.85 - ±0.57	0.72 - 2.54
Lactose. ...	0.23 ± 0.11	0.11 - 0.45	9.19 ± 0.37	8.54 - 9.84	4.25 - ±0.71	2.96 - 5.68
Starch. ...	1.70 ± 0.05	1.61 - 1.80	2.13 ± 0.15	1.88 - 2.39	4.19 ±0.10	3.98 - 4.30
Cellulose ...	0.23 ± 0.07	0.11 - 0.37	4.74 ± 0.49	4.14 - 5.73	2.18 ±0.04	2.13 - 2.27

BBBB

It is seen from thetable 4.21 that the rate of production of butyric acid was less than 1 m mole/hour/lit. which is very low when compared to propionic acid production, except in case of starch which has a rate of 1.7 m mole/hr/lit.

The rate of propionic acid production by glucose, lactose, cellulose and starch were in the descending order of 16.77, 9.19, 4.74 and 2.13 m mole/hr/lit.

The rate of acetic acid production by pure nutrients was also low when compared to propionic acid. The rate of acetic acid production by lactose, starch, cellulose and glucose were in the descending order of 4.25, 4.19, 2.18 and 1.85 m moles/hr/lit. of rumen liquor.

Composition of fresh fatty acids produced from pure nutrients:-

The amount and percentage composition of individual fatty acids freshly produced from glucose, lactose, starch and cellulose was calculated from the data in table 4.20. The result is shown in table 4.22. (See page 81).

It is seen from table 4.22 that percentage of butyric acid produced from glucose, lactose, starch and cellulose were 2.71, 1.94, 21.16 and 3.15% respectively.

Table 4.22.

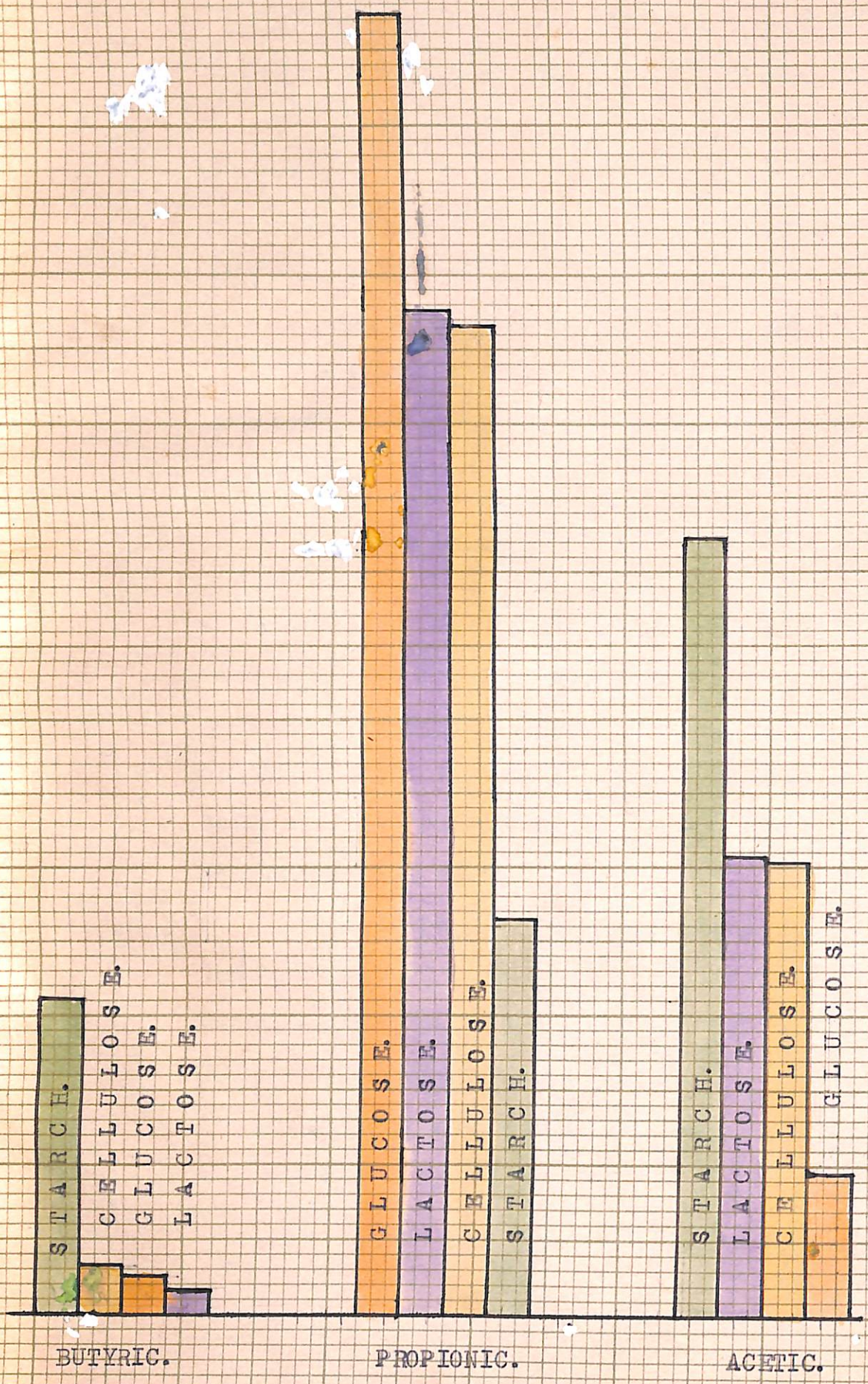
Composition of VFA produced on incubation of pure nutrients.

SOURCE.	Amount of acid (m moles per 1000 ml.)			Percentage composition.		
	Butyric.	Propionic.	Acetic.	Butyric.	Propionic.	Acetic.
Glucose.	2.10 ± 0.4	67.08 ± 1.7	7.42 ± 2.3	2.71 ± 0.47	87.89 ± 3.2	9.60 ± 2.9
Lactose.	0.94 ± 0.4	36.78 ± 1.5	17.01 ± 3.1	1.94 ± 0.73	67.45 ± 3.7	30.61 ± 4.4
Starch.	20.34 ± 0.7	25.61 ± 1.8	50.31 ± 1.3	21.16 ± 0.30	26.56 ± 1.3	52.14 ± 1.2
Cellulose.	5.62 ± 1.8	113.83 ± 11.9	52.41 ± 1.1	3.15 ± 0.77	66.69 ± 1.0	30.39 ± 1.7

??????????????

(Figure 3)

showing percentage composition of fatty acids produced from pure nutrients.



Glucose, lactose and cellulose produced maximum amounts of propionic acid, 87.89, 67.45 and 66.69% respectively, whereas starch which produced 26.56%.

Percentage of acetic acid produced from starch, lactose, cellulose and glucose were in the descending order 52.14, 30.61, 30.39 and 9.60% respectively.

The proportions of fatty acids produced from pure nutrients are presented in the form of Histogram in the figure 3.

S T A T I S T I C A L S T U D Y.

Detailed statistical studies on the percentage composition of fatty acids produced by different foodstuffs were undertaken. A series of correlation studies between the percentage composition of fatty acids and the crude fiber and T.D.N. content of foodstuffs was also studied.

The average percentages of the fatty acids produced from different foodstuffs under study were tested statistically for their significance by applying sine inverse transformation as given in Snedecor (1956). The analysis of variance table 4.23 given below summarizes the results.

Table 4.23.

Analysis of variance table for testing averages of butyric, propionic and acetic acids.

Source of variation.	d.f.	MEAN SQUARES.		
		Butyric.	Propionic.	Acetic.
Total ...	17	41.0	44.0	96.5
Between ...	5	109.8**	88.8*	236.7**
Within ...	12	12.4	25.4	38.1

(*) indicates significance at 5% level.
(**) indicates significance at 1% level.

The above table indicates that the butyric and acetic acid production by different foodstuffs are significant at 1% level ($P < 0.01$); while propionic acid production was significant at 5% level ($P < 0.05$).

To study whether each foodstuff differs with the other in the production of individual fatty acids, the values of critical difference at 5% and 1% were calculated for each of them is shown in table 4.24. (See page 84).

Correlation coefficients were estimated between the crude fiber percentage and total digestible nutrients of the foodstuffs with the acetic, propionic and butyric acid produced from each foodstuff. The data for crude fiber and T.D.N. content of the foodstuffs were taken from I.C.A.R. Bulletin No.25 (1946). The result is shown in table 4.25. (See page 85).

Table 4.24.

Table showing average percentages of fatty acids produced from foodstuffs and their critical difference at 5% and 1%.

FOOD-STUFF.	BUTYRIC ACID.			PROPIONIC ACID.			ACETIC ACID.		
	Average percentage with S.E.	Critical difference.		Average percentage with S.E.	Critical difference.		Average percentage with S.E.	Critical difference.	
		at 5%	at 1%		at 5%	at 1%		at 5%	at 1%
Maize.	27.54 - 1.2	6.32	8.86	42.71 - 9.2	8.94	12.5	29.74 - 9.1	10.9	15.3
Guar meal.	28.67 - 1.2	"	"	38.36 - 7.1	"	"	29.63 - 6.4	"	"
Berseem hay.	18.93 - 1.3	"	"	28.10 - 0.37	"	"	52.96 - 1.6	"	"
Berseem green.	38.18 - 3.6	"	"	27.34 - 1.3	"	"	34.48 - 6.8	"	"
Spear grass hay.	24.60 - 4.2	"	"	19.24 - 1.4	"	"	56.27 - 2.8	"	"

Table 4.25.

Table showing correlation coefficients between crude fiber and T.D.N. content of foodstuffs and fatty acids produced.

VARIABLES.	Correlation coefficient (r).
Crude fiber % X butyric acid %	- 0.68
Crude fiber % x propionic acid %	- 0.81
Crude fiber % X acetic acid %	+ 0.38
T.D.N. X butyric acid.	+ 0.11
T.D.N. X propionic acid.	+ 0.61
T.D.N. X acetic acid.	+ 0.15

Significance of these statistical inferences are discussed in the chapter on 'Discussion'.

CALORIFIC VALUE OF FATTY ACID PRODUCED FROM FOODSTUFFS.

Total fatty acids produced from each foodstuff per day was calculated taking 4 liters as the normal rumen capacity of goat. These figures were corrected for metabolization of fatty acids by microbes according to Mukherjee (1960) at the rate of 23.3% per hour for butyric and 6.6% per hour for acetic acid. Using the calorific values of these acids according to Carroll and Hungate (1954) as 208.4 Cal/mole of acetic, 373.5 cal/mole of propionic, and 504.8 Cal/mole of butyric acid, daily calorific value of fatty acids available from each foodstuffs were calculated. The result is shown in table 4.26. (See page 86).

Table 4.26.

Table showing total Cal. available per day from each foodstuff.

SOURCE.	Butyric (Cal.)	Propionic (Cal.)	Acetic (Cal.)	Total (Cal.)
Maize. ...	119.9	170.7	60.7	350.77
Guar meal. ...	221.5	297.7	145.7	664.9
Berseem hay....	190.6	265.6	259.0	715.2
Berseem green.	124.6	84.1	55.52	264.22
Spear grass hay.	78.74	60.8	93.24	232.78

ESTIMATION OF KETONE BODIES. STANDARD CURVE FOR ACETONE.:-

Standard curve for acetone was drawn as described in the chapter on 'Materials and methods'. The curve is shown in figure (4).

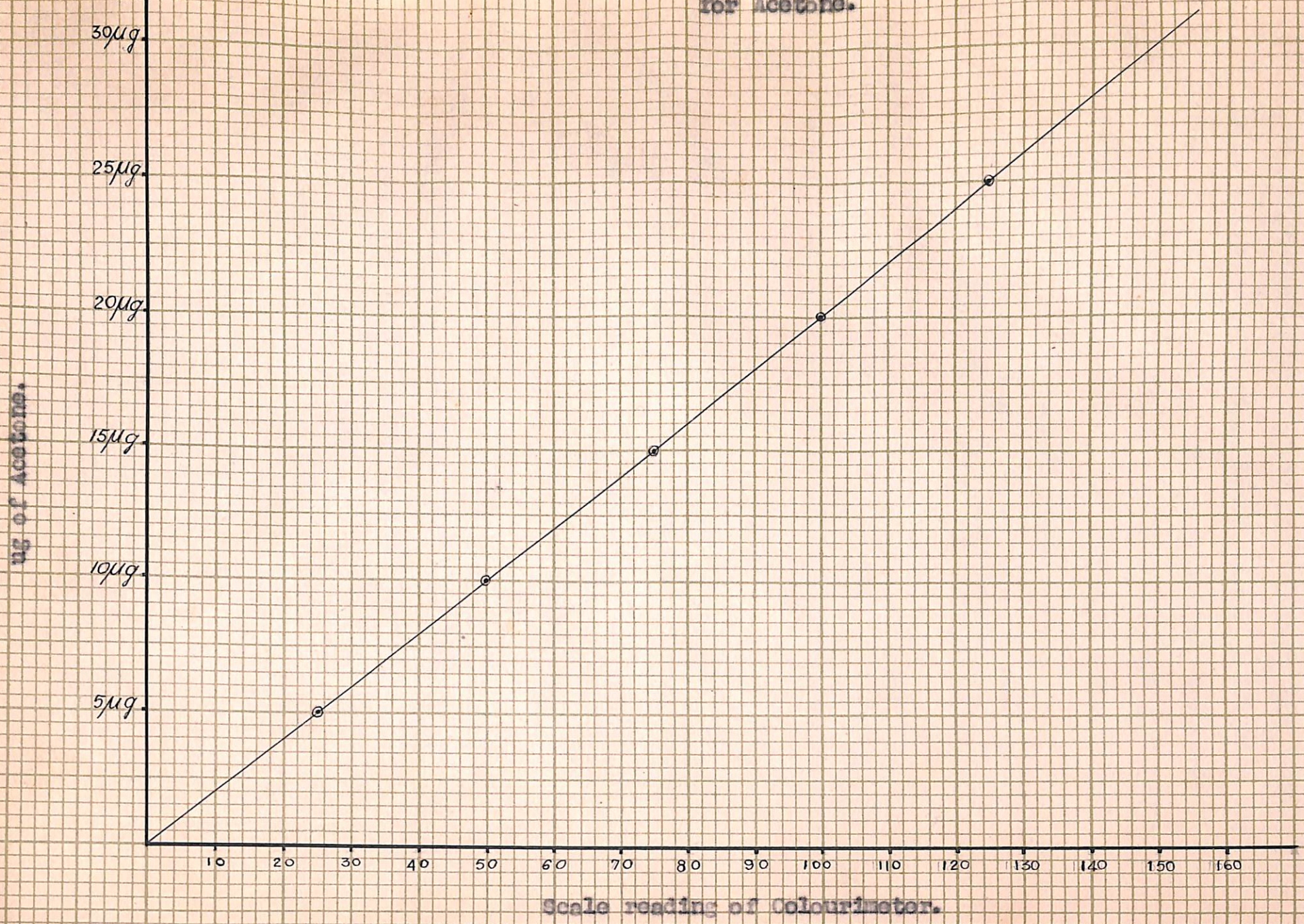
It is seen from the standard curve that every five divisions on the Klett - Summerson photoelectric colourimeter is equivalent to 1 µgm. of Ketone bodies.

'In vitro' incubation of butyric acid:-

To investigate the production of Ketone bodies from butyric acid, several experiments were undertaken in which rumen liquor was incubated for 4 hours and also with the addition of 5, 10 and 20 mgs. of butyric acid to rumen liquor and total Ketone bodies were estimated at the end of 4 hours; as per the

(Figure 4)

Graph showing the standard curve for Acetone.



methods described in chapter on 'Materials and Methods'. It was found that maximum Ketone bodies production was seen by the addition of 10 mg. of butyric acid and does not increase with the further increase in concentration of butyric acid. The result is shown in table 4.27.

Table 4.27.

Table showing total Ketone bodies production from butyric acid on 4 hours incubation. (Expressed as µg/100 ml. of rumen liquor)

Experiment No.	Rumen liquor incubated for 4 hours.	Rumen liquor with 10 mg. butyric acid.	Total Ketone bodies produced from butyric acid.
1.	375.00	625.00	250.00
2.	531.25	748.00	216.75
3.	437.50	656.25	218.75
4.	500.00	781.25	281.25

It is seen from the above table that some Ketone bodies are produced from butyric acid on fermentation with normal rumen content, the quantity does not seem to differ very much in rumen liquor and whole rumen content.

---xXx---

CHAPTER (V).

DISCUSSION.

Chapter (V)

D I S C U S S I O N.

COMPARISON OF 'IN VITRO' METHODS EMPLOYED.

As roughage utilisation is probably the most important rumen function, it is essential for nutritionists to know more about the factors affecting fiber digestion in the rumen. Several types of artificial rumens have been devised for 'in vitro' studies on problems relating to rumen digestion to enable investigation under variety of controlled condition.

Rumen digestion depends almost entirely upon the microorganisms which are present in the rumen. Therefore, in order that the results obtained from any artificial rumen to be significant, the apparatus must be capable of maintaining true rumen microorganisms during the whole fermentation period, simulating as far as possible the natural conditions prevailing in the rumen.

Any 'in vitro' appratus, such as the 'Artificial rumen' has both advantages and disadvantages. The substitution of a simple laboratory device for the intact animal has its main advantages, convenience and œconomy. It also allows greater control and more extensive observations under a wide variety of experimental conditions than does the 'in vivo' method; but it is apparent that conditions

set up artificially must be substantially similar to the environmental conditions in the intact rumen. The limitation is primarily the very real problem of transferring results obtained in the laboratory to the intact animal. Therefore, 'in vitro' devices find their greatest usefulness as pilot device, with realisation that the results obtained do not necessarily duplicate similar functions in the ruminant.

The artificial rumen technique was used in the study since it permitted a quantitative approach to this study of the fatty acids without the usual 'in vivo' interfering factors of absorption or passage of these metabolites from the rumen.

The Carbohydrate constituents of the plant products which are used in ruminants feeding practice range from simple sugars and disaccharides to the high molecular weight plant cell wall components, cellulose and the hemicelluloses. The simple sugars are rapidly attacked with the production of non-volatile acids and the lower straight chain VFA's (Annison and Levis, 1959). Burroughs et. al.(1950 a, b, c and 1951 a, b.) using the artificial rumen technique have made extensive investigations on the mineral requirements of rumen bacteria. It was found that cellulose digestion could be stimulated by addition of a small quantities of salts to the artificial rumen. An "Artificial saliva" containing all essential minerals including trace elements was devised and added

to the artificial rumen. This proved to be a work of basic importance in the development of 'in vitro' rumen fermentation systems.

In the second method, where all the trace elements were provided in mineral mixture, pure cellulose was digested appreciably in 24 hours (Table 4.11), while no digestion could take place during this period by the first method. Since appreciable fermentation of both the foodstuffs and the pure nutrients (glucose and lactose) was observed by the first method of incubation for 4 hours, it is safe to presume that the trace requirements of the microorganisms were being provided from the rumen liquor or / and the foodstuffs. In case of cellulose, however, which requires longer period of incubation, could not be fermented by 24 hours incubation by the first method of incubation, it is reasonable to infer that the trace requirements become critical in later periods.

Advantages of the first method are, it is simple and easy to carry out, it is sufficiently buffered for changes in the pH to a deleterious extent and does not bring any unnatural conditions on the microbiota atleast for short periods of incubation. This method is unreliable for longer periods of incubation.

The second method though complicated is definitely an improvement over the previous method for longer period of incubations and has got definite advantages. The only point to be criticized in this is that a considerable change in the microflora might have occured in the longer fermentation period.

RATE OF VFA. PRODUCTION IN 'IN VITRO'.

Factors controlling the rate of VFA. production:-

Factors controlling the rate of VFA production for any sample of rumen liquor are mainly the period of incubation, chemical and physical nature of the foodstuffs when the pH of the medium does not change deleteriously. The factors are discussed individually below:-

(a) Period of incubation:- The rate of VFA production from the same food depends upon the availability of protein and other essential vitamins and trace elements to the microorganisms in the medium (Burroughs et. al. 1950 a, b, and c; 1951 a, and b.). The rate also varied with the period of incubation (Gray and Pilgrim, 1952). Gray and Pilgrim incubated powdered wheaten hay by rumen liquor from a sheep-fed on the same diet and observed a rate of 6 m moles/hour at 4½ hours, 7.1 m moles on 7½ hours and 3.6 m moles on 19½ hours of incubation. It is seen that maximum rate of production was maintained upto 7½ hours and on 19½ hours, the rate was found to be minimum.

The falling off of the 'in vitro' rate in the above experiment may be due to either lack of substrate or lack of optimum 'in vitro' condition or due to both, in long period of incubation. Balch and Rowland (1957) found in steers rumen, that maximum rate 'in vivo' was reached within first 3 - 4 hours after feeding, at the end of 6 hours it tended to decline.

In view of these findings, an average period of 4 hours of incubation was routinely followed in our experiments, which represented the period of maximum fermentation activity of microbes. As the object in our experiment was only to find out the proportions of three acids produced by different foodstuffs. All incubations have been done for a fixed period (4 hours) and no attempt has been made to find out the rate of VFA production with increasing time of incubation.

(b) Chemical nature of foodstuffs:- The rate of fermentation is also affected by the chemical nature of the foodstuffs, specially with respect to the quantitative presence of easily available source of Nitrogen and soluble Carbohydrates.

(i) N F E. The rate of VFA production is primarily affected by the nature of the Carbohydrates present. Each carbohydrate constituents of the ruminant diet is degraded to some extent in the rumen. Easily digestible carbohydrates like starch, cane sugar etc. are rapidly attacked with the consequent reduction in the digestibility

of the fiber (Swift et. al. 1947; Watson et. al. 1949; and Head, 1953). This may be explained due to the preferential attack on the simpler Carbohydrates by the bacteria.

But, it has also been shown that a certain amount of easily available carbohydrates like starch is essential for the proper utilisation of fiber, the former provides energy for the bacteria in the early stages of fermentation (Maynard and Loosli, 1956). The addition to the roughage ration of moderate amounts of pure protein (Hoflund et. al. 1948) or protein rich feeding stuffs (Burroughs et. al. 1949) had, in the absence of starch, no effect on the digestibility of cellulose.

(ii) Nitrogen:- Although the nature of carbohydrate of the foodstuff is the important controlling factor in the determination of both the rate and the proportions of the acids, the Nitrogen requirements of the fermenting bacteria must also be met simultaneously.

As the bacteria multiply, they synthesize protein to construct, their own bodies obtaining raw material from the ingested food. For this purpose, they can utilise the Nitrogen from amides, ammonium salts and even nitrates, as well as the protein itself. Therefore, a certain minimum amount of Nitrogen either in the form of protein or N.P.N. substance is essential for the growth and multiplication of microorganisms. In the absence of a source of

Nitrogen, the rate of fermentation is being reduced. McDonald (1952) has demonstrated that there was an increase in the rate of VFA production from starch when small amounts of casein are also added.

Williams et. al. (1953) found that a minimum amount of protein is necessary for proper microbiological breakdown of fiber. Maynard and Loosli (1956) stated that the replacement of poor quality hay by alfalfa has been shown to stimulate microbiological activity, suggesting that the latter hay may supply specific vitamins or other factors needed for the best growth of bacteria. The same explanation appears to hold good for the faster fermentation rate observed with Berseem hay in our experiment.

The above discussion gives us clue that a certain ratio between Nitrogen and Carbohydrate is needed by the rumen microbes for the proper utilisation of crude fiber. This is also supported by the work of Lewis and McDonald (1958) who have shown that active fermentation was encouraged in the presence of both supplements of easily available source of carbohydrates and Nitrogen and that growth and synthetic reactions were increased by a more balanced system. It appears, therefore, that there is a considerable interdependence between carbohydrates and protein digestion in the rumen.

(c) <u>Physical nature of foodstuffs:-</u>

Extent of digestion of foodstuffs depends primarily on the physical nature of the nutrients presented to the microorganisms (Baker et. al. 1959).

These investigators have reported that the 'in vitro' cellulose digestibility of cotton linters could be increased slightly by grinding to a fine powder in a ball-mill. Thus any effect on digestion due to particle size, must be either in the rate of digestion or in the duration of 'lag phase'. Dehority (1961) found that ball-milling cellulose increases the overall rate of cellulose digestion by reducing the 'lag phase' upto 6 hours from 12 hours. Thus to determine if a true difference in the rate of digestion occurs, comparison should be made when maximum digestion is occuring, and ther substrate concentration is non-limiting.

In our experiments, the foodstuffs which were used in incubations were all finely powdered with pestle and mortar, after drying. In case of green berseem, however, it was finely chopped with scissors and appreciable fermentation was observed.

The effect of particle size on the rate of fermentation was investigated. It is interesting to note that when Berseem hay and Jowar leaves chopped about half-an-inch in length were incubated, could not at all be fermented even upto 12 hours. While the same foodstuffs when powdered after drying in pestle and morter, appreciable fermentation occured in 4 hours only. This finding has a direct bearing on the importance of chaffing the roughages before feeding and more important on the proper mastication by the ruminant.

Rate of VFA. production from foodstuffs:-

The rate of ~~VFA.~~ production of VFA from the same foodstuff varied appreciably between incubations, although the duplicates of each incubation agreed very closely. This variation between incubations may be due to either lack of optimum conditions for 'in vitro' fermentation, or to inherent variations in the factors controlling the rate of fermentation such as types and the numbers of the microflora present.

The data of the present work are compared with those obtained by other workers from steers, sheep and goat, is shown in table 5.1. (See page 97).

It is interesting to note that maximum rate of VFA production was from Berseem hay, though same quantity of all foodstuffs were incubated for the same period of 4 hours. Maize which contains mostly starch had a rate of only 10.6 m moles/hr/liter which is much lower compared to berseem hay with a rate of 26.4 m moles/hr/liter. Our observations may, to some extent, appear not to agree to those observed by Balch and Rowland (1957) and many others who found an increased rate of VFA production after feeding low hay diet with high concentrated than those containing normal amounts of hay. This may be due to the fact that in their experiments, a mixture of concentrates and hay was being fermented and as such, it is understandable that the concentrate could have been preferentially attacked while in our experiments the two ingredients were incubated separately.

Table 5.1.

Rate of production of VFA. in 'in vitro' fermentation of rumen content with various materials as compared to our data on foodstuffs.

Materials.	Animal.	Rate per hour.	Reference.
Whole rumen content from hay-fed steer. ...	Steer.	11.8 m moles per Kg.	Carroll and Hungate. (1954)
Alfalfa hay incubated with rumen liquor. ...	Steer.	23.8 mg./100 ml.	Stewart and Schultz. (1958)
Rumen liquor from sheep fed on wheaten hay. ...	Sheep.	8.7 m moles.	Gray and Piligrim. (1952)
Whole rumen content from sheep fed on hay and concentrates. ...	Sheep.	8.2 m moles per Kg.	Mukherjee. (1960)
Whole rumen content from hay and concentrate fed sheep fasted overnight. ...	Sheep.	4.1 m mole per Kg.	
Bagasse fermented with rumen liquor from slaughtered goats.	Goat.	9.8 m moles per lit.	Maitra. (1962)
Rumen liquor from slaughtered goats incubated with food-stuffs. ...	Goat.		
(a) Berseem hay. ...	-do-	26.4 m moles/lit.	
(b) Guar meal. ...	-do-	20.65	-do-
(c) Maize. ...	-do-	10.6	-do-
(d) Spear grass. ...	-do-	8.8	-do-
(e) Berseem green. ...	-do-	8.7	-do-

hhhh

However, our results are in agreement with those of McNaught (1951); and Stewart and Schultz (1958) who found that 'in vitro' incubation of corn meal with steers rumen content, yielded lower levels of VFA than alfalfa hay. Phillipson (1952) also observed in his preliminary investigations with sheep, that concentrations of total VFA was lower than would be expected from the high starch content of the ration.

Possible reasons for the lowered VFA production from maize may be:

(1) Physical:- Due to differences in the degree to which they are subjected to grinding before incubation.

(2) Chemical:- Maize being a starchy food, is being fermented, to lactic acid (Phillipson, 1952) which later on is being converted to propionic acid. Phillipson (1952) stated that the first stage of lactic acid formation proceeds at a greater rate than the subsequent conversion of lactate to VFA. The period of incubation being a shorter period of 4 hours, there was, perhaps not much conversion of lactate to VFA. this process being slower one.

The possible explanation for the faster rate with Berseem may be found in the fact that the NFE of berseem (about 38% on D.M. basis) might contain soluble sugars simpler than starch which would naturally be attacked at a very fast rate, as have been found by us with glucose and lactose.

FERMENTATION OF GROUNDNUT SHELLS
'IN VITRO'.

With the idea of exploring of its use for maintenance for goats, preliminary 'in vitro' incubation of powdered groundnut shells was undertaken to determine the extent of its fermentation if any as evidenced by production of VFA's.

The criteria for digestibility being that with increasing period of incubation, there should be continuous increase in total VFA. though the rate may recede after certain interval. On incubation of groundnut shells for different periods, there was no increase in total VFA after 3 hours (Table 4.3.). The slight increase in total VFA observed at the end of 3 hours was found to be due to release of VFA already existing in shells (table 4.4.).

It is an established fact that the soluble sugar in small amounts furnish available source of energy to microorganisms in the rumen and the bacterial population is thus increased, resulting into improved utilisation and breakdown of crude fiber. But if the ration contains enough of soluble sugar, then the utilisation of crude fiber is hampered as the microbes attack the soluble sugar preferentially to crude fiber (Burroughs et. al. 1949; Arias et. al. 1951; Hershberger et. al. 1955 and Kane et. al. 1959). As groundnut shells contain only 14% of

NPN. so in this case addition of 250 mg. of glucose also did not bring about digestion of groundnut shells (Table 4.5.).

The ruminant possesses no salivary enzyme for the degradation of cellulose nor is there any such enzyme secreted by the rumen. Thus the degredation of cellulose is clearly a function performed by the flora found in the rumen. Burroughs et. al. (1951) have found that rumen flora are extensively sensitive to changes in the environment and have got varying requirements. To provide more favourable conditions groundnut shells was incubated by the second method (Table 4.6.) indicates that even by this method groundnut shells could not be digested; although it is clear from the table 4.11 that 'in vitro' conditions were suitable for the cellulolytic organisms to thrive by the second method.

The cause of its not being fermented was not further investigated.

Though groundnut shells contain 62% of crude fiber, rumen microbes should have fermented it. The possible reason for its not being fermented is that shell is the hardest part which acts as a protective covering to groundnut seeds. Polysaccharides are attacked by the microbes at rates which roughly reflect their solubility and ease of accessibility, the most interactable materials being cellulose and hemicellulose, which exist in close association with lignin which covers the former.

It appears that in this, lignification may have advanced to a great degree. The lignin is not only indigestible itself, but it also lowers the digestibility of the cellulose and other complex carbohydrates (Maynard and Loosli, 1956), though lignin was not estimated in this case. The above reasoning gives appealing explanation for its indigestibility 'in vitro'.

RATE OF VFA PRODUCTION FROM PURE NUTRIENTS.

Glucose and lactose because of their easily soluble nature were found to be highly fermentable. The average rate of VFA production being 18.9 and 13.7 m moles/hr/liter with a range of 18.4 - 19.3 and 12.8 - 15.0 m moles per hr/liter respectively (table 4.8). It is quite apparent from the above results that the rate of production of VFA between incubations and replicates of the same incubations agreed very closely in contrast to the wide variation observed in case of foodstuffs (table 4.1). This increased production of VFA from glucose and lactose is also inferred from lowered pH 5.5 and 5.7 (table 4.8) observed at the end of 4 hours incubation which varied inversely with the VFA production. This statement is supported by the findings of Waldo and Schultz (1956) who found correlation coefficient of r = -0.57 between total fatty acids and pH which was highly significant (P 0.01). Our pH changes are in general agreement with results of Hinders and Ward (1961) who observed similar pH changes in different foodstuffs incubated 'in vitro'.

Though starch is known to be readily fermentable, in our case we found that the rate of VFA production was not found to be so during 4 hours incubation. This finding is supported by statement of Annison and Lewis (1959) that the fermentation of starch in rumen is slow and complex process accompanied by multiplication of the microorganisms concerned.

Lowered production of VFA from starch may be due to ingestion of starch grains by some protozoa especially the smaller oligotrich citrates of genus Entodinium, which are retained in the gastric sac until they are digested. A storage polysaccharide, amylopectin was detected within 5 minutes of adding rich starch to a suspension of starved Isotricha (Gutierrez, 1955).

Another reason for this low VFA production from starch may be that fermentation of starch appears to have taken place in atleast two stages in a manner postulated by Elsden (1945-46), the first stage leading to the formation of lactic acid and the second being further fermentation of lactic acid with the production of VFA. Phillipson (1952) stated that the first stage of lactic acid fermentation proceeds at a greater rate than the subsequent fermentation of lactate to VFA.

In view of the above reasoning and because of short period of incubation of 4 hours, the second explanation cited above appears to fit well in the facts.

Another point in favour of this is that; PH recorded at the end of 4 hours incubation was 6.3 (table 4.8)

which was lower than control of pH 6.6, in which rumen liquor alone was incubated for 4 hours. This reduction in pH in case of starch is more than what could be expected from the production of 9.6 m moles of VFA per liter from starch in 4 hours. This low pH observed may be due to the accumulation of lactic acid, though lactic acid was not estimated in our case. Similar finding was also noted by Phillipson (1957) in lambs fed maize only.

In long term incubation of starch for 12 hours, the rate of VFA production was 8.0 m moles/hour/liter with a range of 7.7 - 8.5. The probable reason for this higher rate may be due to (1) longer 'lag phase' and (2) conversion of lactic acid formed in the earlier stage to VFA. (Phillipson, 1952).

The fact that maize containing a large amount of starch was fermented much faster during the first 4 hours incubation compared to pure starch, may be due to the higher protein content in the fermentation mixture in its case, and also due to presence of simple sugars in it.

It is interesting to note that powdered Berseem hay produced more VFA (26.4 m moles/hr/lit.) than even pure glucose. Whether this is due to the higher Nitrogen content in the former is a matter of conjecture.

In our experiments with food-stuffs essential requirements of the microbes were met from the incubated foodstuffs. However, when pure cellulose was incubated by first method, because of the lack of trace requirements, cellulose could not be digested. Second method which provided trace elements

in the form of mineral mixture could digest cellulose with the production of 7.2 m moles of VFA/hr/lit.

The pH changes during incubation by second method are characterised in that they give sufficient indication of the extent of fermentation ~~of the extent of fermentation~~ of cellulose with time without actually estimating the VFA produced. It is seen from table 4.4 (P) that the reduction in pH in the control and experimental flasks upto 6 hours was similar, for 12 hours onwards, there was greater reduction in pH in the cellulose flask than in the flask which contains rumen liquor only. This indicates that digestion of cellulose is started only after 6 hours and thereafter it gradually increased upto 24 hours. This finding agrees very well with that of Dehority (1961) who found a minimum lag phase of 6 hours before being digested.

COMPARISON OF THE VFA OF RUMEN LIQUOR AND OF THE FRESH VFA'S PRODUCED BY 'in vitro' INCUBATION.

The composition of VFA produced by ruminal fermentation depends upon the diet of the animal and its carbohydrate content.

A comparison of the composition of the VFA before and after incubation of the rumen liquor (table 4.14) shows no significant differences. This is of particular importance because the samples analysed for separation was from different animals, whose feeding habits before collection of the rumen samples were not known.

The close agreement in the proportions of the 3 acids in the rumen content of these animals probably may be due to the fact that the animals from which rumen content has been obtained were in the fasting condition just on the day before slaughter, which nullifies the effect of any feed which the animal might have taken prior to fasting, and left in the rumen more or less similar substrate consisting mostly of the more resisting fractions of the carbohydrate whose fermentation later on resulted in almost similar proportions of the fatty acids.

The close agreement in the composition of the VFA between the replicates of rumen liquor incubated with different foodstuffs and pure nutrients (table 4.14and 4.18) indicates the maintenance of similar incubation conditions. This does not necessarily indicate that the conditions were optimum for 'in vitro' fermentation.

PERCENTAGE COMPOSITION OF FATTY ACIDS PRODUCED FROM FOODSTUFFS.

The present study was designed to estimate the proportion of the three acids produced from roughages and concentrates on 'in vitro' incubation with goat's rumen content. The object of this study was only to know whether foodstuffs differ in the production of three acids or not. As we know that individual fatty acids vary in their efficiency for utilization by the ruminant for productive purposes (Armstrong and Blaxter, 1957 b.), propionic and butyric acids are more efficiently utilised than acetic

acid for lipogenesis. So any food which produces more of propionic and butyric acid is better utilised than that which produces more of acetic acid. It is proposed that a simple determination of the molar proportions of the VFA will serve as a valuable guide to the efficiency of rations for various productive purposes.

The inherent limixtations in 'in vitro' incubation work stand in the way of strict comparison between different experiments even with the same foodstuff. That the pattern of microflora even from different individuals of the same species may differ to a considerable extent, explains lack of reproducibility of these experiments. However, the general trend can always be compared with advantage.

BUTYRIC ACID:-

The percentage of butyric acid produced from Bersoem green, guar meal, maize, spear grass and berseem hay were in the descending order 38.18, 28.64, 27.54, 24.60 and 18.93% respectively (table 4.18). It is seen that in general when compared to incubated rumen liquor which produced 12.2% butyric, the butyric acid percentage from foodstuffs were higher. Elshazly (1952); Belasco (1954); Woodhouse et. al (1955) and Davis et. al (1957) all have shown that high protein diets increase the butyric and higher fatty acids. On the contrary, our results did not show any correlation between the butyric acid with the protein content of foodstuffs. Befause guar meal which is known to contain 40% protein produced only 28% butyric acid, while maize which contains 8% protein produces 27% butyric

which is almost equal to guar meal.

The most probable reason for this high percentage of butyric acid may be found in the fact that during the shorter period of incubation of 4 hours, mostly starch and other soluble sugars if any were being fermented along with breakdown of the proteins, the quantum of latter being much less compared to that of soluble carbohydrates. That the butyric acid percentage was very similar to that obtained by us from pure starch after 12 hours incubation - the earliest period when appreciable fermentation took place - lends additional support to the above hypothesis.

Another contributory factor for the higher proportion of butyric acid may be due to the increased production of butyric and higher fatty acids by sunthesis from lower acids by the Gram negative large Coccus described by Elsden and Lewis (1953) and of Clostridium butyrium as observed by Masson (1950).

However, our results are in agreement with those of Mukherjee (1960) who noticed similar high percentage of butyric acid in the fresh VFA produced in sheep under very similar conditions of incubation.

The persistently higher value for the butyric acid obtained by us as compared to that generally found 'in vivo' may be explained by the fact that butyric acid is absorbed from the rumen at a relatively faster rate (Pfander and Phillipson, 1953).

PROPIONIC ACID:-

The percentage of propionic acid produced from maize, guar meal, berseem hay, berseem green and spear grass hay were in the descending order of 42.77, 38.36, 28.10, 27.34 and 19.23% respectively. The percentage of propionic acid production by different foodstuffs agreed very well with the accepted general trend that with increase in the soluble carbohydrate in foodstuffs, there is an increase in the propionic acid production. Maize which is known to contain maximum soluble carbohydrates produced 42.72% propionic acid which was highest. This trend found by us agreed with 'in vivo' findings of Phillipson (1952) in sheeps rumen and 'in vitro' findings of Stewart and Schultz (1958) with steers rumen content.

ACETIC ACID:-

The percentage of acetic acid produced by spear grass hay, berseem hay, berseem green, maize and guar meal were 56.27, 52.97, 34.48, 29.74 and 29.60% respectively. The percentage in acetic acid production by different foodstuffs also agreed very well with the general trend that with increase in the crude fiber content there is an increase in the percentage of the acetic acid. Spear grass hay which is known to contain maximum crude fiber content produced 56.27% acetic acid which was highest. Similarly, the two concentrates - maize and guar meal - produced relatively much less of acetic acid. This is in agreement with the finding of Stewart and Schultz (1958).

Gray et. al (1951) obtained two different composition of the VFA from 'in vitro' fermentation of wheaten hay and leucerne hay, the latter yielding relatively more of propionic acid. All these fermentation experiments were, however, of longer duration ranging from 48 - 112 hours and as such the overall fermentation pattern might have changed appreciably during the process as compared to the shorter period of incubation of ours.

On statistical analysis of the fresh VFA production they were all found to be ~~mutix~~ statistically significant between the foodstuffs. On the contrary, Carroll and Hungate (1954) who have also carried out incubation experiments for a shorter period of 4 hours, using steer rumen content, however, did not find any appreciable difference in the composition of the VFA production with hay alone and hay and high grain diets.

A probable reason for this similarity in the composition of the VFA produced may be that all his experiments were conducted on rumen samples from fed animals and as such may have contained appreciable proportions of the more readily fermentable carbohydrate fractions. Their data on different feeds are shown in table 5.2 for comparison. (For table 5.2, See page 110).

Table 5.2.

Carroll and Bungate's data on 'in vitro' incubation of rumen contents from steers.

FEED.	% composition of VFA (Initial)			% composition of VFA. produced.			Rate of VFA production m moles/hr/Kg.
	Acetic.	Propionic.	Butyric.	Acetic.	Propionic	Butyric.	
Hay.	62.9	23.0	14.1	60.3	21.1	18.6	14.2
Grains.	68.1	25.4	16.5	63.4	22.6	14.0	23.5
Pasture.	67.6	21.0	11.4	60.2	23.6	16.5	9.5

It is seen from the above table that wide variation in the rate of~~mr~~ fermentation was observed with different feeds, but the composition of the VFA produced during incubation even with samples from a grain diet, did not show any clear difference from that produced from hay. The molar percentage composition of VFA mixtures in the rumen of sheep in the experiments of some of the workers have been shown in table 5.3.

Table 5.3.

Results of proportions of fatty acids present in rumen in fasted and fed sheep.

Physiological state.		Acetic %	Propionic %	Butyric %	Reference.
Fasted.	...	69.4	16.7	13.9	Gray and Pilgrim (1952)
Fed.	...	61.9	23.5	14.6	
Fasted.	...	67.0	20.0	13.0	Pfander and Phillipson (1953)
Fed.	...	65.0	23.0	10.0	
Fasted.	...	74.0	15.0	11.0	Mukherjee. (1960)
Fed.	...	67.5	18.0	14.5	

Table 5.3 indicates that relatively higher proportion of acetic acid in all cases when compared to its proportion in the VFA formed. In all cases, it exceeded more than 65% of the total, while in our 'in vitro' experiments with foodstuffs, 56% of acetic acid in spear grass hay was the highest. Similar low percentages of acetic acid in 'in vitro' fermentation has also been noted by Mukherjee (1960).

This discripancy between the 'in vivo' and 'in vitro' results may be due to the fact that both propionic and butyric acids are absorbed relatively at a faster rate by the rumen wall, particularly when the rumen pH is in the acid range of 5 - 6 (Daniell et. al, 1945; Gray, 1947; and 1948; and Pfander and Phillipson, 1953). Another probable contributory factor may be the increased rate of conversion of butyrate to acetate observed by Mukherjee (1960) in his 'in vitro' experiments to take place appreciably to the extent of 29.3%. The unexplained observation of Gray (1947) of the sudden lowering of the proportion of propionic and butyric acids with the intake of food by sheep receiving a mixture the three acids into its emptied rumen may also be another contributory factor.

STATISTICAL INFERENCE.

By analysis of variance when the fatty acids produced from different foodstuffs were found to differ significantly, to actually find out which of the foodstuffs were significantly different in producing the fatty acids, the average percentage for them were compared with critical

difference. The values of the critical difference are shown in table 4.24.

BUTYRIC ACID:-

Butyric acid production from Berseem green was highest (38.18%) which differed with the rest of the foodstuffs studied at 1% level ($P < 0.01$). The average percentages for butyric acid from guar meal, maize and spear grass hay did not differ significantly. Guar meal and maize differed at 1% level in the production of butyric acid with berseem hay. There was also no difference in the butyric acid percentage between spear grass hay and berseem hay.

(b) PROPIONIC ACID:-

There was no difference in the propionic acid production between maize and guar meal at 5% level as is to be expected because both are known to contain high amounts of soluble carbohydrates. Berseem hay, berseem green and spear grass hay were found to have no significant difference in propionic acid production. This is to be expected because of their similar high content of crude fiber. Maize and guar meal differed in the propionic acid production with the rest of the foodstuffs at 5% level. At 1% level only maize differed with the ~~xxxxxx~~ rest of the foodstuffs.

(c) ACETIC ACID:-

Acetic acid production as found to be just the reverse of the propionic acid production. There was no difference in the acetic acid production in spear and berseem hay but they differed with the rest of the

foodstuffs at 1% level. There was no difference in the acetic acid production between the berseem green, maize and guar meal.

From the above discussion, it is apprent that the concentrates which contained more of soluble carbohydrates, maize and guar meal produced more of propionic acid and less of acetic acid and they belonged to one group. While spear grass and berseem hay which contained more of crude fiber produced just the reverse, i.e. more of acetic and less of propionic acid only. Berseem green which produced highest amount of butyric acid was just in between roughage and concentrates in the production of acetic and propionic acid.

The VFA data are in general agreement with previously published results of others (Tyznik and Allen, 1951; Card and Schultz, 1953; Balch et. al. 1955; Stewart and Schultz, 1958 and Hinders and Ward, 1961).

CORRELATION STUDY:-

Statistically, it was observed that not all foodstuffs differed in the production of individual fatty acids. Certain foodstuffs tend to produce the same propirtion of fatty acids. The above study and Blaxter's (1956) discussion of the fiber content of the ration on the rumen VFA rations, prompted a study of these interrelationships between the crude fiber and TDN. content of the foodstuffs and their ratios of VFA produced (Table 4.25). Correlation coefficients between crude fiber

content of foodstuffs with the butyric, propionic and acetic acids were -0.68, -0.81 and -0.38 respectively. It is seen that the crude fiber content of the foodstuffs was negatively correlated with the production of butyric and propionic acids and positively correlated with the acetic acid. That is with increase in crude fiber content of the foodstuffs there was decrease in the proportion of butyric and propionic acid and a proportionate increase in the acetic acid production. Though the correlation coefficients for acetic and butyric acids were not statistically significant, but they tend to show the trend.

The crude fiber VFA relationships are in agreement with observations cited and discussed by Blaxter (1956) and also those of Elldot and Loosli (1959). Though the actual and value varied but the trend was the same in both cases.

The correlation coefficient between T.D.N. content of foodstuffs and production of butyric, propionic and acetic acid were 0.11, 0.61 and 0.15 respectively. As all the fatty acids are produced from total digestible nutrients of the foodstuffs so the correlation coefficients with the T.D.N. were all in positive as expected. There was greater positive correlation between TDN. and propionic acid of 0.61 which just approached significance at 5%. So the conclusion is that with increase in TDN. content of foodstuffs, there was increase in all the fatty acids production, but there was greater increase in pro-

propionic acid than of butyric or acetic acids.

As these estimated correlation coefficients were based on very limited data, it would be incorrect to adduce quantitative significance to these results and should be taken to indicate the trends of the processes involved.

PERCENTAGE COMPOSITION OF FATTY ACIDS PRODUCED FROM PURE NUTRIENTS.

A number of simpler sugars occur in grasses and other ruminant foodstuffs and become available during the breakdown in the rumen of dietary polysaccharides. Simple sugars are actively metabolized by both the protozoa and the bacteria in the rumen. A clue to the nature of processes involved in the foodstuff may be obtained from studies on pure nutrients that make up the foodstuffs. The pure nutrient studied are glucose, lactose, starch and cellulose (tables 4.20 and 2.2).

Glucose and lactose were incubated for 4 hours and starch and cellulose were incubated for 12 hours and 24 hours by the first and second method respectively. The data for the percentage composition of fresh VFA produced from one nutrient with the other cannot be compared with the other except glucose and lactose, which produced almost the same proportion of the fatty acids. For this reason only, the data for pure nutrients were not subjected to statistifal study.

Elsden's (1945 - 46) data for the VFA produced 'in vitro' from cellulose with sheep were 42.1% acetic, 54.5% propionic and 3.4% butyric acid. Our results are in close agreement with that of Elsden in the proportion of fatty acids produced from cellulose, except for the fact that the percentage of propionic acid in our case was little higher. Elsden's data for glucose are acetic 23.20%, propionic 51.20, and butyric 25.60. This, however, do not agree with our data. The discrepancy could not be explained. However, the trend both in case of cellulose and glucose was the same.

CALORIFIC VALUE OF FATTY ACIDS PRODUCED FROM FOODSTUFFS.

Since the fatty acids produced in the rumen are considered to be utilised mostly for the production of energy in the body (Armstrong and Blaxter, 1956). It is useful to form an estimate of the daily calorific intake from this source. From our 'in vitro' rates of the VFA production from different foodstuffs, total VFA produced per day from each foodstuffs were calculated on two assumptions: firstly, assuming that the rates of production of VFA is same throughout the day; secondly, taking the capacity of goats rumen content to be 4 liters and constant throughout the day. Calculated on the calorific values of the fatty acids according to Carroll and Hungate (1954) as 208.4 Cal/mole of acetic, 373.5 Cal/mole of propionic and 504.8 Cal/mole of butyric acid,

BUTYRIC ACID:-

The percentage of butyric acid produced from pure nutrients did not exceed 4% except in the case of starch which produced 21% of butyric acid. This high proportion of butyric acid observed in case of starch agrees very well with the finding by us with maize, which is mostly starch also produced 27% of butyric acid.

The probable reason for the high butyric acid percentage with starch and maize may be due to the presence of Cl. butyricum, which is known to grow more favourably in presence of starchy rations (Elsden, 1945). This organism is known to produce more of butyric acid in pure culture as already discussed under maize.

PROPIONIC ACID:-

The propionic acid produced from pure nutrients was found to be more than 65% of the total, except starch which produced as low as 26%. The cause for which could not be explained. It is interesting to note that glucose produced the highest proportion of 87% of propionic acid.

BUTYRIC ACID:-

The proportion of acetic acid produced by different pure nutrients varied much glucose produced lowest amount of 9% acetic acid. Starch produced highest amount 52% of acetic adid. Lactose and cellulose were intermediate and produced 30% of acetic acid.

Elsden's (1945 - 46) data for the VFA produced 'in vitro' from cellulose with sheep were 42.1% acetic, 54.5% propionic and 3.4% butyric acid. Our results are in close agreement with that of Elsden in the proportion of fatty acids produced from cellulose, except for the fact that the percentage of propionic acid in our case was little higher. Elsden's data for glucose are acetic 23.20%, propionic 51.20, and butyric 25.60. This, however, do not agree with our data. The discrepancy could not be explained. However, the trend both in case of cellulose and glucose was the same.

CALORIFIC VALUE OF FATTY ACIDS PRODUCED FROM FOODSTUFFS.

Since the fatty acids produced in the rumen are considered to be utilised mostly for the production of energy in the body (Armstrong and Blaxter, 1956). It is useful to form an estimate of the daily calorific intake from this source. From our 'in vitro' rates of the VFA production from different foodstuffs, total VFA produced per day from each foodstuffs were calculated on two assumptions: firstly, assuming that the rates of production of VFA is same throughout the day; secondly, taking the capacity of goats rumen content to be 4 liters and constant throughout the day. Calculated on the calorific values of the fatty acids according to Carroll and Hungate (1954) as 208.4 Cal/mole of acetic, 373.5 Cal/mole of propionic and 504.8 Cal/mole of butyric acid,

the daily calorific value of fatty acids available to the animal from maize, guar meal, berseem hay, berseem green and spear grass hay works out to be 374.0, 742.2, 793.3, 305.8 and 261.6 Cal/day. These values does not include the metabolization of the acids by rumen microorganisms. Correcting the above values for metabolization of acids at the rates presented by Mukherjee (1960) 23.3% per hour for butyric and 6.6% per hour for acetic acid, reduces the daily calorific value to be 350.77, 664.9, 715.2, 264.22, 232.78 Cal/day respectively.

The total Cal. available per day from each foodstuffs depends on rate of VFA production as also on the proportion of the fatty acids produced. Our calculation are based on assumptions which are highly theoretical. Neither the rate nor the rumen contents remain the same throughout the day. So the data obtained by us on the total Cal. available per day from each foodstuff do not necessarily reflect the actual figures.

The data for daily calorific value as calculated by Mukherjee (1960) from the results of Carroll and Hungate (1954) for steers to be 430 Cal. Mukherjee (1960) from his 'in vivo' rates of absorption of fatty acids calculated the daily calorific value for fasting sheep to be 261 Cal.

This value is likely to be minimal inx view of the fact that both the production and absorption rates tend to be minimal in fasting state. In Carroll and Hungate's experiment, the rates of production of the acids were estimated on samples from fed animals and as such the rates must have been perceptibly higher than that expected from samples from fasting animals. The value obtained by Schambye as quoted by Phillipson and Cuthbertson (1956) range between 300 - 600 Cal/12 hours digestion cycle for sheep. The daily calorific value obtained in our data fall within the range noted by other workers as mentioned above.

KETONE BODIES AS A PROBABLE METABOLIC PRODUCT OF BUTYRIC ACID BY RUMEN MICROBES.

The formation of Ketone bodies as an intermediate in fatty acid oxidation is well known and the conditions conducive to its formation and accumulation in the system with the symptoms of Ketosis are well established.

'In vitro' experiments of Pennington (1952) have indicated that butyric acid is being utilised by the rumen epithelium, with the production of ketone bodies.

Mukherjee (1960) in his 'in vitro' experiments on interconversion of labelled fatty acids found that with propionate, whole of the initial activity was recovered in the fasting animal, indicating very little, if any, loss through metabolisation. With butyrate, however, 35% of the initial activity could not be recovered. This loss he attributed to its metabolization by the rumen microbes to

compounds other than fatty acids.

The above findings have prompted us to investigate the unaccounted activity with butyrate as observed by Mukherjee (1960), as to whether metabolization of butyric acid by the bacteria results in production of Ketone bodies or not.

Our 'in vitro' experiments with added butyrate revealed that on an average 241 µg. of ketone bodies/100 ml. of rumen liquor are produced from butyric acid during 4 hours incubation.

Our result in table 3.3 indicates that butyric acid used was free from acetone, so the ketone bodies produced on incubation with butyric acid, may be due to metabolization by the bacteria.

The percentage conversion of butyric acid to ketone bodies by the rumen bacteria in 4 hours was found to be 0.5% as against 70 - 90% conversion by liver cells 'in vitro' as observed by Loloir and Munoz (1939).

In view of the wide differences in the percentage conversion of butyric acid to ketone bodies and because of the inconclusive observations made by us, a final answer for the unaccounted activity of Mukherjee (1960) must await further investigations under more controlled conditions.

C O N C L U S I O N.

The work here in described has demonstrated that the proportions of the three fatty acids produced differ from different foodstuffs. These differences were statistically significant between the concentrates and roughages but not within each group. Concentrates known to be high

in TDN. content tend to produce more of propionic and butyric acids and roughages which are high in crude fiber content produced more of acetic acid. This explains the differences in the relative nutritive value of concentrates and roughages for productive purposes observed by Kellner (1920).

Fuller explanation of the low productive value of roughages have come from the work of Armstrong and Blaxter (1957) who found differences in the efficiency of utilisation of different fatty acids. They have determined the heat increments of the VFA's individually and in mixtures. The heat increments of propionic acid and butyric were only about 50% that of acetic acid. They also found that the heat increments of the acid mixtures are those to be expected on the basis of direct proportionality of response to the individual acids, above maintenance datum. These workers concluded that the net energy content of the 3-carbon and 4-carbon acids is greater than that of the 2-carbon acids. Therefore, it is expected that concentrates which produced more of propionic and butyric acids, increase the amount of net energy available, while roughages which produced more of acetic acid decrease the net energy available, provided the digestibility was not affected.

This finding has revealed one of the many hidden mysteries of the rumen which has kept in dark, the pioneer workers in the early forties of this century in the field of nutrition.

-oOo-

Chpter (VI)

SUMMARY.

Chapter VI.

S U M M A R Y.

1. Liquid:-

Liquid partition Chromatography with celite 535 as the inert page, 2 Mphosphate buffer of pH 6.5 as the stationary phase and Chloroform - butanol mixtures as the mobile phase, was found to separate mixtures of acetic, propionic and butyric acids quantitatively, even when the acids were present in amounts of 5 - 10 micromoles each. Recovery was found to be cent per cent.

2. Critical estimates of the probable experimental errors in the estimation and separation of VFA from rumen liquor was made and was found to be negligible.

3. The 'Artificial rumen' technique was used in this study for fermentation of foodstuffs and pure nutrients. Two methods of incubations were followed. The first method was found to be more suitable for short period of incubation and second method was used for longer period of incubation. The advantages and disadvantages of these techniques are discussed.

4. Maize, guar meal, berseem hay, berseem green and spear grass hay, all finely powdered except berseem green which was finely chopped were fermented for 4 hours

by the first method. The average rate of VFA production was found to be 10.6, 20.65, 26.37, 8.7 and 8.8 m moles/hr/liter of rumen liquor respectively. Factors affecting the rate of VFA production are discussed.

5. The percentage composition of VFA produced from different foodstuffs are determined. Roughages tend to produce more of acetic acid and concentrates more of propionic and butyric acid.

6. Statistical analysis of the fatty acids produced from different foodstuffs were found to be highly significant. Critical difference study tended to group the foodstuffs into concentrates and roughages in which fatty acidx proportions produced between the groups were statistically significant while those within each group were not.

7. A series of correlation coefficients were estimated between crude fiber and T.D.N. content of foodstuffs and VFA's produced from them. Crude fiber content was found to be positively correlated with the acetic acid production while with butyric and acetic acid, there was negative correlation. TDN. content of foodstuffs was positively correlated with all the fatty acids, but there was greater positive correlation with propionic acid than with acetic acid and butyric acid.

8. Poudered groundnut shells could not be fermented in vitro by both the methods upto 24 hours; even after the addition of 250 mg. of glucose.

9. Glucose and lactose are fermented in 4 hours. Starch is fermented in 12 hours while cellulose is fermented in 24 hours by the second method. A lag phase of 4 hours for starch and 6 hours for cellulose is found before being fermented. The average rate of VFA production was 18.9, 13.7, 9.6 and 7.2 m moles/litor of rumen liquor.

10. The percentage composition of VFA produced from pure nutrients are determined.

11. The daily calorific ~~mk~~ value of the fatty acids available to the animal are calculated for the different foodstuffs studied.

12. Butyric acid was found to be metabolized by the rumen microbes with the production on an average of 241 µg. of ketone bodies per 100 ml. of rumen liquor in 4 hours.

13. The differences in the relative nutritive value of concentrates and roughages for productive purposes are discussed in the light of our data on the proportion of fatty acids produced from different foodstuffs.

14. The effect of physical nature of the foodstuffs on fermentation was studied. Bersoem hay and Jowar leaves chopped to about half-an-inch, could not be fermented even upto 12 hours of incubation while some foodstuffs when powdered finely could be fermented in 4 hours only.

-xxx-

REFERENCES.

axter, K.L.,	(1956) :	J. Dairy Sci.	39.	1396.
ach, K., Borek, E., d Rittenberg, D.	(1946) :	J. Biol. Chem.	162.	441.
eding, E., and Yale, H.W.	(1951) :	Ibid.	193.	411.
rroughs, W., Gerlaugh, P., gington, B.H., and Bethke, R.M.	(1949) :	J. Ani. Sci.	8.	9.
rroughs, W., Frank, N.A., rlaugh, P., and Bethke, R.M.	(1950a):	J. Nutr.	40.	9.
rroughs, W., Headley, H.G., thke, R.M., and Gerlaugh, P.	(1950b):	J. Ani. Sci.	9.	513.
rroughs, W., Long, J., rlaugh, P., and Bethke, R.M.	(1950c):	Ibid.	9.	523.
rroughs, W., Arias, C., Paul, P., Gerlaugh, P., and thke, R.M.	(1951a):	Ibid.	10.	672.
rroughs, W., Latona, A., Paul, P., Gerlaugh, P., and thke, R.M.	(1951b):	Ibid.	10.	693.
rd, C.S., and Schultz, L.H.	(1953) :	J. Dairy Sci.	36.	599.
rroll, E.J., and Hungate, R.E.	(1954) :	Appl. Microbiol.	2.	205.
nsden, R., Gordon, A.H., d Martin, A.J.P.	(1944) :	Biochem. J.	38.	224.
wie, A.T., Folley, S.J., rench, T.H., and Popjak, G.	(1951) :	Ibid.	49.	610.
nielli, J.F., Hitchcock, M.W.S., rshall, R.A., and illipson, A.T.	(1945) :	J. Exptl. Biol.	22.	75.
avis, R.F., Woodhouse, N.S., eeny, M., and Becker, G.H.	(1957) :	J. Dairy Sci.	40.	75.
hority, B.A.,	(1961) :	Ibid.	44.	687.
euel, J., Harry, Jr., ulls, S., Joseph, Hallman, F., ois, and Cutler, H. Charles.	(1935) :	J. Biol. Chem.	112.	15.
chstein, H.C.,	(1933) :	Ibid.	102.	591.
lliot, J.M., and Loosli, J.K.	(1959) :	J. Dairy Sci.	42.	843.
lsden, S.R.,	(1945) (1946) :	J. Exptl. Biol.	22.	51.

Elsden, S.R.,	(1946) :	Biochem. J.	40.	252.
Elsden, S.R., Hitchcock, M.W.S., Marshall, R.A., and Phillipson, A.T.	(1946b):	J. Exptl. Biol.	22.	191.
Elsden, S.R., and Lewis, D.,	(1953) :	Biochem. J.	55.	183.
El-Shazly, K.,	(1952) :	Ibid.	51.	640 & 647.
Emery, R.S., Smith, C.K., and Huffman, C.F.	(1956) :	J. Ani. Sci.	15.	854.
Eusebio, A.N., Shaw, J.C., Leffel, R.C., Lakshmanan, S., and Doetsch, R.N.	(1959) :	J. Diary Sci.	42.	692.
Forbes, E.B., Braman, W.W., Kriss, M., and Swift, R.W.	(1931) :	J. Agric. Res.	43.	1015.
Forbes, E.B., Braman, W.W., Kriss, M., and Swift, R.W.,	(1933) :	Ibid.	46.	753.
Gray, F.V.,	(1947) :	J. Exptl. Biol.	24.	1.
Gray, F.V., and Pilgrim, A.F.	(1951) :	Ibid.	28.	83.
Gray, F.V., Pilgrim, A.F., and Weller, R.A.	(1951) :	Ibid.	28.	74.
Gray, F.V., Pilgrim, A.F.,	(1952) :	Ibid.	29.	54.
Gray, F.V., Pilgrim, A.F., Rodda, H.J., and Weller, R.A.	(1952) :	Ibid.	29.	57.
Gray, F.V.,	(1953) :	Ibid.	30.	204.
Greenberg, L.A., and Lester, D.	(1944) :	J. Biol. Chem.	154.	177.
Gutierrez, J.,	(1955) :	Biochem. J.	60.	516.
Halse, K., and Velle, W.	(1956) :	Acta, Physiol. Scand.	37.	380.
Head, M.J.,	(1953) :	J. Agricul. Sci.	43.	281.
Hershberger, T.V., Bentley, O.G., and Moxon, A.L.	(1955) :	J.Ani. Sci.	14.	725.
Hershberger, T.V., Bentley, O.G., Cline, J.H. and Tyznik, W.J.	(1956) :	J. Agric. Food Chem.	4.	952.

Hibbs, J.W., Conrad, H.R., and Pounden, W.D.,	(1954) :	J. Dairy Sci.	37.	724.
Hinders, G., Raymond and Ward M. Gerald.	(1961) :	Ibid.	44.	1129.
Hoflund, S., Quinn, S., and Clark, R.	(1948) :	Onderstepoort. J. Vet. Sci.	23.	395.
Hungate, R.E.	(1942) :	Biol. Bull.	83.	303.
Hungate, R.E.	(1950) :	Bacteriol. Review.	14.	1.
Hungate, R.E., Dougherty, R.W., Bryant, M.P., and Cello, R.M.	(1952) :	Cornell Vet.	42.	423.
James, A.T., and Martin, A.J.P.	(1952) :	Biochem. J.	50.	679.
Jarrett, I.G., and Potter, B.J.	(1950) :	Nature, Lond.	166.	515.
Johns, A.T.,	(1951) :	J. Gen. Microbiol.	5.	337.
Kane, E.A., Jacobson, W.C., and Damewood, P.M.	(1959) :	J. Dairy Sci.	42.	849.
Kellner, O.,	(1900) :	Landw. Vers. Sta.	53.	1.
Kleiber, M., Black, A.L., Brown, M.A., and Tolbert, B.M.	(1952) :	J. Biol. Chem.	197.	371.
Leloir, L.F., and Munoz, J.M.	(1939) :	Biochem. J.	33.	734.
Lewis, D., and McDonald, I.W.	(1958) :	J. Agric. Sci.	51.	108.
Louw, J.G., Williams, H.H., and Maynard, L.A.	(1949) :	Science.	110.	478.
Maitra, D.N.,	(1962) :	Studies on 'in vitro' fermentation of Bagasse and its utilisation by goats. M.Sc. (A.H.) Thesis, Magadh University.		
Marston, H.R.,	(1948) : a	Biochem. J.	42.	564.
Marston, H.R.,	(1948b):	Austr. J. Sci. Res. series.B,	1.	93.
Masson, M.J.,	(1950) :	Brit. J. Nutr.	4.	VIII.
Maynard, A.L., and Loosli, K.J.	(1956) :	Animal Nutrition - 4th edition. McGraw-Hill Book Co., Inc.		

McAnally, R.A., and Phillipson, A.T. (1942) : J. Physiol. 101. 13.

McClymont, G.L., (1952) : Austr. J. Sci. Res. series B, 5. 374.

McDonald, I.W., (1948a): Biochem. J. 42. 584.

McDonald I.W., (1952) : Ibid. 51. 86.

McNaught, M.L., (1951) : Ibid. 50. i.

Menon, U.K., Krishnamurthy, C.R., and Miss. Thankan Bastin. (1962) : Ind. Vet. J. 38. 380.

Moyle, V., Baldwin, E., and R. Scarishriskr, R. (1948) : Biochem. J. 43. 308.

Mukherjee, D.B., (1960) : Application of Isotope technique in the study of fermentation in the rumen of sheep. Ph. D., Thesis (Aberdeen).

Pant, H.C., Rawat, J.J., and Roy, A., (1962) : Ind. J. Dairy Sci. 15. 167.

Pearson, R.M., and Smith J.A.B., (1943) : Biochem. J. 37. 153.

Pennington, R.J., (1952) : Ibid. 51. 251.

Pennington, R.J. (1954) : Ibid. 56. 410.

Pennington, R.J., and Sutherland, T.M., (1956) : Ibid. 63. 618.

Peterson, M.H., and Johnson, M.J., (1948) : J. Biol. Chem. 174. 775.

Pfander, W.H., and Phillipson, A.T. (1953): J. Physiol. 122. 102.

Phillipson, A.T., (1942) : J. Exptl. Biol. 19. 186.

Phillipson, A.T., and McAnally, R.A., (1942) : Ibid. 19. 199.

Phillipson, A.T., (1947) : Ibid. 23. 246.

Phillipson, A.T., (1952) : Brit. J. Nutr. 6. 190.

Phillipson, A.T., and Cuthbertson, D.P., (1956) : Modern concepts of rumen digestion and metabolism.

pjak, G., French, T.H., nter, G.D., and Martin, A.J.P.	(1951) :	Biochem. J.	48.	612.
tter, R.J.,	(1952) :	Nature - London.	1770.	541.
inn, L.J.,	(1943) :	Onderstepoort J. Vet. Sci. and A.H.	18.	91.
msay, L.L., and Wipatherson, W.I.	(1945):	J. AOAC.	28.	644.
id, R.L.,	(1950) :	Nature.	165.	448.
id, R.L., and Lederer, H.,	(1951) :	Biochem. J.	50.	60.
id, R.J., Hogan, J.P., and riggs, P.K.,	(1957) :	Austr. J. Agric. Res.	8.	691.
nger, A.I.,	(1912) :	J. Biol. Chem.	12.	511.
itzman, E.G., and Benedict, F.G.	(1938) :	Nutr. Physiol. adult ruminant Carnegie Institute of Washington Publ. No.494.		
chambye, P.,	(1951a):	Nord Vet. Med.	3.	555.
chambye, P.,	(1951b):	Ibid.	3.	748.
chambye, P.,	(1955a):	Ibid.	7.	961.
chultz, L.H., Smith, V.R., nd Lardy, H.A.,	(1949) :	J. Dairy Sci.	32.	817.
en, K.C.,	(1946) :	Imp. Coun. Agric. Res. Misc. Bul.25.		
esham, P.A.,	(1935) :	Ind. J. Vet. Sci.	5.	355.
mith, E., Lester.	(1942) :	Biochem. J.	36.	XXII
mith, J.A.B., and Baker, F.,	(1944) :	Ibid.	38.	496.
chultz, L.H., and Smith, V.R.,	(1951) :	J. Dairy Sci.	34.	1191.
haw, J.C., Leffel, E.C., oetsch, R.N., Lewis, T.R., obinson, R.R., Senger, M.E., nd Brown, W.H.,	(1957) :	J. Dairy Sci.	40.	835.
haw, J.C., Robinson, R.R., enger, M.E., Lakshman, S., nd Lewis, T.R.	(1959) :	J. Nutr.	69.	235.
haw, J.C., Ensor, W.L., ellechea, H.F., and Lee, S.D.,	(1960) :	Ibid.	71.	203.
nedecor, W.G.,	(1956) :	Statistical methods. 5th edition. Iowa State College Press, Ames, Iowa.		

Stewart, W.E., and Schultz, L.H.,	(1958) :	J. Ani. Sci.	17.	737.
Swift, R.W., Thacker, E.J., Black, A., Batzler, J.W., and James, W.H.,	(1947) :	Ibid.	6.	432.
Syn, E.A.,	(1938) :	Cited in Biol. Rev. (1944).	19.	41.
Ta ppeiner, H.,	(1883) :	Z. Biol.	19.	228.
Tyznik, W., and Allen, N.N.,	(1951) :	J. Dairy Sci.	34.	493.
Waldo, D.R., and Schultz, L.H.,	(1956) :	Ibid.	39.	1453.
Warner, A.C.I.,	(1956) :	J. Gen. Microbiol.	14.	733.
Watson, C.J., Davidson, W.M., Kennedy, J.W., Robinson, C.H., and Muir, G.W.,	(1949) :	J.Agric. Sci.	29.	400.
Wegner, M.I., Booth, A.N., Bohstedt, G., and Hart, E.B.	(1940) :	J. Dairy Sci.	23.	1123.
Williams, V.J., Hottle, M.C., Moir, R.J., and Underwood, E.J.	(1953) :	Austr. J. Biol. Sci.	6.	142.
Woodman, H.E.,	(1930) :	Biol. Rev.	5.	273.
Woodman, H.E., and Evans, R.E.,	(1938) :	J. Agric. Sci.	28.	43.
Woodhouse, J.S., Davis, R.R., and Beck, G.H.,	(1955) :	J. Dairy Sci.	38.	605.