"In Vitro"
Studies on the Effect of Tannins of Pipal Leaves (Ficus Religiosa) and Sal Seeds (Shorea Robusta) and Added Tannic Acid on Volatile Fatty Acids Production by Goat's Rumen Micro-Organisms.

Thesis
Submitted to the Rajendra Agricultural University, Bihar
in partial fulfilment of the requirements for the degree of
MASTER OF SCIENCE (ANIMAL HUSBANDRY)
1971

BY
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BIHAR VETERINARY COLLEGE,
PATNA
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IN MEMORY OF MY LATE MOTHER
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Patna

The 29th February, 1972.

I certify that this thesis has been prepared under my supervision by Dr. Mahendra Singh, a candidate for the Degree of M.Sc. (A.H.) with 'Animal Nutrition' as major subject, and it incorporates the results of his independent study.

( D. B. MUKHERJEE )
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(Mahendra Singh)
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CHAPTER-I

INTRODUCTION
INTRODUCTION

Ruminants are closely associated with Homo Sapiens since the dawn of civilization. They have long had a special role in man's economy, providing milk, milk products, meat, wool, leather, bone, horn and transport. The main species are cattle, buffalo, sheep, goat, reindeer and yak, together with the closely allied camel, Vicuna and Llama. The nutrition of these numerous animals is, therefore, of great importance. But the detailed knowledge of rumenology has come into light only during the present century with rapid advancement in its physiology and Biochemistry.

There was a great mystery about the rumen. Hoard (1906) stated that the rumen of a cow was the darkest place on earth. But many of these mysteries are being resolved by the probing light of science and with the application of new methods and techniques in studying rumen activities, particularly through the use of the 'permanent fistula technique' (Colin, 1886).

A mixed population of bacteria and protozoa become established with the development of rumen. The microbial population exist in a remarkable symbiosis with the host animal. The rumen simulates a large vat-like fermentation structure providing suitable environment for the continuous multiplication of the microbes.

The rumen is essentially an anaerobic highly reducing system at a slightly acid but buffered pH at a temperature of nearly 39°C and a gas phase composed mainly of CO₂, Methane and Nitrogen. In such an environment a very specialised microbial population develops. The continuous multiplication of the microbes is ensured by a periodic intake of food, continuous flow of saliva, passage of contents along with digestive tract, and absorption of the end products of microbial metabolism through the ruminal wall.

As the symbiotic relationship is developed to the highest degree in ruminants, the rumen bacteria digest the coarse carbohydrates in roughages and also synthesize high quality protein and valuable B-Vitamins and Vitamin K for the host.
The potential source of chemical energy to the living being is food materials as proximate principles. The chemical energy is known to be primarily liberated in three phases - the hydrolysis of food during digestion → the anaerobic glycolysis → decarboxylation of carbohydrate and deamination of protein, followed by entry of the above products in the tricarboxylic acid (TCA) cycle to produce \( \text{CO}_2 \) and \( \text{H}_2\text{O} \).

As a result of various intermediary reactions, and due to microbial action in the rumen, various acids and gases are formed as end-products. Starch, pentoses and cellulose are most probably hydrolysed to monosaccharides and then fermented. Phillipson (1947) pointed out that cellulose is broken down to cellubiose \( \rightarrow \text{glucose} \rightarrow \text{pyruvate} \rightarrow \text{lactate} \) and ultimately to volatile fatty acids (VFA).

The work of Sir Joseph Barcroft and his collaborators (Eladen and Phillipson, 1948) has shown that in ruminants, the end products of bacterial dissimilation of dietary carbohydrates are volatile fatty acids (VFA), mainly acetic, propionic and butyric acids. A number of investigations have shown that the relative proportions of the individual VFA produced depends upon, amongst other factors, upon the nature of the diet, species of the animal and even the individual of the same species (Phillipson, 1947).

It is now well established that these acids constitute the major source of energy to the ruminants, since only a small proportion of the ingested carbohydrates escape degradation in the rumen.

The ultimate usefulness of a food-stuff as a source of energy can be predicted, not only by knowing how much of it is apparently digested, but also on the chemical nature of the products of digestion which are finally absorbed into the body.

If the major metabolites arising from bacterial dissimilation of carbohydrates which reach the tissues of the ruminants are the volatile fatty acids, then food must differ in their nutritive value in terms of energy for productive
purposes (Phillipson and Cuthbertson, 1956). As both food-stuffs and the individual
VFA vary in their energy values, it is logical to expect that different food-stuffs,
which differ in their energy values, must also differ in the proportions of the
individual fatty acids produced on their fermentation, and that, a simple determina-
tion of this proportion may serve as a valuable index of the efficiency of rations
for various productive purposes.

The total VFA concentration in the rumen and the amount of individual
acids present, are dependent on the diet. Recently, considerable information on this
subject has accumulated but the results obtained by different investigators are rarely
strictly comparable due to the fact that the pattern of VFA production in the rumen
is dependent on both the composition of the ration and the feeding regime. 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 the
lower fatty acids. However, a new chapter in ruminant nutrition was opened with the
introduction of Elsdon’s Silica-jel partition chromatography. Later, the works of
Armstrong et al. (1957a & b) indicated that the efficiency of utilization of the
three fatty acids by the ruminants as the source of their energy varies considerably
acetic acid being least efficient, next is butyric acid while propionic acid is
utilized most efficiently.

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 can not be determined directly because of the follow-
ing 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, intake of
food and water. Secondly, due to absorption of the fatty acids through the rumen
wall (Mc Anally & Phillipson, 1942; Barcroft et al., 1944 a). Thirdly, due to the
metabolism of the acids, particularly butyric by the rumen wall itself(Pennington,
1952). Fourthly, because of the appreciable interconversion of butyrate into
acetate (Mukherjee, 1960).

Rates of production can, however, be estimated by fermenting the food-stuffs 'in Vitro', which can only be approximate, as it imposes lot of unnatural conditions on the microbes.

Mukherjee (1960) for the first time gave a solution for this long awaited problem by the application of Isotope dilution-technique 'in Vivo'. The rates of production of the acids are as follows: Acetic 4.79, Propionic 1.10 and Butyric 0.6 m.mol./hr./kg. of rumen content respectively in fasting sheep previously fed on oats and hay.

Barcroft, Mc Anally & Phillipson (1944 b) have shown that absorption of lower VFA occurs from the rumen itself. Danelli et al. (1945) have studied the mechanism of absorption from the rumen, by the rumen wall of these fatty acids—acetic, propionic and butyric. According to them the absorption of the lower fatty acids from the rumen is influenced by pH of the rumen content and the maximum absorption occurs at pH 5.8. The determination of the rates of absorption of the different fatty acids from the rumen is also a difficult process because of the difficult surgical procedures involved. Mukherjee (1960) intelligently and accurately estimated for the first time 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—Acetic 38.8, propionic 9.66, and Butyric 4.22 m. moles/hr/kg. rumen content 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 microbial fermentation of the carbohydrates being short chain fatty acids, mainly acetic, propionic and butyric as against glucose in simple stomached animals, one is naturally inquisitive to know the fate of these acids.
Propionic acid is a precursor of glycogen (Echstein, 1933; Deuel et al., 1935). The administration of propionic acid to phloridzinised dogs results in the formation of equivalent amounts of glucose in the urine (Ringer, 1912). It has been shown that it enters the tricarboxylic acid cycle by carboxylation to succinate (Pennington and Sutherland, 1956).

Butyric is more controversial. It is generally known to be ketogenic. It is metabolized by rumen wall resulting in the production of ketone bodies (Pennington, 1952). Potter (1952) showed that it can cause a rise in the level of blood glucose, lactate, and pyruvate in the sheep.

Acetic acid is quantitatively the most important acid produced in the rumen. It is an important metabolic intermediate and precursor of wide variety of compounds. Acetic acid is utilized by liver (Leloir and 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 moiety of acetic acid has been traced also to cholesterol (Bloch et al., 1946) and milk casein (Kleiber et al., 1952).

The short chain fatty acids are quantitatively much more important than glucose as a source of energy to the ruminant. Carrolland Hungate (1954) have calculated that in cattle some 6000-12000 calories of energy are available from the acids produced by fermentation in the rumen and this was found to meet 70% of the maintenance energy requirements in steers. Phillipson and Cuthbertson (1956) have calculated from the results of Schambye (1951 a,b, 1955 a) that at least in the form 600-1200 calories of energy of VFA could be absorbed every 24 hours in the sheep. Armstrong and Blaxter (1956) calculated the maintenance requirement of sheep to be 1200 cal./day and on this basis about 65% 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 with from VFA.
The study of ruminant's nutrition is of particular importance in relation to the conditions prevailing in our country when grazing is sparse or grazing material tends to become more and more indigestible, cognisance must be taken not only of the requirements of the animal itself but also of the specific demands and deficiencies of the microflora since they are responsible to direct the course of fermentation in rumen.

In our country the pasture and fodder are subjected to tremendous changes due to floods and drought. There is a great scarcity of feeds and fodders during famine periods. On the other hand, there are a number of edible tree leaves and seeds which in times of scarcity can very well be utilized for feeding cattle, sheep and goat but it has not been tapped to the fullest extent. Nomin and Ray (1943) and Majumdar et al. (1967) have studied about the chemical composition and nutritive values of such fodder leaves and the influences which the locality, season and the stages of maturity exert on them.

As there is acute shortage of feeds and fodders for livestock, specially during famine, various tree leaves, seeds, fruits and other Agro-industrial by-products have been tried successfully on ruminants and poultry (Curasson, 1951; Bose et al., 1952; Hossain, 1959; Mia et al., 1960 b; Majumdar and Nomin, 1960; Hossain, 1961; Jayal and Kehar, 1962; Patel et al., 1962; Ayre-Smith, 1963; Patel et al., 1967; Johri et al., 1967; Sharma et al., 1968 and many others). Majumdar (1967) estimated that the present resources can meet only 1/3 of the total requirement of livestock population.

Pipal tree (Ficus religiosa) is a very common Indian tree found abundantly throughout the country. Recently, many workers have tried successfully the leaves of pipal tree as a fodder for ruminants, especially during famine for the maintenance and production. (Patel and Patel, 1957; Kehar and Johri, 1959; Hossain, 1960; Mathur, 1960; Mia et al., 1960 a).
Sal tree (Shorea robusta) is a common Indian forest tree and is abundant in the forests of Bihar, Bengal, Orissa, U.P., Madhya Pradesh, Maharashtra, Himachal Pradesh and Tripura. Recently attention has been directed to use decorticat-
ed Sal seed and its cake (a byproduct in soap industry) in cattle, pig and poultry feed as a cheap source of carbohydrate replacing a part of the energy rich feeds in the ration (Panda and Pradhan, 1967; Nayak et al., 1967; Saxena, 1967; Patnaik, 1966-67; Panda et al., 1969; Pal, 1969; Murty and Khan, 1969). It has been reported that about 48,000 maunds of Sal fruits can be collected per square mile of forest area (c.f. I.V.R.I. Associateship thesis, 1969 by R.N.Pal). Ramamurthy et al. (1967) reported that about 40,000 tons of Sal seeds are available annually from the Sal forests of Orissa. Perhaps, larger quantities are available from Sal forests in other States.

But a limiting factor in the utilization of tree leaves and seeds by the ruminants is in the wide prevalence of tannins in them. Tannins are widely distributed in plants and their leaves and seeds in varying concentrations and forms some of whom are having a very appreciable amount of tannins present, viz.

Sal seed (Shorea robusta), Babul leaves and seeds (Acacia arabica), carob beans and pods (ceratonia siliqua), Lespedeza sericea, Shin oak (Quercus incana), Sorghum grains (Sorghum Valgare), Tick clovers (Desmodium spp.), and to some extent in Pipal leaves (Ficus religiosa), Banana leaves and stems (Musa spp.) and Bargad leaves (Ficus bengalensis) etc.

The detrimental effects of tannins upon digestibility of the protein, growth rate, milk production and palatability by feeding trial experiments have been reported by various workers in Cattle (Herman et al., 1953; Hawkins, 1955; Hawkins and Autrey, 1957; Osage and Becker, 1958; Volcani and Roderig, 1961; Droni and Volcani, 1962; Volcani and Levi, 1962; Droni and Volcani, 1963; Pal, 1969; Murty and Khan, 1969; Mc Ginty, 1969; Donnelly and Anthony, 1969; Zelter et al., 1970) sheep (Wilkins et al., 1955; Bissel and Weir, 1957; Osage and Becker, 1958;
Dollahite et al., 1962; Leroy et al., 1964), Pig (Husby and Presthege, 1952; Becker et al., 1955; Neto, 1964), Poultry (Chang and Fuller, 1964; Vohra and Kratzer, 1964; Alumot et al., 1964; Vohra et al., 1966; Fuller et al., 1966; Potter et al., 1967, Potter, 1969; Clandinin and Heard, 1968; Conner et al., 1969; Kaushal et al., 1971), rats (Joelyn and Glick, 1969; Glick and Joelyn, 1970; Tamir and Alumot, 1970; Mitjavila et al., 1970) and rabbit (Pigeon et al., 1962).

Most of the workers have observed that the digestibility of protein was either nil or greatly reduced. Siu (1951), Oslage and Becker (1958), and Basaraba(1960) have reported that natural tannins and tannin containing substances have long been known to inhibit decomposition process of organic matters. Tagari et al., (1963) have reported that gallotannic acid inhibited cellulolysis and tannin fraction of carob pod extract significantly affected proteolysis and protein biosynthesis by the rumen microflora 'in vitro' with sheep inoculum. Volcani et al. (1963) have reported that carob pod meal and its various extracts inhibited cellulolysis and deamination 'in vitro'. It was also observed 'in vitro' after prolonged feeding of carob meal by Y. Rabi(Cited by Tagari et al., 1965, Appl. Microbiol., vol.13,p-437).

Tannins have an astringent taste and precipitate proteins by formation of insoluble tannates. It would appear, therefore, that any effect of tannin on the quantity of forage consumed by livestock would be due to the astringent taste and that any effect on digestibility would be associated with the property of tannins which cause precipitation of proteins.

Tannins are a group of astringent, aromatic, acidic compounds found in various plants and trees. They precipitate alkaloids, mercuric chloride and heavy metals; form deep blue or black solutions (Ink) with ferric solutions; and their strongly alkaline solutions absorb O₂ rapidly.

Tannic acid (C₆H₂₄O₉ = 322.2), tannin, Gallotannic acid, digallic acid, are yellow amorphous powder or lustrous crystalline scales, soluble in water,

Tannins are derivatives of polyhydroxy benzoic acids which are widely distributed in the vegetable kingdom. They owe their generic name to the fact that they are the active constituents of materials, such as oak bark, which are used in the tanning of skins. Tannins occur in many vegetable drugs, and are present in galenicals prepared from them. Most of the tannins are very complex substances which can only be isolated in the pure state with the greatest difficulty. Their chemical constitutions are rarely known with certainty, but it is well established that many of them are glycosidal. One of the simplest tannins (Glucogallin, in Chinese rhubarb) has been synthesized, and is a condensation product of glucose (1 mol.) and gallic acid (1 mol.).

Tannic acid—the basic component of all tannins is a name reserved for the tannin extracted from oak galls. Tannic acid is a brownish powder, consisting of thin, glistening scales. It has a characteristic odour and strongly astringent taste and is freely soluble in water, alcohol and glycerine, but very sparingly soluble in dry ether. The aqueous solution has an acidic reaction. Many mineral acids and salts precipitate tannic acid from its aqueous solution. Its aqueous solution forms precipitates with solutions of albumin, alkaloids and tartar emetic, and gives a deep bluish-black colour with ferric chloride. The formula of tannic acid is sometimes given as C$_{14}$H$_{10}$O$_{9}$, but this is only an approximate empirical formula.

When tannic acid is boiled with dilute Sulphuric acid it undergoes hydrolysis with formation of glucose and gallic acid:

$$C_{76}H_{52}O_{46} + 10H_2O \rightarrow C_6H_{12}O_6 + 10 C_6H_2(OH)\_3 \text{Coo H.}$$

Glucose     Gallic acid

The term tannin is variously employed by different writers, sometimes to denote a particular substance better described as Gallotannic or Digallic acid, and sometimes as a collective term for a whole group of substances having certain characteristics in common. In order to prevent confusion it is proposed here to use the word 'tannin' in the latter sense.

Since many of the plants contain natural tannins and its detrimental effects have been reported by many workers, the present problem was undertaken to study 'in vitro' the effects of tannins of herbage (Pipal leaves and Sal seeds) and also of gallotannic acid on the course of microbial digestion of carbohydrates in the rumen. So this work was designed to ascertain the detrimental effects, if any, of the tannins on carbohydrate utilization by the ruminants as also the effects of its extraction from the feed on this process. Thus it will explore the possibilities of better utilization of tannin containing subsidiary feeds like sal seeds and pipal leaves, specially during famine conditions.
CHAPTER II

REVIEW OF LITERATURE
REVIEWS OF LITERATURE

Rumenology is a very vast field of scientific research in the sphere of ruminant nutrition. There are numerous branches of studying ruminant nutrition. The field of carbohydrate metabolism is itself a vast one. Therefore, aspects related to the present work and of practical importance have been reviewed briefly. The various aspects reviewed are as follows:

Artificial rumen technique, Volatile fatty acids in the rumen, rate of VFA production in the rumen, factors affecting the production and pattern of VFA in the rumen and the effects of Tannins in the nutrition of livestock, Poultry and Laboratory animals.

Artificial rumen technique:

Since long scientists have been in search of some suitable technique to study 'in vitro' the activities of the rumen micro-organisms. But our quantitative knowledge of this rumen activity and of its role in nutrition has developed largely in the last thirty years, particularly through the use of the 'permanent rumen fistula' technique described in 1886 by the French Physiologist Colin. As long as 1883 Tappeiner showed that the microorganisms of the rumen fermented cellulose with methane, carbon dioxide and volatile fatty acids as end-products.

Thus the recent developments of 'Permanent rumen fistula technique' and the 'Artificial rumen technique' have provided important research tools for studying the activities of rumen microbes and as such have contributed greatly to our knowledge of rumen functions. But the modern developments were stimulated by Barcroft during the second world war. The pioneer work has been reviewed by Elsdon and Phillipson in 1948.

The methods used in the beginning merely involved incubation of rumen contents in a vessel to which a substrate was added and the disappearance of the latter or the appearance of the end-products were observed. Further improvements have been aimed at reproducing the 'in vivo' conditions as closely as possible.
particularly regarding salt concentration, buffering power, anaerobiosis and the nature of the substrate. In this technique an attempt was made to impose upon the withdrawn rumen sample conditions which duplicate those within the animals. So far as these are known, the cellulolytic bacteria (Hunghée, 1950) and Protozoa (Hunghée, 1942) of rumen are very sensitive to changes in its environment.

The early attempt at developing an 'in vitro' system for studying rumen microbial activities consisted of primarily incubating the strained rumen fluid in glass vessels with various substrates and measuring microbial activities in several ways.

Wegner et al. (1940) criticised the 'Artificial rumen technique' in that there was no movement and diffusion. They used whole rumen content in artificial rumen technique.

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 are vitally linked to various products derived from the bacterial metabolism.

Pearson and Smith (1943) and Smith and Baker (1944) used undiluted samples of rumen ingesta strained through muslin in earlier artificial rumen experiments. No elaborate attempt was made to duplicate exactly the rumen contents as their experiments were of short duration.

Elsden et al. (1946) adopted the procedure of incubation of rumen contents 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 of rumen functions.

Marston (1948 a) improved the technique by attempting to regulate the pH with the addition of suitable buffer and maintaining an anaerobic environment.
Louw et al. (1949) made an important improvement by using semipermeable vessels which dialysed the fermentation products by diffusion resembling more closely to 'in vivo' conditions.

Huhtanen et al. (1954) have made further modifications by using a small cellophane sac suspended in a 4 oz. screw-cap jar containing a solution similar in mineral composition to sheep's saliva. The sac containing the substrate and rumen fluid was held in position by screwing the cap on to the sac. The whole artificial rumen was small enough to be placed in an incubator. The outside bath consists of approximately 100 ml. of Mc Dougall's artificial saliva.

Bentley et al. (1954) studied the factors needed by rumen microorganisms for cellulose digestion 'in vitro'. They carried out 'in vitro' fermentation experiments with rumen microorganisms in liter low actinic Erlenmeyer flasks which were arranged in a thermostatically controlled water-bath (32°-35°C). To permit individual gassing of the flasks with Carbondioxide, the flask stoppers were fitted with two tubes and the one, which was used to admit CO₂ extended to the bottom of the flask. The other tube, which extended only through stopper, served as vent. The length of the fermentation was from 24 to 48 hours and no additions other than sodium carbonate, were made to the medium during the experiment.

Warner (1956) made further improvements by using 'cellophane dialysing sac' containing 50 ml. rumen liquor and substrate and suspended in a complex mineral solution similar in composition to rumen liquor and incubated at 39°C in an atmosphere of nitrogen and carbondioxide up to 8 hours. Provision was made for periodic removal of samples and addition of substrate. He got predictable results by reproducing as closely as possible conditions simulating in the living animal.

Protasejna (1956) devised a new method of studying digestion of carbohydrates, Proteins and fat in animals with fistulae. He developed an
apparatus in which starch, egg-protein or fat can be prepared in the form of stiff sticks of 3 cm long. Such sticks were enclosed in a perforated capsule suspended in the digestive tract through fistulae.

Fina et al. (1958) developed an artificial rumen technique for studying rumen digestion 'in vivo'. A porcelain tube, permeable to VFA but not to bacteria, connected to a glass frothing tube and gas escape mechanism, was suspended in the rumen of steer with a fistula. Maintenance of anaerobiosis was assisted by blowing oxygen-free nitrogen through the connected manifold. The inoculum for the tube was prepared by straining rumen fluid, allowing particles to separate out at 40°C, and using relatively particle-free layer of liquid. Tubes containing cellulose but no inoculum showed no digestion, tubes containing 500 mg cellulose and inoculum showed complete digestion in 48 hours. By adding successive increments of cellulose, digestion at a continuous rate was maintained for 10 days.

Davey et al. (1960) developed an improved artificial rumen designed for continuous control during prolonged operation. A stream of nitrogen and CO₂ was bubbled through the rumen contents in a dialysis sac, capacity about one liter, and then over the continuously changing physiological saline in the outer compartment. Tubes fitted into the two compartments allowed for removal of samples, addition of substrates, and recording of pH and temperature. The apparatus was run for periods of about 14 days and bacteriological and chemical analyses of the contents of the 'rumen' sac were compared with those of the rumen contents of the cow from which the initial artificial rumen inoculum had been taken. The artificial rumen was 'fed' daily on the same hay. The amount of inoculum taken was proportional to the relative volumes of the natural and artificial rumen. A proportional volume of artificial saliva was also added. The counts of bacteria, amounts of volatile fatty acids and D.M. digestion coefficients were similar in the cow and artificial rumen. In the artificial rumen pH values were somewhat lower.

The dialysis sac tended to become unserviceable after 10 days so on extended runs the rumen contents were changed to a fresh sac after 7 days.
It was concluded that in all essentials the artificial rumen closely simulates the activity of the natural rumen.

El-Shazly et al. (1960) compared the all-glass, semipermeable membrane and continuous flow types of apparatus for 'in vitro' rumen fermentations. On the basis of cellulose degradation and production of total volatile fatty acids and ammonia during 30 hours, the all-glass apparatus of Bentley et al. (1954 a), a semi-permeable membrane type of Warner (1956) and an apparatus like the last, but with dialysis in a continuously flowing mineral solution, were compared. There was little difference between them and the all-glass one was preferred for its simplicity.

Doneyer et al. (1960) outlined an 'in vitro' fermentation technique in which 90 ml. centrifuge tubes were used as fermentation vessels. 700 mgs. of substrate and 50 ml. of an inoculum-nutrient-buffer solution containing urea, glucose, biotin, para-aminobenzoic acid, casein hydrolysate and valeric acid, were used. To this solution 20 ml. of phosphate buffer extract was added per tube. Screw clamps were used on each tube for adjusting the flow rate of CO₂ to 150 bubbles per minute. The samples were fermented in a water-bath at 39°C.

Tilley et al. (1960) devised an 'in vitro' technique in which one gram sample of substrate in 75 ml. centrifuge tubes fitted with gas release valves were used. The inoculum-nutrient-buffer solution consisted of 40 ml. of artificial sheep's saliva (McDougall, 1948) and 10 ml. of strained rumen fluid per tube. The tube contents were flushed with CO₂, and immediately stoppered and fermented at 39°C in an incubator.

Stewart et al. (1961) developed continuous culture as a method for studying rumen fermentation. The fermentation vessel consisted of an 8-litre glass tank with a plastic lid, inlet and outlet tubes, sampling tube and stirrer. Substrate, ground hay or hay with concentrate, suspended in artificial saliva, was added intermittently by a solenoid-operated valve and culture was removed through
a second valve which operated when the volume reached a predetermined point. The dilutions were carried on for about 24 hours when mechanical failure usually terminated the experiments. The apparatus was initially flushed with C O₂ but was not completely airtight and gas formation by the culture was relied on to keep conditions anaerobic. The production of volatile fatty acids and the numbers of oligotrich protozoae and coliform bacteria were studied. The results suggested that normal rumen conditions were simulated in the apparatus and that it could be used for more detailed investigations.

Baumgardt et al. (1962) developed a simplified artificial rumen procedure for the repeatable estimates of forage nutritive value. The artificial rumen consisted of 125 ml. Erlenmeyer (Conical) flasks capped with rubber stoppers and equipped with a bunsen valve. They used 30 ml. of C O₂-saturated Mc Dougall's 'artificial saliva' during their 'in vitro' studies. This glass system was compared to the semi-permeable membrane and no appreciable difference was found in cellulose digestion or VFA patterns.

Gray et al. (1962) devised an artificial rumen. A gauze container, gently shaken in a glass vessel held at 40°C, holds rumen contents and artificial saliva is added continuously to the vessel at the same rate as fluid is drawn off. A mixture of 95% N₂ and 5% C O₂ is passed through the gas space. Regenerated cellulose tubing is wound around the gauze container and a dialysing medium, physiological saline, is pumped through at an appropriate rate. To test the system, acetic, propionic and butyric acids labelled with 14C were introduced into the rumen of a sheep and 2 hours later a sample of rumen contents was withdrawn and placed in the apparatus. During the next 3 hours the percentage decline in the specific activity of each acid was about the same 'in vivo' as 'in vitro' but fermentation appeared to be slower 'in vitro'.

Pant et al. (1962) adopted the 'Glass Artificial Rumen' technique as accepted by Barnett and Reid (1957 a) for carrying out 'in vitro' experiments.
Each GAR was used simultaneously in pairs and each pair was connected in series to a carbon dioxide cylinder. Rumen contents were strained through a double folded fine muslin cloth. Each GAR was charged with 25 ml. of mineral mixture of Burroughs et al. (1950 a) along with 110 ml. of water and allowed to reach a temperature of 39°C by standing in a thermostatic water-bath overnight. In the morning prior to collection of rumen liquor CO₂ was bubbled into all the flasks at a rapid rate for about 3-5 minutes to ensure anaerobic condition. CO₂ was subsequently bubbled continuously at a rate of about 160 bubbles per minute. 25 ml. SRIL obtained 2 hours after feeding was added to each flask of the pair used for incubation.

Tilley and Terry (1963) developed a two-stage technique for the 'in vitro' digestion of forage crops. Ground forage, 0.5 gm. was dried at 100°C for 1 to 2 days then transferred to 90 ml. centrifuge tubes fitted with Bunsen valves. After adding 40 ml. buffer and 10 ml. strained rumen liquor from sheep on a ration of hay, the tubes were gassed with CO₂ to pH 6.7 to 6.9 which was maintained, if necessary by adding N (Normal) Na₂CO₃ after 6 and 24 hours incubation. After 48 hours 1 ml. 5% HCl and 2 ml. N Na₂CO₃ were added and the tubes were centrifuged for 15 minutes at 1800 rpm. The supernatant fluid was replaced by 50 ml. Pepsin solution and was left with occasional shaking for 48 hours at 38°C. The insoluble residue was washed and dried at 100°C. In routine estimation of digestibility two standard forages of high and low digestibility relative to the unknown material were used. Accuracy depends on the maintenance of anaerobic conditions and constant pH during fermentation.

Barnes et al. (1964) compared 'in vitro' rumen fermentation methods to study the magnitude and rate of cellulose digestibility of forages and the variability between different 'in vitro' rumen fermentation techniques. The different methods compared were of Donefer et al. (1960), Baumgardt et al. (1962), Tilley et al. (1960), Tilley and Terry (1963) and the last 'in vitro' system consisted
of one gm. samples of substrate in 75 ml. centrifuge tubes fitted with gas release valves. The buffer-nutrient solution for each fermentation tube was 25 ml. of CO₂-saturated artificial saliva (Mc Dougall, 1948), to which enough urea and glucose had been added to supply 0.05% of each in the total volume in the fermentation flask. 21 ml. of strained rumen fluid per tube served as inoculum. The ratio of buffer to rumen fluid was identical with that in the method of Baumgardt et al. (1962) and fermented at 39°C in an incubator.

Differences were found between fermentation periods and an interaction between periods and methods occurred. This interaction was primarily the result of changing rates of cellulose digestion between time periods. Differences were found between Bromegrass hay and alfalfa substrates but not between the two alfalfa samples. Differences between methods were obtained after 6 and 12 hours fermentation periods but not after 18, 24 and 48 hours periods.

The primary factors which could have contributed to differences between methods were size of substrate, nutrient media, preparation of inoculum, maintenance of anaerobiosis and type of fermentation vessel. The variability between duplicate samples decreased as the length of fermentation period increased. Differences also existed between methods in the magnitude of the within-trial variability.

Looper et al. (1966) developed an 'in vitro' procedure for studying starch digestion by rumen microorganisms in which washed cell suspensions of rumen microorganisms in a buffered nutrient medium at pH 6.8 were incubated with purified water in soluble maize starch for 8 hours at 39°C. Incubators were pyrex tubes with CO₂ bubbled continuously through the medium. Starch was estimated gravimetrically by filtering the contents of the fermentation tubes through Gooch crucibles with asbestos fiber pads and estimating loss on ignition after drying.

Czerkawski (1967) developed a procedure of incubation inside the bovine rumen in which rumen liquor in semipermeable sacs were incubated inside the bovine rumen. It was found practicable, using one animal, to incubate
simultaneously samples of strained diluted rumen liquor in three identical vessels, each of 100 to 120 ml. capacity, for periods of up to 23 hours. The pH value of the reaction mixture varied little compared with the pH changes that occurred inside the rumen, and the sampling technique was satisfactory.

The microbial growth measured by increase in turbidity and in concentration of protein, was greatest when the rumen liquor inoculum constituted about 20% of the reaction mixture and when the protozoa and large food particles were removed. The apparatus was used to study microbial growth and hydrogenation of fatty acids in rumen liquor.

Brent (1967) developed a continuous flow 'in vitro' fermentation system of the dialysing, continuous flow type, designed to sample automatically both fermentation liquor and dialysate. It was used in the study of the utilization of soya-protein and several NPN sources and control to which no NPN was added. Of these N sources only soya-protein and 1,3-dimethyl urea appear to be hydrolysed to an extent sufficient to be useful to the ruminant. This system duplicates closely in quality and quantity reactions 'in vivo' and end-products, and also maintains an active protozoal population throughout the five day fermentation periods.

Asfjes and Nijhof (1967) devised a simple artificial rumen giving good production of VFA. The apparatus is a glass vessel, volume 18 liters with a plastic lid. Through a hole in the lid a plunger at a speed of 5000 r.p.m. is attached to a nylon gauze bag filled with hay. The glass vessel is fitted with outflow and kept in a constant temperature waterbath at 40°C. There are also inlet holes for gas and introduction of artificial saliva which is automatically controlled by a titrator connected to a pH meter. When pH dropped below 6.7 artificial saliva was introduced. At the start of the experiment the vessel was filled with 10 litres of solution containing 26.6 gm. Na₂HPO₄ and 117.6 gm. Na HCO₃. The bag attached to the plunger contained 400 gm. hay. When temperature equilibrium was reached another bag of nylon gauze containing rumen contents
from a cow consuming similar hay was introduced and placed at the bottom of the vessel. The artificial rumen was fed twice daily at 9 A.M. and 5 P.M. The bags remained there for 2 days after which they were removed after pressing out as much as possible of the rumen content. This experiment lasted 7 weeks. The VFA produced was normal but the protozoal population was lower than in the natural rumen. More than 50% of the hay dry matter was digested in artificial rumen. The production of VFA was estimated to be 200 gm. per 24 hours. The concentration of acetic acid in the artificial rumen was lower and that of propionic and butyric was higher than in normal rumen content.

Griffiths (1967) evaluated an improved artificial rumen technique for the study of rumen fermentation. The results obtained with a permeable artificial rumen were compared with measurements 'in vivo' with a cow. The pH and production of NH₃, VFA's and lactic acid were measured. The artificial rumen was satisfactory for measuring treatments differences but not accurate enough for quantitative measurements. There were significant correlations between the molar proportions of the volatile fatty acids found by both methods but there was no significant correlation between 'in vivo' and 'in vitro' methods for pH and NH₃ production.

**VOLATILE FATTY ACIDS IN RUMEN**

In recent years the importance of the formation of volatile fatty acids (VFA) in the rumen for optimum nutrition of ruminants has become increasingly evident. The major fatty acids such as acetic, propionic and butyric acids are now considered as energy sources and key anabolic intermediates rather than waste-products of digestion.

Propionic acid, in particular, has received special attention as an intermediate in the biosynthesis of glucose and other carbohydrate material in the ruminant (Schultz, 1952; Pennington, 1952; Elsden and Phillipson, 1948).

Because of the importance of the volatile fatty acids (VFA)
and their close relationship to cellulose digestion, it was considered necessary to review briefly the literatures available on VFA formation and cellulose digestion both 'in vitro' and 'in vivo'.

Tappeiner as long as 1883 for the first time demonstrated that the 'in vitro' fermentation of cellulose in the rumen of ox resulted in the formation of large amounts of volatile fatty acids and concluded that it contained at least 50% acetic acid. But little nutritional value was ascribed to these acids by him. Because 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) reported that glucose was a normal intermediary in cellulose fermentation, and glucose was the product which was absorbed.

Later on, Woodman and Evans (1938) admitted that lower volatile fatty acids were among the principal end-products of cellulose fermentation. Phillipson and Mc Anally (1942) observed that hexoses were rapidly fermented to lactic acid in the sheep rumen and this metabolite was consequently converted to lower volatile fatty acids mainly propionic acid. However, the development of new chromatographic technique by Consden et al. (1944) led Elsdon (1946) and later Moyle et al. (1948) to design reliable methods for characterising the volatile fatty acids arising from rumen fermentation.

Elsdon (1945-46) for the first time separated volatile fatty acids and stated that under natural conditions acetic, propionic and butyric acids are the main acids present and acetic acid predominates in concentration. The results are shown in Table 2.1.

Marston (1942 b) showed that there is little doubt that the main sources of energy to ruminants are the fatty acids arising from the dissimilation of carbohydrates in the rumen.

Gray et al. (1951) conducted 'in vitro' incubation of wheaten hay and lucerne hay for varying length of time using sheep rumen liquor and concluded that there was remarkable production of acetic acid in earlier stages but in
later stages larger amounts of the higher fatty acids, particularly propionic acid was formed. The results are shown in table 2.1.

Bentley et al. (1951) studied 'in vitro' the effects of feeding poor quality hay on the biochemical functions of rumen microbes and reported 30% drop in the VFA production. Gray et al. (1952) found that large amounts of higher fatty acids are produced when wheaten hay was incubated 'in vitro' for longer period. 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.

Hibbs et al. (1952) observed in calves raised on high roughage system that the total VFA increased with the development of rumen function and the blood sugar level decreased from 80–100 mgs./100 ml. to 45–65 mgs./100 ml. during the first week. Phillipson (1952) observed large concentration of lactic acid which fermented mainly to propionic acid in the rumen of lambs fed a ration of hay, flaked maize and maize gluten meal. Results are shown in table 2.1.

El-Shazly (1952) showed that the amino acid of an acid hydrolysate of casein were attacked under anaerobic conditions by washed suspension of rumen organisms and the reaction products were ammonia, CO₂ and volatile fatty acids in equimolecular amounts. The results are shown in table 2.2.

Fauconneau et al. (1953) reported that the mean proportions of VFA in the killed sheep were acetic 65.7, propionic 19.9 and butyric 14.5 percent and after 48 hours incubation 60.6, 28.0 and 11.5 percent respectively. The values for carbohydrate digestion and total VFA formation were almost identical with those obtained 'in vivo' although the proportions of the fatty acids differed. About 83% of the carbohydrates lost could be accounted for as VFA. The production of VFA was about 153.4 to 263.4 gms. per kg. alfalfa hay on dry matter basis.

Carroll and Hungate (1954) conducted 'in vitro' experiments by incubating whole rumen content of steers for short periods under conditions which
# Table 2.1

Table showing VFA production 'in vitro' on various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Animal</th>
<th>Acetic</th>
<th>Propionic</th>
<th>Butyric</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Cellulose</td>
<td>Sheep</td>
<td>42.10</td>
<td>54.50</td>
<td>3.40</td>
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<tr>
<td>Glucose</td>
<td>&quot;</td>
<td>23.20</td>
<td>51.20</td>
<td>25.60</td>
<td>Elsden (1945-46)</td>
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<tr>
<td>Dried grass</td>
<td>&quot;</td>
<td>52.50</td>
<td>34.50</td>
<td>13.00</td>
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</tr>
<tr>
<td>Wheaten hay</td>
<td>&quot;</td>
<td>46.00</td>
<td>41.00</td>
<td>13.00</td>
<td></td>
</tr>
<tr>
<td>Lucerne hay</td>
<td>&quot;</td>
<td>59.00</td>
<td>27.00</td>
<td>14.00</td>
<td></td>
</tr>
<tr>
<td>Grain</td>
<td>Steer</td>
<td>63.40</td>
<td>22.60</td>
<td>14.00</td>
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</tr>
<tr>
<td>Pasture</td>
<td>&quot;</td>
<td>60.20</td>
<td>23.30</td>
<td>16.50</td>
<td>Carroll and</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hungate (1954)</td>
</tr>
<tr>
<td>Hay</td>
<td>&quot;</td>
<td>60.30</td>
<td>21.10</td>
<td>18.60</td>
<td></td>
</tr>
<tr>
<td>Hay and Concentrate</td>
<td>Fasting Sheep</td>
<td>73.55</td>
<td>15.00</td>
<td>11.45</td>
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</tr>
<tr>
<td>Hay and Concentrate</td>
<td>Non-fasting Sheep</td>
<td>41.60</td>
<td>24.00</td>
<td>34.40</td>
<td>Mukherjee (1960)</td>
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<tr>
<td>Flaked Maize</td>
<td>&quot;</td>
<td>30.50</td>
<td>27.80</td>
<td>41.70</td>
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<tr>
<td>Substrate</td>
<td>Animal</td>
<td>Rumen VFAs (molar proportion)</td>
<td>Reference</td>
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</tr>
<tr>
<td>----------------------------</td>
<td>--------</td>
<td>-------------------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetic</td>
<td>Propionic</td>
<td>Butyric</td>
<td>Higher Acids</td>
</tr>
<tr>
<td>Flaked maize</td>
<td>Sheep</td>
<td>51.0</td>
<td>42.0</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Flaked maize</td>
<td></td>
<td>60.0</td>
<td>35.0</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Flaked maize</td>
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<td>59.0</td>
<td>23.0</td>
<td>14.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Concentrate + 50 Gm Casein</td>
<td></td>
<td>56.9</td>
<td>23.7</td>
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<td>5.3</td>
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<tr>
<td>Frozen grass</td>
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<td>22.6</td>
<td>15.9</td>
<td>2.3</td>
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<td>Dried grass</td>
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<td>57.7</td>
<td>22.2</td>
<td>9.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Silage</td>
<td></td>
<td>53.2</td>
<td>25.4</td>
<td>8.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Wheaten hay</td>
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<td>64.7</td>
<td>23.5</td>
<td>9.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Wheaten hay</td>
<td></td>
<td>65.4</td>
<td>22.3</td>
<td>8.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Wheaten hay</td>
<td></td>
<td>69.6</td>
<td>16.0</td>
<td>11.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Lucerne hay</td>
<td></td>
<td>66.9</td>
<td>22.3</td>
<td>6.7</td>
<td>3.9</td>
</tr>
<tr>
<td>Groundnut Cake</td>
<td></td>
<td>60.0</td>
<td>26.0</td>
<td>11.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Maize</td>
<td></td>
<td>46.0</td>
<td>32.0</td>
<td>18.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Casein</td>
<td></td>
<td>60.0</td>
<td>22.0</td>
<td>10.4</td>
<td>5.6</td>
</tr>
<tr>
<td>Hay</td>
<td></td>
<td>81.0</td>
<td>13.0</td>
<td>4.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Hay (2 lb.) Concentrate (24 lb) Cow</td>
<td>40.6</td>
<td>36.5</td>
<td>10.7</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>Hay (16 lb) + Concentrate (20 lb)</td>
<td>57.1</td>
<td>23.7</td>
<td>12.0</td>
<td>7.2</td>
<td>Balch and Rowland (1957)</td>
</tr>
<tr>
<td>Free Grazing</td>
<td></td>
<td>67.5</td>
<td>18.2</td>
<td>11.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Silage</td>
<td></td>
<td>73.7</td>
<td>16.8</td>
<td>6.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Hay and Concentrate Sheep</td>
<td></td>
<td>71.3</td>
<td>16.0</td>
<td>12.7</td>
<td></td>
</tr>
</tbody>
</table>
Simulated those of the rumen. The results are shown in Table 2.1.

Annison (1954) stated that occasionally small amounts of formic acid as well as some branched-chain acids are present. At present, the major fatty acids such as acetic, propionic and butyric acids are considered as energy sources and key metabolic intermediates rather than waste products of digestion. The results are shown in Table 2.1.

Francois et al. (1955) demonstrated the formation of volatile fatty acids in the rumen from the digestion of pure starch, cellulose, soluble sugar, green fodder and lucerne hay alone or with raw or cooked potatoes, fodder beet or beet molasses. Lactic acid was produced from soluble sugars, beet and molasses but not from starch, potato or green fodder. The combination of hay and beet or molasses tended to increase the VFA formation at the expense of lactic acid.

Belasco (1956) conducted 'in vitro' experiments and reported that cellulose digestion was better alone than with high levels of dextrose. Starch, dextrose and cellulose at high levels, influenced the total VFA production as well as the proportions of butyric proprionic, valeric and acetic acids.

Balch and Rowland (1957) observed low levels of acetate and high levels of propionate and higher acids in the rumen fluid of cows fed predominantly on flaked maize presumably due to the heating of the corn during flaking process. The ratio of acetic to propionic acid decreased with decrease in the ratio of fibrous to starchy concentrates. Results are shown in Table 2.2.

Devis et al. (1957) observed increase in the amounts of all volatile fatty acids excepting 'higher acids' as a result of high level of protein intake in the rumen of dairy cows. The percentage of butyric acid in the total VFA in the rumen decreased with an increase in protein intake.

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

Elliot et al. (1957) conducted trials to study the effects on
rumen VFA concentration, by replacing part of the hay in the ration with corn or hay crop silage. In the third trial, corn silage was compared with hay crop silage as the sole roughage. Corn silage increased total VFA concentration slightly but not significantly when used as a part of the roughage. The relative proportions of the acids were unaltered. The total VFA concentration did not increase when hay crop silage was fed as part of the roughage but the relative proportions of butyric and higher acids increased and that of propionic and acetic acids decreased. But these differences were statistically non-significant. Corn silage as the sole roughage resulted in a significantly higher total VFA concentration, a significantly greater relative proportion of propionic acid, and significantly smaller relative proportions of butyric, acetic and higher acids than did hay crop silage used as the only roughage.

Jamieson (1959) estimated ruminal total VFA and their molecular percentage composition in monthly samples from adult sheep grazing the same pasture throughout the year. The molecular proportions of acetic acid were more frequent in spring and autumn, when the crude protein content of the herbage was high. The amount of total VFA, though variable, appeared to be independent of the molecular proportions of individual fatty acids.

Lampila (1959) reported that VFA production 'in vitro' increased rapidly from pH 5.0 to a maximum at about 6.2 in the rumen of cows. Varus (1959) observed rise in VFA concentration and fall in pH of rumen contents after feeding to a greater extent, lasting longer, in sheep by deviating the flow of saliva either through an oesophageal fistula or by anaesthesia than in normal sheep.

Lampila and Poijervi (1959) incubated rumen liquor of Ayrshire cows 4 hours after feeding with fodder beet, hay and concentrate and showed that at pH 6.85 butyric acid accounted for 15% of the VFA and at pH 5.30 for 34%.

Church and Petersen (1960) reported increased VFA production with increased substrate concentration. The proportions of dry matter and cellulose digested were related inversely to substrate concentration and directly to the
proportion of rumen fluid in the fermentation mixture.

Mukherjee (1960) estimated for the first time the extent of interconversion of the three ruminal acids quantitatively both 'in vivo' and 'in vitro' and found to be not more than 3% excepting in case of butyrate to acetate. He estimated volatile fatty acids in fasting and non-fasting sheep kept on hay and concentrate diet and also on flaked maize. The results are shown in table 2.1. 

Ward et al. (1961) reported that the level of total VFA in the different parts of the gastro-intestinal tract of full fed beef heifers were as follows: rumen 11.902, abomasum 1.292, small intestine 1.943, caecum 12.155 and colon 11.214 mEq/100 ml.

Omar et al. (1962) investigated to chart the distribution and concentration of intra-ruminal VFA in lambs of different ages. Total VFA concentration varied from 3.1 to 10.5 mEq/100 ml. ruminal fluid from three weeks lambs that had eaten pastures or dry feed and from 1.4 to 1.9 mEq/100 ml. ruminal fluid from 2 – 3 weeks lambs that had not yet consumed grass or dry feed. Raun et al. (1962) estimated total VFA production on the dietary factor of 80% concentrates: 20% roughage and 50% concentrate: 50% roughage and found that total VFA levels and pH were higher in the 50% concentrate: 50% roughage ration. Additions of sodium bicarbonate and Calcium carbonate did not produce any significant effect either on rumen total VFA or on rumen pH.

Matsumoto et al. (1963) observed fluctuations in the concentration of total VFA between 50 and 100, being lower on the more fibrous diet, in the goat rumen. The molar proportions of the fatty acids varied slightly with time, but on the more fibrous diet the proportions of acetic acid was higher and that of butyric was lower than on the low fiber diet. There was little or no appreciable effect on the concentrations of acetic, propionic and butyric acids with the rise of pH by 0.5 with the addition of 6 gm. of urea into the rumen.

Pant et al. (1963a) investigated seasonal variation in the rumen
microbial activity of buffalo. The seasons were divided according to the nature of grazing material available to the animals: (i) 15th July to 15th September—when there was abundant green pasture, (ii) December—when grazing was negligible as the pasture grass was sparse and dry, and (iii) 15th January to 15th February—when grazing material was like December but 8 lbs. green berseem was given as a supplement to each buffalo. Microbial activity during the first season was found to be significantly more than the other seasons. Total VFA, ammonia and total soluble nitrogen concentration 2 hours after feeding was found to increase in all the seasons but the maximum increase occurred in the first season.

Thompson et al. (1964) conducted experiments in steers fed different feed-stuffs and observed that the steers receiving long hay had a significantly higher molar percentage of acetate with a corresponding lower percentage \( (C_2/C_3 = 1.34) \) than steers fed no hay (1.02) or ground hay (1.03). They also had a significantly lower total VFA concentration (126.5 μmoles/ml.) than in steers fed no hay (155.7 μmoles/ml.) or ground hay (153.7 μmoles/ml.). Rumen fluid of steers fed flaked corn contained significantly less total VFA (132.5 μmoles/ml.) than in steers on ground corn ration (158.1 μmoles/ml.).

Davison et al. (1964) reported that the total VFA in the rumen increased significantly \( (P<.01) \) with experimental rations varying from 100% hay to 100% concentrate. This significant increase was observed with each increment of concentrates up to 60% of the ration, it then decreased with each succeeding increase of concentrate up to 100% in the ration.

Ichhponani and Sidhu (1965) reported that the concentrations of propionic, butyric and total volatile fatty acids were significantly higher in the rumen of Buffalo than those in Zebu cattle when both were fed on wheat straw + concentrate mixture. However, the concentration of acetic acid was not significantly different in buffalo and cattle. When the animals were fed on green Bajra + cowpea mixture, the concentrations of acetic, propionic and total volatile fatty acids were higher in the rumen of the buffalo than in Zebu cattle.
Yang and Thomas (1965) observed the distribution, secretion and absorption of organic and inorganic constituents in the gut of calves fed on diets high and low in fiber and found that the rumen contained 55-80% of the total VFA and the large intestine and caecum 11-36%. Absorption from the omasum has been reported from 32-45% of each VFA in the rumen.

Lampila and Poutsainen (1966) observed the concentration of VFA from the dorsal and ventral sacs of rumen to be 69.8 and 66.8% respectively. The pH value from all the four regions of rumen were found to be 5.93, 6.17, 6.32 and 6.4 respectively.

Fenner et al. (1967) conducted trials in sheep and cows to find out the effects of intake on digestibility and on the levels of metabolites in the rumen and showed that increased intake raised total VFA up to 3 hours after feeding and then decreased. The proportions of acetic acid in VFA decreased up to 3 hours and subsequently increased. The proportion of propionic and butyric acids was inversely related to that of acetic acid.

Weller et al. (1968) observed the production of VFA in sheep fed pure cellulose as sole carbohydrate in its diet along with gluten and inorganic salts for several months. Cellulose digestion was 84% and the VFA produced was 6–7 moles/Kg. dry matter consumed. Production of propionic acid represented 34–42% of the total VFA produced.

Devuyyst et al. (1968) studied the effects of addition of carbohydrates to the basal ration of sheep, on the pH changes and VFA content of rumen fluid. They observed that the addition of glucose and starch affected the proportions of VFA in that the starch decreased production of acetic and butyric acid and glucose decreased the molar concentration of propionic and butyric acids.

They further observed that the results were also affected by time interval between intake of food and collection of the sample. Arrival of food in
the rumen decreased pH and increased the concentration of ammonia and VFA. The effect of starch was slower but more lasting than glucose having a stronger but shorter effect than starch.

Sharma et al. (1968) reported variations in VFA concentration with different forage having urea as concentrate. The proportion of acetic was higher and of butyric lower after the hay than after the silage feeding. The proportion of propionic acid was slightly affected by the forage.

Verma et al. (1968) observed the mean concentration of total VFA during the digestion in the rumen of cattle and buffalo fed on common Indian feeds such as Elephant x Bajra, Chari and wheat straw and the total VFA concentrations were 81.7, 74.7 and 70.7 μ moles/ml. respectively. The buffalo produced higher mean VFA concentrations than the cattle on all the three feeds. In both the species, individual and total VFA concentrations increased immediately after feeding and a peak concentration was recorded after about 6 hours of feeding.

Ulyatt and Henderson (1968) observed in sheep grazed on pasture mainly of perennial ryegrass and white clover that the total VFA increased from 10.8 μ moles/100 ml. to 17.5 μ moles/100 ml. from 9 AM to 9 PM and then declined again during night. The proportions of propionic and n-butyric acids increased but that of acetic decreased with an increase in the total VFA.

Sutton (1968) studied the fermentation of soluble carbohydrates in rumen contents of cow fed on diets containing a proportion of hay and found that glucose and fructose were almost completely fermented but the other carbohydrates fermented more slowly. Changes in the proportions of VFA in the rumen indicated marked differences among the carbohydrates in the proportion of VFA produced. In ‘invitro’ fermentation, acetic acid was predominant specially from galactose and pentoses.

Kumeno and Mishimatsu (1968) measured the production of VFA and disappearance of dry matter ‘in vitro’ using washed suspension of rumen micro-
organisms and Mc Dougall’s “synthetic saliva” for four days at 39°C bubbling carbon dioxide continuously. Production of VFA and disappearance of dry matter ‘in vitro’ were closely correlated with total digestible nutrients estimated from trials with sheep.

Stilinovic and Cer (1968) observed the effect of different amounts of meadow hay on the molar proportions of volatile fatty acids in rumen fluid of cows 6–12 hours after feeding and found that there were few significant differences between rations with different amounts of hay in the molar proportions of VFA. At 12 hours after feeding the ration with 10 Kg. hay produced relatively more propionic and less butyric acid than that with 8 kg. and that with 6 kg. hay gave intermediate values. Acetic acid was little affected by time after feeding or by ration.

Faichney (1968) studied the production and absorption of volatile fatty acids from the rumen of sheep fed on ground pelleted lucerne or oat straw or a mixture of whole wheat grain + lucerne chaff and calcium carbonate and estimated the proportions of the digested energy absorbed as volatile fatty acids as 33.6, 42.4 and 33.2% for the three diets in the above order. On lucerne diet the difference between the mean molar proportions of volatile fatty acids (VFA) absorbed and the volatile fatty acids (VFA) in the rumen was significant for butyric acid and almost significant for acetic acid. The differences were not significant for the other diets.

Esdale et al. (1968) measured ruminal VFA production in Jersey cow with a rumen cannula fed on a maize silage or a lucerne hay, using a ‘continuous infusion isotope dilution technique’. Daily ruminal production of acetate, propionate and butyrate for the silage diet was 19.6, 6.7 and 4.6 moles, and for the hay 19.6, 5.2 and 1.9 moles. Total ruminal VFA production in moles per kg. dry matter intake was 8.8 with silage and 6.8 with the hay.

Fritz et al. (1968) compared the effect of fresh and wilted lucerne silage on the formation of VFA in the rumen of sheep and found that wilted lucerne promoted propionic acid production and reduced the acetic:propionic acid ratio in
the rumen significantly.

Fritz (1968) observed the effect of different feeds on fermentation in the rumen of cows and found that there was an increase in the proportion of propionic and butyric acids and a fall of the acetic : propionic acid ratio with rising proportion of concentrates in the ration. Green maize but not maize silage had a similar effect. Green lucerne stimulated acetic acid production and increased the concentration of all volatile fatty acids in the rumen.

Ichhponani et al. (1969) studied the 'in vitro' production of volatile fatty acids by rumen inoculum of Buffalo and Zebu cattle using green Berseem alone, green Berseem + wheat straw (1 : 1 on D.M basis), wheat straw + concentrate mixture and green Bajra + cowpea mixture. The Buffalo inoculum gave consistently higher production of VFA in all feed combinations tested than Zebu cattle on corresponding feed combinations. Total VFA production was in the following order: green Berseem, wheat straw + concentrate mixture, green Berseem + wheat straw, green Bajra + cowpea mixture.

Storry and Sutton (1969) investigated the effect of changes from low roughage to high roughage diets on rumen fermentation in cows. The concentration of lactic acid and the proportion of propionic and Valeric acids decreased and the pH and the proportion of acetic acid increased, to values typical for high roughage diets. The changes in proportions of rumen volatile fatty acids were associated with increased concentrations in blood plasma of acetic and beta hydroxy - butyric acids.

Rao and Mukherjee (1969) estimated the rate of production and percentage composition of VFA produced on 'in vitro' fermentation of glucose, lactose starch and cellulose with rumen liquor of goats.

A lag phase of about six hours was observed with cellulose before it could be fermented while the rest of the carbohydrates could be fermented
Sutton (1969) studied the fermentation of soluble carbohydrates in rumen contents of cows given diets containing a large proportion of flaked maize. The proportion of VFA in the rumen before addition of carbohydrates, varied widely but on an average acetic acid constituted about 52% and n-butyric acid about 13% of the total. In 'in vitro' experiments, mean proportions of carbohydrates fermented ranged from about 60% of the pentoses to about 65% of sucrose and glucose. Acetic acid constituted about 40% propionic acid 45-55% and n-butyric acid 1-7% of the VFA's produced from galactose and pentoses.

Chawdhry (1970) conducted studies to find out the VFA production on Barley straw + Rumvite Block and lucerne hay diet fed to Border Leicester Romney cross ewes and observed higher m. moles per 100 ml of VFA from lucerne hay when compared with barley straw long + Rumvite Block feeding.

Margaret et al. (1970) observed in rumen of cattle that large ciliate populations and high proportions of butyric acid can be produced in animals fed exclusively on a barley diet by suitable adjustment of the intake and the methods of feeding. It is postulated that the ciliate population may be largely responsible for the high butyric acid concentration.

Gupta et al. (1970) reported the mean initial and day's average concentration of VFA in adult ruminants fed wheat bhusa and paddy straw enriched with urea and molasses. In the first period with controlled feeding of a mixture of wheat bhusa, 1.5% of urea and 10% molasses, the mean initial and day's average of VFA's were found to be 7.5 and 2.3 m. eqv./100 ml of rumen fluid respectively. In the second period, i.e. on ad lib feeding of the same mixture, the VFA concentration improved to 9.7 and 2.8 m.eqv/100 ml respectively. In the third period, i.e. on increasing the level of molasses to 15% and feeding the mixture ad lib, the concentration of VFA was 8.5 and 8.3 m.eqv/100 ml respectively.
feeding a mixture of paddy straw, 1% urea and 10% molasses, the VFA concentra-
tion was 8.5 and 9.0 m. equiv/100 ml. respectively.

Whitelaw et al. (1970) observed VFA production in two heifers given
a diet containing 85% barley and 15% protein-mineral-vitamin supplement. Daily
intake of the diet was restricted to 5.1 kg, which was given in three equal feeds.
In one heifer (no. 794), VFA production was measured over a 24 hours feeding cycle;
in the other (no. 832), a single 6 hour feeding period was measured.

Simultaneous measurements of rumen fluid volume and outflow allowed
estimates to be made of VFA production and absorption in the entire rumen during
each feeding period. In heifer no. 794, total VFA production was 18.2 moles/day
of which approximately 85% was absorbed directly from the reticulo-rumen.

Rate of VFA production in the rumen:

The rate of VFA production in any sample of rumen content either
'in vitro' or 'in vivo' is dependent upon various factors which have been described
later, but the single most important one being the nature of the food fermented.
A complicating factor in its measurement 'in vivo' is the extensive absorption of
these acids by the rumen wall. As a result most of the works on the estimation
of rate of VFA production has been done 'in vitro'.

Gray and Pilgrim (1952) fermented powdered wheaten hay 'in vitro' by
rumen liquor from a sheep and estimated a rate of 8.1 m. moles between 2-4\(\frac{1}{2}\) hours
and 8.7 m. moles between 4\(\frac{1}{2}-7\frac{1}{2}\) hours followed by a sharp fall in the rate of
production probably due to lack of substrate.

Carroll and Hungate (1954) estimated 'in vitro' the rate of produc-
tion of VFA at a rate of 8.3 m. moles per hour per liter on a hay fed steer when
the sample was drawn 16 hours after feeding, but the 13 hour's sample gave a rate
of 12.5 m. moles. Apparently the rate tended to fall off between 13 and 16 hours.
Stewart and Schultz (1958) incubated 'in vitro' various feeds using steer's rumen content and found that fresh hand-clipped legume mixed grass caused marked depression of propionic acid fermentation in comparison to hay. Molasses consistently decreased acetic acid production. Beet pulp significantly increased acetic acid production and corn meal increased propionic acid production. Results are shown in Table 2.3.

Rao (1963) fermented different feed-stuffs 'in vitro' and estimated the rate of VFA production as 26.4, 20.65, 10.6, 8.8 and 8.7 mmoles per liter per hour from Berseem hay, Guar meal, maize, spear grass and Berseem green respectively.

Gray et al. (1965) measured the rate of VFA production in the rumen of sheep both 'in vitro' and 'in vivo'. The production of acetic, propionic and butyric acids was measured in an artificial rumen of the dialysing type both by direct analysis and by the 'Isotope dilution technique'. Estimates of production by the latter method agreed closely with the direct measurements when due allowance was made for small losses of the radioactive labels from their respective acids.

Rates of production of the three acids in the rumen of the sheep were measured by the 'Isotope dilution technique'. The small losses of $^{14}$C from the acids were measured in the same material fermented in an artificial rumen which was shown to produce a mixture of the acids in the same proportions as the fermentation in the rumen itself.

Roy (1970) studied 'in vitro' the effects of delignification on the digestibility of roughage in cattle and determined the rates of VFA production as 4.6 and 9.3 mmoles per liter per hour from untreated paddy straw and alkali-treated straw respectively. The effect of delignification was found to improve the rate of VFA production.
<table>
<thead>
<tr>
<th>Material:</th>
<th>Animal</th>
<th>Rate per hour</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruminal liquor from sheep fed on wheaten hay.</td>
<td>Sheep</td>
<td>8.7 m.moles</td>
<td>Gray and Pilgrim(1952)</td>
</tr>
<tr>
<td>Whole rumen content from Steers fed on Hay</td>
<td>Steer</td>
<td>14.2 m.moles/kg.</td>
<td></td>
</tr>
<tr>
<td>Grain</td>
<td>Steer</td>
<td>23.5 m.moles/kg.</td>
<td>Carroll and Hungate (1954)</td>
</tr>
<tr>
<td>Pasture</td>
<td>Steer</td>
<td>9.5 m.moles/kg.</td>
<td></td>
</tr>
<tr>
<td>Alfalfa hay incubated with rumen liquor.</td>
<td>Steer</td>
<td>23.8 mg/100 ml</td>
<td>Stewart and Schultz(1958)</td>
</tr>
<tr>
<td>Whole rumen content from sheep fed on hay and concentrate.</td>
<td>Sheep</td>
<td>6.1 m.moles/kg.</td>
<td></td>
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<tr>
<td>Whole rumen content from sheep fed on hay and concentrate and fasted overnight.</td>
<td>Sheep</td>
<td>4.2 m.moles/kg.</td>
<td>Mukherjee (1960)</td>
</tr>
<tr>
<td>Whole rumen content from sheep fed on flaked maize (non-fasting)</td>
<td>Sheep</td>
<td>12.0 m.moles/kg.</td>
<td></td>
</tr>
<tr>
<td>Ruminal liquor from slaughtered goats incubated with foodstuffs</td>
<td>Goat</td>
<td>26.4 m.moles/liter</td>
<td></td>
</tr>
<tr>
<td>Berseem hay</td>
<td>Goat</td>
<td>20.65 m.moles/liter</td>
<td>Rao (1963)</td>
</tr>
<tr>
<td>Guar meal</td>
<td>Goat</td>
<td>10.6 m.moles/liter</td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>Goat</td>
<td>2.8 m.moles/liter</td>
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<tr>
<td>Spear grass</td>
<td>Goat</td>
<td>2.7 m.moles/liter</td>
<td></td>
</tr>
<tr>
<td>Berseem green</td>
<td>Goat</td>
<td>2.7 m.moles/liter</td>
<td></td>
</tr>
<tr>
<td>Ruminal liquor from slaughtered cattle incubated with foodstuffs</td>
<td>Cattle</td>
<td>4.6 m.moles/liter</td>
<td>Roy (1970)</td>
</tr>
<tr>
<td>Untreated paddy straw</td>
<td>Cattle</td>
<td>9.3 m.moles/liter</td>
<td></td>
</tr>
<tr>
<td>Alkali-treated paddy straw</td>
<td>Cattle</td>
<td>9.3 m.moles/liter</td>
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</table>
Mukherjee (1960) for the first time by the application of 'Isotope dilution technique', determined 'in vitro' the rate of production of VFA in fasting sheep previously fed on oats and hay to be 4.2 m. moles per kg. per hour.

Gray et al. (1966) measured the rates of VFA production 'in vivo' by the application of 'two Isotope dilution technique' in sheep fed at 12 hourly intervals on a mixture of wheaten hay and lucerne hay. Approximately 5.0 moles of volatile fatty acids were produced per kg. of dry matter. This same rate of production was found for retentions of 360, 450 and 675 gms. dry fodder fed at 12 hourly intervals.

Weller et al. (1967) measured by 'Isotope dilution procedure' the rate of production of volatile fatty acids both individual and total in the rumen of sheep fed at 12 hour intervals on roughage diet (lucerne and wheaten hay). Their findings support the view that the mixture of acids produced in the rumen were similar in composition to those present in the rumen fluid throughout the feeding-cycle. The composition of the acids initially formed in the rumen were acetic 77-83%, Propionic 15-18% and butyric 1-7% according to the diet given and the time elapsed after feeding.

Whitelaw et al. (1970) estimated the rate of VFA production in heifers given diet containing 85% barley and 15% protein-mineral-vitamin supplement. In one of the heifers, the rate of VFA production reached a maximum of 52-62 m. moles/liter/hour 15 minutes after each feeding and declined throughout the remainder of the feeding period.

FACTORs AFFECTING THE PRODUCTION AND PATTERN OF VFA:

Since in Herbivora 20% or more of the ration may consist of substances that can be digested only by the action of microorganisms and which are of varying complexity and chemical composition, the factors which may govern this activity are obviously of large importance. The quantitative relations involved in the
microbiotic decomposition of carbohydrates vary according to the kind and number of the microorganisms present, which in turn are under the influence of the character of the food. There is also evidence that the character of the roughage, irrespective of its fiber content, has an influence on the nature of the bacterial flora and on their activity.

The variations in the extent of crude-fiber digestion in the various animal species are due to the fact that the varying opportunities are presented for the action of microorganisms. The anatomical differences in the digestive tract enable the ruminants to digest at least 50% of the crude-fiber of most feeds. The degree of breakdown of crude fiber is dependent on its chemical and physical nature. The nature and amount of crude-fiber present are important factors governing the extent of the digestibility of the various nutrients in the ration.

Because of the importance of VFA and their close relationship to cellulose digestion, the various factors affecting cellulose digestion have also been reviewed briefly. The factors affecting VFA production from any rumen sample are chiefly the period of incubation, physical and chemical nature of the foodstuffs, the species of animal, feeding regime and the environmental temperature.

**PERIOD OF INCUBATION**

There are differences in the degree of breakdown of crude-fiber and VFA production depending upon the period of incubation 'in vitro' and the period elapsed after feeding 'in vivo'. Some of the factors have been reviewed here briefly.

Gray and Pilgrim (1951) reported that the rate of VFA production varied with the period of incubation. They incubated powdered wheaten hay with 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/hour at 7½ hours and 3.6 m.moles/hour on 19½
hours of incubation. It was seen that the maximum rate of production was maintained up to $7\frac{1}{2}$ hours and the rate was minimum at $19\frac{1}{2}$ hours.

Balch and Rowland (1957) observed in steers that the maximum rate of VFA production 'in vivo' was reached within first 3-4 hours after feeding and it tended to decline at the end of 6 hours.

El-Shazly et al. (1963) observed 'in vitro' with different food-stuffs that the rate of production of total VFA and total organic acids was greatest after 6 hours but then declined. Total organic acids exceeded total VFA but not after 24 hours.

Rao and Mukherjee (1969) observed a lag phase of about 6 hours with cellulose before it could be fermented, while the rest of the carbohydrates (Glucose, lactose and starch) could be fermented appreciably in 4 hours.

Ichhponani (1969) found a fall in the rate of VFA production after 36 hours of incubation from the flasks containing green berseem + wheat straw, wheat straw + concentrate mixture and green Bajra + cowpea mixture as substrate.

El-Shazly et al. (1969) observed the effect of periodical renewal of medium on VFA production and cellulose digestion 'in vitro' as criteria of energy content and found that the production of volatile fatty acids from legumes was high early in the fermentation periods and became lower in later periods. Grasses behaved in an opposite manner. There were significant negative correlations between VFA production and D.E. per kg. D.M. for legumes at later intervals of fermentation, correlation coefficients for grasses lost their significance at later periods of fermentation. D.E. per kg. D.M. could best be predicted for legumes from the VFA produced in the second 12 hour fermentation period or from the sum of the VFA produced in second, third and fourth 6-hour periods of fermentation.
Roy (1970) observed an increase in VFA production 'in vitro' with the increasing period of incubation in case of untreated and alkali-treated paddy straw using cattle inoculum.

**PHYSICAL NATURE OF FOOD-STUFFS:**

For a given species and animal, the extent of digestion of food-stuffs and the VFA production depends primarily on the physical nature of the nutrients presented to the microorganisms. Some of the factors have been reviewed here briefly.

Baker et al. (1959) reported that 'in vitro' digestibility of cellulose of cotton linters could be increased slightly by grinding to a fine powder in a ball-mill.

Sullivan and Hershberger (1959) observed 'in vitro' an increase in digestibility of cellulose after reduction in lignin content and consequent increase in VFA production.

Dehority (1961) observed that Ball-milling of cellulose increased the overall rate of cellulose digestion by reducing the lag phase up to 6 hours from 12 hours.

Dehority et al. (1962) reported that the lignification decreased the fermentation of hemicellulose in roughages but after Ball-milling the fermentation increased as the physical barrier was removed by Ball-milling.

Rao (1963) reported about the effect of particle size on the rate of fermentation and VFA production 'in vitro' and found that when Berseem hay and Jowar leaves chopped about half an inch in length were incubated, could not be fermented at all even up to 12 hours while the same food-stuffs when powdered after drying in mortar and pestle appreciable fermentation occurred in 4 hours only.
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Ørskov et al. (1968) investigated the extent of fermentation and efficiency of VFA formation in rumen of lactating cows fed on lucerne hay and a mixture of ground maize and Soyabean meal at hay : concentrate ratio of from 100 : 0 to 20 : 80 and at or near voluntary intake. Fermentation varied from 71% on the hay diet to 40% on a completely pelleted 20% hay and 80% concentrate diet. They concluded that the composition and particle size of the diet and the feed intake were the principal factors affecting fermentation.

CHEMICAL NATURE OF FOOD STUFFS:

The production of VFA is affected by the chemical nature of the food-stuffs, specially with reference to the quantitative presence of easily available source of NFE (soluble carbohydrates), Nitrogen, minerals and vitamins.

NITROGEN-FREE EXTRACT (N.F.E.)

VFA production is primarily affected by the nature of the carbohydrates present. Each carbohydrate constituents of the ruminants 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 due to the preferential attack on the simpler carbohydrates by the rumen microbes.

But it has also been shown that certain amount of easily available carbohydrates like starch is essential for the proper utilization of fiber, which provides energy for the activities of the microbes in the early stages of fermentation (Waynard and Locsli, 1962). In the absence of starch, addition of moderate amount of protein to the roughage ration (Hoflund et al., 1949) or protein - rich food-stuffs (Burroughs et al., 1949) had no effect on the digestibility of cellulose.

Burroughs et al. (1949) observed that cellulose digestion was depressed by feeding enough starch to steers. This was due to the fact that the rumen
microbes attack the soluble carbohydrates preferentially to crude fiber (Arias et al., 1951; Hershberger et al., 1955; and Kane et al., 1959).

Tyznik and Allen (1951) reported a relative increase in propionic acid production and a decrease in acetic acid production in cows on a restricted roughage ration.

Hungan et al. (1952) found that starch as well as glucose resulted in a large production of lactic acid and a consequent modification in the rumen fermentation products.

Card and Schultz (1953) reported significant differences in the percentage of each of the three prominent acids due to general type of ration.

Hibbs et al. (1954) working with calves found differences in proportions of acids with different proportions of grains added to roughage ration.

Belasco (1954) using 'in vitro' methods reported that the total production and relative proportions of the volatile fatty acids are dependent on the type and amount of carbohydrate used. He found generally higher production of VFA/starch than with cellulose.

Belch et al. (1955) found that when rations that are high in concentrate and low in roughages are fed to cattle the ratio of acetic acid 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 concentration is enhanced if a cooked concentrate such as bread is used (Shaw et al., 1957) or if the concentrate is heated before being fed (Busehio et al., 1959).

Hershberger et al. (1956) found that when starch was fermented, VFA mixtures were produced with lower acetate and propionate 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. Waldo and Schultz (1956) found that butyric acid showed an increase in concentration as well as an increase in percentage of total VFA 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 the total VFA. Acetic acid showed similar concentration on all levels of grain feeding with a decrease as a percentage of the total VFA with grain feeding.

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 increase 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 predominantly of uncooked concentrates.

Eusebio et al. (1959) showed that feeding of preheated concentrates 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. But it was 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 in the molar proportions of acetate and increase in the molar proportions of propionate.

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

Maynard and Loosli (1962) suggested that addition of easily digestible carbohydrates such as starch, cane sugar and molasses to the ration of cattle reduced the digestibility of fiber and this observation was explained on the ground that the bacteria attack the simpler carbohydrates by preference.
Chou and Walker (1964) studied the effect on the rumen composition of feeding sheep diets supplying different starches. They observed variations in rumen composition of sheep fed lucerne or wheat as the sole diet and found that total VFA's were lower on the lucerne diet - 39.9 m. moles/liter, than on the wheat diet - 69.4 m. moles/liter, with which less acetate and more propionate were found.

They further studied the effects of whole rice, broken maize or potato (raw or cooked and dried) on composition of rumen contents and compared with the previous experiment. They observed that total VFA's varied from 21 to 89 m. moles/liter being lowest on the potato diet and highest on the wheat and maize diets. A relatively high concentration of propionic acid was found on the wheat diet while butyric acid was highest when raw potato was given.

Ichhponeni and Sidhu (1966) conducted 'in vitro' experiments to study the factors affecting the digestion of cellulose in important coarse roughages, viz. wheat and paddy straw, maize and Jowar stalks, which are commonly used as cattle feed in India. The starch was added in the 'artificial rumen' flasks at the graded levels of 0.0, 0.1, 0.25, 0.5, 1.0 and 1.5 percent. Small depressions in cellulose digestion occurred at lower levels, but were much more pronounced as the level of starch in the incubation fluid was increased from 0.5 to 1.5%. Starch has no effect when incubated with a forage of good quality like green berseem.

The digestion of cellulose from the forage and the production of TCA/N increased with the increase of ureas/N from 0 to 50 mgs./100 ml. of the incubation fluid. The addition of valine, proline and methionine singly or in combination stimulated the digestion of cellulose. The supplementation of poor quality roughages with green berseem increased their digestibility.
NITROGEN

Although the nature of carbohydrates of the food-stuffs is the important controlling factor in the determination of both the rate and the proportions of the acids, the nitrogen requirements of the fermenting microbes must also be met with simultaneously.

As the bacteria multiply, they synthesize protein to construct their own bodies obtaining raw materials from the ingested food. For this purpose, they can utilize the nitrogen from amides, ammonium salts and even nitrates, as well as protein itself. Therefore, a certain minimum amount of nitrogen either in the form of protein or non-protein nitrogenous (NPN) substance is essential for the growth and multiplication of microorganisms. In the absence of a source of nitrogen the rate of fermentation is reduced.

McDonald (1952) has demonstrated that there was an increase in the rate of VFA production from starch when small amounts of casein was also added.

Williams et al. (1953) found that a minimum amount of protein was necessary for proper microbial breakdown of fiber.

Elshazly (1952); Belasco (1954); Woodhouse et al. (1955) and Davis et al. (1957) all have shown that high protein diets increase the butyric acid and higher fatty acids due in part to deaminated amino acid fragments of the meal proteins.

Balch and Rowland (1957) observed that 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.

Lewis and McDonald (1958) 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.
MINERALS AND VITAMINS...

Numerous experiments have shown that ration composition can influence microbial activity in the rumen of cattle and sheep. Certain unidentified factors have been shown to stimulate the activity of rumen microorganisms 'in vitro' (Burroughs et al., 1950 & 1951; Hungate 1950, Huntanen et al., 1952.) that Burroughs et al. (1950a, b and c; 1951 a and b) stated that cellulolytic organisms require a number of vitamins and trace minerals in the medium for efficient 'in vitro' fermentation of cellulose.

Bentley et al. (1953) found that a combination of B-Vitamins, purine, uracil and alfalfa ash increased cellulose digestion 'in vitro'.

Heil et al. (1953) found that biotin and Vitamin B₁₂ increased cellulose digestion in their 'in vitro' experiments.

Wasserman et al. (1953) reported that an isolated culture of rumen microorganisms has complex vitamin and other growth factor requirements.

Bentley et al. (1954) demonstrated the importance of certain B-Vitamins, purines and uracil for rumen microorganisms activity and thus provided additional evidence for the existence of undetermined nutritional factors which are required by the rumen microorganisms for growth 'in vitro'. Since these factors have a marked effect on cellulose digestion, it has been convenient to refer to them as "Cellulolytic factors" with the full realisation that there may be additional specific effects of these materials on rumen microorganisms activity 'in vitro', e.g. ammonia utilization.

Maynard and Loosli (1962) stated that the replacement of poor quality hay by alfalfa hay has been shown to stimulate microbial activity suggesting that the latter hay might have provided specific vitamins and other factors needed for the best growth of the microorganisms.
Giesecke (1968) observed the effect of lactic acid on volatile fatty acids in the rumen of sheep fed hay or hay and concentrates. Lactate in all conditions shifted the molar proportions of VFA from acetate towards propionate so that the acetate:propionate ratio fell from about 4 to 2.6. Protein in the concentrates seemed to promote that effect. A single dose of lactate was more effective on hay diet and a continuous infusion on that of hay and concentrates.

**SPECIES VARIATION:**

Pant et al. (1963 b) reported about the species variations in the total VFA concentrations in buffalo, sheep and goat immediately before morning feed as 62.9, 65.7 and 73.5 m. eqv./liter respectively.

**ENVIRONMENTAL TEMPERATURE:**

So far very little work has been done on this aspect. Environmental temperature necessitates consideration only when 'in vivo' experiments are conducted on this aspect.

Kelley et al. (1967) studied the effect of environmental temperature on ruminal VFA levels with controlled feed intake in Holstein cows and found significantly different proportions of acetic, propionic and total VFA when two higher temperatures were compared. But the values in the cold were not significantly different from those at 18.2°C. At 1.6°C mean amounts of acetic, propionic and total VFA/liter rumen fluid were 99.4, 37.6 and 153.1 m. eqv., at 18.2°C they were 47.2, 10.6 and 66.3 m. eqv. As the temperature rose the molar percentage of acetic acid increased and that of propionic acid fell.

**EFFECTS OF TANNINS IN THE NUTRITION OF LIVESTOCK, POULTRY AND LABORATORY ANIMALS:**

Tannins are a group of substances having 'tannic acid' as the basic material present in all. These are widely prevalent in vegetable kingdom
which forms the major portion of the ruminant's diet and as such tannin is a limiting factor in the utilization of tannin containing food-stuffs for livestock nutrition. Tannins have long been known to cause poisoning and other detrimental effects in animals. Various workers have studied about the detrimental effects of tannins, tannic acid and tannin containing substances by feeding experiments in various classes of livestock, poultry and laboratory animals. Some of the works done in this field have been reviewed here briefly.

Bondi and Meyer (1944) reported about the low digestibility and nutritive value of carobs (High tannin containing) for ruminants and chicks.

Kratzer and Williams (1951) observed that growth of chicks was reduced significantly when they were fed ration containing as little as 5% ground carobs.

Siu (1951) reported that natural tannins and tannin containing substances have long been known to inhibit decomposition processes of organic matter.

Husby and Presthegge (1952) observed that pigs fed carobs lost appetite and condition after 2 weeks but at 20% level of carob-meal in the ration they grew but did not look as well as the others.

Herman et al. (1953) observed that a ration containing about 12% tannins to which a further addition of 5% tannic acid was made had no effect on growth, food consumption or milk yield of cows and there was no digestive disturbances. They thought that tannic acid was apparently converted to tannates in the mouth and part of digestive tract and these are further broken down to non-astringent gallic and pyrogallic acids.

Url et al. (1954) have shown that at higher levels of addition of the ground carobs to chick-ration growth depression increased and mortality occurred.
Hawkins (1955) conducted palatability and digestibility trials with lespedeza hay and alfalfa hay in calves and found that the tannin affects palatability by its astringent taste and digestibility by precipitation of protein as insoluble tannates. He concluded that probably some of the tannin in the lespedeza was already combined with some of the protein in the plant as insoluble tannate, or that tannin added to the alfalfa ration was inactivated in the upper part of the gut before it could affect the protein.

Wilkins et al. (1955) observed that as the tannin content of herbage increased, the amount consumed by the sheep declined very appreciably. They concluded that tannins have a definite effect on palatability.

Becker et al. (1955) conducted digestibility trials in pigs with rations containing 2/3, 1/2 and 1/10th of the total as carob-meal and concluded that larger proportions of carob-meal greatly reduced digestibility of the rest of the ration, especially that of protein but with 10% carob-meal the reduction of the digestibility was slight.

Bissell and Weir (1957) conducted digestibility trials in deer and sheep by feeding oak and alfalfa hay and found poor digestibility for crude protein and crude fiber both in sheep and deer.

Hawkins and Autrey (1957) conducted feeding experiments in lactating cows using sericea (Lespedeze Cuneata) hay and alfalfa hay for milk production and showed that alfalfa hay had a higher value for milk production than sericea hay and supplementation of the latter with urea did not improve its value for milk production. They concluded that there were indications that sericea contained a milk depressing substance.

Bejovic et al. (1957) reported poisoning of cattle by tannic acid after exclusive feeding in early spring on buds and newly opened oak leaves.
Oslage and Becker (1958) conducted digestibility trials in sheep and observed that digestibility of protein of carob-meal was nil and that of lucerne hay protein 71%; the larger amounts of carob-meal significantly reduced the digestibility of the lucerne hay, that of its protein 50% but with smaller proportions of carob-meal the reduction of digestibility was slight.

Bejovic (1958) reported about the detrimental effects of oak leaves feeding in cattle and also the same changes when calves were given by mouth solutions of 2 to 10% of tannic acid, and 5 liters of the strongest solution caused death in 22 hours and this corresponded to the amount which an animal might obtain from oak leaves.

Oslage and Becker (1958) conducted digestibility trials in lactating cows using carob pods and observed low digestibility of protein. They also reported that natural tannins and tannin containing substances have long been known to inhibit decomposition processes of organic matter (Volcani and Roderig, 1961, Drori and Volcani, 1962).

Bornstein and Lipstein (1959) showed that higher levels of ground carobs in chick-rations caused growth depression and mortality.

Basaraba (1960) reported about the inhibitory effects of tannin and tannin containing substances on the decomposition processes of organic matters and concluded that tannins of various origin have different effects on the decomposition of organic matter.

Dollahite et al. (1962) studied the toxicity of gallic acid, pyrogallol, tannic acid and Quercus havardi in the sheep, goat and rabbit and found that tannic acid was absorbed from the gastro-intestinal tract into the blood stream in sheep, goat and rabbit. In rabbits it was rapidly detoxified or excreted. In rabbits, the lesions produced by all the polyphenols studied and those produced by Quercus havardi had a marked similarity. However, the lesions produced by tannic
acid fed in multiple doses more nearly resembled those produced by Q. havardii. These findings suggested that tannins in Shin oak might be involved in its toxicity.

Pigeon et al. (1962) isolated a hydrolyzable tannin from Quercus havardii. The oral multiple median lethal dose (LD 50) for the isolated tannin in the rabbit was 6.9 gm. per kg. per day for 5 days. The most prominent lesion observed in rabbits given the oak tannin was a hemorrhagic gastritis including ulcers and an exudate on the mucous membrane. Gallic acid was identified as the polyhydroxy phenol moiety of the oak tannin indicating that the tannin is a hydrolyzable tannin of the gallic tannin class.

Volcani and Levi (1962) and Volcani and Drori (1962) have studied the effects of carob pods on the growth of calves and concluded that the detrimental effects of carob pods in calf nutrition were due to lowered digestibility of all ration components.

Volcani et al. (1963) reported that carob pod meal and its various extracts inhibited cellulolysis and deamination 'in vitro' and it has been also observed 'in vivo' after prolonged feeding of carob meal by Y. Rabi (cited by Tagari et al., 1965).

Bornstein et al. (1963) conducted trials for improving the nutritive value of carobs for chicks and found that a substance extractable with hot water and which depressed appetite possibly a plant phenol was responsible for inhibition of growth. Extraction with hot water increased feed consumption and counteracted inhibition of growth to some extent. Diets including pods soaked in a hot water solution of ferric chloride or calcium chloride were slightly better than other diets containing carobs.
Leroy et al. (1964) studied the effect of tannic acid on deamination of protein in artificial rumen with sheep inoculum. Water or an aqueous preparation of tannin was added at 13% or 23% to oil cakes, 5 of groundnut and 6 of Soya, which were then dried. Ammonia with equal nitrogen and cellulose content were tested in artificial rumen. Ammonia-N and other soluble N first rose, then were fairly constant for 12 to 14 hours. They were not significantly different between oil cakes untreated or with water, or between 13% or 23% tannin solution, but were significantly higher without than with tannin. In earlier tests 6% tannin was not enough.

With pepsin and trypsin 'in vitro' a 13% tannin solution did not affect cellulose digestion of lucerne hay. Enzyme solubility of nitrogen from either Groundnut cake or Soya cake was significantly less, about 6% with water than with untreated cakes, and not significantly less with 13 or 23% solution than with water. Work then done on sheep with rumen fistula sometimes confirmed that tannins prevented bacterial deamination of feed without upsetting digestive processes.

Chang and Fuller (1964) studied the effect of tannin content of grain sorghums on their feeding value for growing chicks and observed that growth was depressed in proportion to the amount added from 0.5 to 2% tannic acid in the sorghum grains. Growth of chickens given diets with similar energy and protein and containing different varieties of sorghum varied inversely with the tannin content of the sorghum. Supplements of choline and Methionine hydroxy analogue prevented the depression of growth caused by 1% tannic acid. When twice as much choline and Methionine hydroxy analogue as usual were added to the diet chickens given sorghum with 2.0% tannin grew as well as those given sorghum with 0.2% but when choline and methionine were ommitted from the diet growth of chickens was retarded by sorghum with 2.0% tannin.

Vohra and Kratzer (1964) conducted experiments with day-old chickens and found that part of the cereals in the diet can be replaced by 70% ground carob pods (ceratonia siliqua ) when the diet had 5 to 10% Soysbean oil but with 4% ground carobs growth was retarded even when the diet had 13% Soyabean oil.
Alumot et al. (1964) conducted trials to elucidate the cause of the growth-inhibiting effect of carob meal in chick rations, with special emphasis on estimating its productive energy value and found that the presence of tannin-like compounds affected appetite and resulted in reduced feed intake. But the low caloric value of carobs was the major factor responsible for growth depression in chicks.

Neto (1964) conducted digestibility trials with pigs replacing 20 to 60% of maize by carob beans and found reduced gains in body weight.

Henis et al. (1964) reported that carob pod extract and its tannin as well as gallotannic acid at very low concentrations, have been shown to inhibit saprophytic cellulolytic bacteria and modify their colonies, especially cellul vibrio.

Tagari et al. (1965) conducted 'in vitro' experiments to study the effect of carob pod extract on cellulolysis, proteolysis, deamination and protein biosynthesis. They used microorganisms from rumen fluid of sheep fed on lucerne hay, concentrates and cotton seed hulls. The incubations were carried on for 48 hours in containers to which cellulose and minerals were added. Carob pod extract was a hot water extract and tannins and sugars were prepared from the extract. They observed that cellulose decomposition was almost completely arrested by the addition of carob extract to a final concentration of 1.5 mgs./ml. However, whereas the addition of gallotannic acid resulted in a similar effect, the tannin fraction of the carob extract alone did not inhibit cellulolysis significantly. They compared the effect of carob extract on cellulolysis with that of its sugar content, and found that all the three fractions had a similar effect on cellulolysis, with a 12 mgs./ml concentration being almost completely inhibitory.

Fowler and Richards (1965) reported about 'Acorn' poisoning in cow and sheep. They found that all parts of the oak tree, including acorn, contain tannins which are toxic agents in this genus.

Bornstein et al. (1965) conducted trials to estimate the metabolizable and productive energy of carobs for the growing chicks. They observed that the energy value of the experimental diets was significantly reduced when the proportion of the
Carob increased. There was a significant decrease in growth and efficiency of feed conversion with 20 or 45% carob meal as the reduced nutritional density of the diet did not induce a corresponding increase in intake of feed. The reduced energy of diets was due to the low energy content of the carob meal.

Zorita and Sanz (1965) conducted feeding experiments with Acorn meal in poultry and found that weight and efficiency were worse with 30% or 40.5% acorn meal but were not significantly affected by smaller amounts.

Vohra et al. (1966) investigated the growth depressing and toxic effects of tannins in chicks and found that different plant tannins, tannic acid, D-catechin, gallic acid or pyrocatechol 1% in diets based on maize, Soyabean meal and fish meal significantly reduced body weight of chickens 3-weeks old and with 5% tannic acid 70% of the chickens died between 7 and 11 days of test, and 0.5% significantly reduced body weight. Addition of 0.1% methionine or ornithine or 0.5% choline or betaine did not prevent the depression of growth caused by 1% tannic acid. Body weight, efficiency of feed conversion, metabolizable energy of the diet and retention of N were significantly less with 2% tannic acid. There was increase in blood cholesterol but the absorption of fat was unchanged.

Fuller et al. (1966) conducted trials to evaluate the feeding value of grain sorghums in relation to their tannin content and found that grain sorghums containing 1.6% or more tannin depressed growth of chickens when given as 50% of the diet.

Mc Cosker and Hunt (1966) reported poisoning of cattle with Acacia salicina which contained 35% tannin in bark and 14% in leaves.

Burns (1966) reported that chopping or macerating the green forage of Sericia lespedeza reduced the amount of tannins and consequently the associated cellulose inhibition effects.

Fuller et al. (1967) conducted experiments to ascertain the effects of tannic acid on chickens and found that growth was depressed significantly by 1% tannic acid and slightly by 0.5%. The toxicity of 1% tannic acid was reduced by 0.18%
methionine hydroxy analogue. Increasing amounts of choline with each amount of
methionine hydroxy analogue had no effect on growth rate with either amount of
tannic acid. Depression of growth caused by tannic acid was reduced slightly by
0.2% methionine hydroxy analogue without extra betaine or choline. Choline or
betaine reduced toxicity of tannic acid only when methionine hydroxy analogue was
given. Methionine hydroxy analogue and arginine had an additive effect in reducing
the growth depressing effect of tannic acid.

Potter et al. (1967) studied the effect of tannic acid on egg
production and egg-yolk mottling in laying hens. Egg production was significantly
reduced by 2% but not by 1% tannic acid. Egg-yolk mottling was significantly
increased by either amount of tannic acid. Yolks of eggs from hens given tannic
acid were coloured olive green. The white was not affected.

Delort and Zelter (1968) reported that treatment with tannins
strongly inhibited the deamination of many proteins without adversely affecting
digestibility of the diet, rumen microflora cellulolytic activity and VFA production.
They concluded that tannins reduce the degradation of Proteins in the rumen.

Potter (1968) studied the toxicity of tannic acid and the mechan-
isms for its detoxification in chickens. Tannic acid was fed to young chickens at
levels of 0.0.5 and 1.0% of their diet. Choline, methionine (as the hydroxy analogue,
Ca-salt = MAH), arginine and ornithine were added to the diets singly and in various
combinations at each level of tannic acid fed. The growth rate of chicks was signi-
ficantly depressed when either level of tannic acid was fed, the depression being
more severe at the highest level. Supplemental choline and methionine were partially
affective in alleviating the tannic acid induced growth depression. The combination
of MAH, choline and arginine (or ornithine) further reduced the toxicity of tannic
acid fed at 1.0% level and completely alleviated the effects of 0.5% tannic acid in
a manner suggesting an additive effect of these nutrients.

Clandinin and Heard (1968) reported that both prepress-solvent and
solvent extracted rape seed meal contained tannin as quercitannic acid ranging from
2.71 to 3.0% and found that this tannin content was enough to affect adversely the growth of chickens and the M.E. value of diets.

Murty and Khan (1969) used 'in vitro' and 'in vivo' digestion techniques to study the concentration of ruminal ammonia in buffaloes using Groundnut cake with and without the addition of Salseed cake. The addition of Salseed cake to G.N. Cake was reported to reduce significantly the ruminal ammonia production. The reduction in ammonia level in the rumen coincided with the lower excretion of urinary nitrogen and high nitrogen balance from G.N. Cake protein.

Pal (1969) reported on the basis of observation made during a feeding trial with Haryana bullocks that Salseed cake was unpalatable to cattle. But, it was consumed in appreciable quantities when offered in admixture with G.N. Cake. It was further observed that when Salseed was fed as the sole source of concentrate to the bullocks, the digestibility of protein was nil, and that of other nutrients was also less in comparison to the animals to which G.N. Cake alone was fed as sole source of concentrate. A comparison of the average D.M. intake in group I (with Salseed cake as the sole concentrate) and group II (with G.N. Cake as sole concentrate) showed that supplementation of the wheat bhussa ration with G.N. Cake instead of Salseed Cake, improved intake and that the same was bettered when salseed cake was offered simultaneously with G.N. Cake. During the experimental feeding period extending over a month, the animals supplemented with salseed cake alone lost 9% of their live weight on an average, while in the groups supplemented with G.N. Cake alone and in combination with Salseed cake the animals gained 3 and respectively 4% live weight. The inclusion of salseed cake with G.N. Cake increased the total D.M. intake in the ration but it had adverse effect of bringing about depression in average D.M. digestibility from 51 to 46%, and that in wheat bhussa supplemented with salseed cake alone averaging about 45% only. The digestibility of organic nutrients followed the trend of D.M. digestibility.

Conner et al. (1969) studied some nutritional aspects of feeding sorghum grains of high tannin content to growing chickens. The grains of 3 varieties
of sorghum with different tannin contents were given to growing chickens as 70% of their diet in 3 experiments. Tannic acid was given also as 0.1% or 1.0% of the grain component in one of the experiments. Growth was retarded as tannin content of grain increased. This retardation produced by an increase in plant tannin or addition of tannic acid could be only partly alleviated by supplementation with large amounts of methionine and choline. Increase of tannic acid or plant tannin seemed to depress feed intake and had a toxic effect. Addition of tannic acid significantly depressed fat content of liver and weight of testis. Additional methionine plus choline prevented the depression of liver fat but did not prevent the reduction in testis weight.

Joslyn and Glick (1969) studied the comparative effects of gallotannic acid and related phenolics on the growth of rats. Weanling male Long Evans rats were given freely water and diets containing two types of gallotannic acid, three types of condensed tannins, ellagic acid, gallic acid or catechin at 5% and from 2 to 10% for periods up to 30 days. Food intake and growth were reduced by catechin and gallic acid but less than by tannic acid. Tannic acid at 5, 8 and 10% caused a higher mortality in a shorter time than did other compounds but mortality was not necessarily correlated with growth depression. Ellagic acid had no adverse effects 'in vivo' and 'in vitro'.

Mc Ginty (1969) conducted 'in vivo' and 'in vitro' digestibility trials in cattle to know the factors affecting digestibility of varieties of sorghum grains and found that the factors causing poorer digestion was in the pericarp. When pericarp from the poorly digested varieties was added to fermentation tubes with endosperm of any variety gas production was less, and the soluble material of the pericarp, extracted with organic solvents and water, had the same effect. Tannic acid also reduced gas production. The decrease caused by poorly digested pericarps could be lessened by polyethylene glycol. When soluble material from the pericarp was added to centrifuged rumen fluid there was a significant increase in the amount of N-precipitated.
Donnelly and Anthony (1969) studied dry matter digestibility with low and high tannin varieties of Sericea leaves and stems by Lusk's method in which ground material in Nylon bags was placed in the rumen of bullocks. Average crude protein percentage of the low and high tannin varieties was 15.9 and 16.0 respectively. They observed significantly different dry matter digestibility. The digestible D.M for high tannin variety was 63.4% and that of low tannin variety was 71.8%. No correlation was found between digestible D.M and Protein.

Zeltey et al. (1970) studied 'in vitro' the behaviour of some proteins tanned with tannin from chestnut wood or some aldehydes (Formaldehyde, glutaraldehyde, glyoxal). Proteins of ground nut, soya bean, linseed, rape seed and sunflower seed oil meals, skimmed milk powder or dried lucerne meal or casein were complexed with the different tanning agents, chestnut wood extract, formaldehyde, glutaraldehyde or glyoxal in an artificial rumen. The minimum amount of each tanning agent which would prevent protein degradation depended on the original physico-chemical properties and the heat treatment applied to the protein. The original values for enzymic solubility of the protein were not affected by formaldehyde or glyoxal but were reduced by 5% by chestnut extract or glutaraldehyde. The minimum dose of each aldehyde which would completely protect the protein decreased cellulytic activity of an inoculum of rumen contents on a wheat straw substrate by 13 to 20%, while doses which gave 90% protection reduced cellulytic activity by 3%. Chestnut extract did not have this negative effect on the cellulytic activity of rumen inoculum.

Tamir and Alumot (1970) studied the growth depressing effects of carob-tannins and also estimated the levels of insoluble nitrogen in the digestive tract of rats.

Male albino rats were given for 15 days diets containing green carobs, ripe carobs, ripe carobs without sugar, a hot water extract of green carobs, tannins from green and ripe carobs, an ethyl acetate extract of green carobs, a control diet or the control and sucrose. The decrease in growth of rats given
green carobs or ethyl acetate-soluble polyphenols was thought to be caused by decreased food intake. Tannins from ripe carobs also caused significant decrease in growth while those from green carobs had less effect. The faeces and digestive tract contents from rats given tannins from green or ripe carobs or ethyl acetate extracts of carobs contained more nitrogen than those of control rats. The increase was in the insoluble N-fraction and was correlated with the degree of growth depression. The activities of digestive enzymes in the caecum were increased in all the experimental groups.

Glick and Joslyn (1970) observed food intake depression and other metabolic effects of tannic acid in the rat and found that tannic acid depressed food intake and growth when diets containing 4-5% tannic acid was given and on diets containing 8% tannic acid 90% mortality occurred in weanling rats.

Rats weighing 130 and 200 gms. tolerated large amounts of tannic acid better than weanling rats and partly adapted to the tannic acid diet after a few days. When 40% casein was given with 5% tannic acid, food intake and weight gain were almost double to those with 20% casein and 5% tannic acid. Neither 1% choline hydrochloride nor 1.5% methionine had any effect on growth rate when given with the 1.5% tannic acid diet.

They also studied the effect of tannic acid and related compounds on the absorption and utilization of proteins in the rat and found that rats given diets containing 2 to 8% tannic acid, 2 to 6% gallic acid, 2% D-catechin, 5% ellagic acid or 5% condensed tannins from grape seeds and quebracho, for 12 to 53 days. Nitrogen in faeces was more than control values with 2% tannic acid and continued to increase with larger amounts of tannic acid. Condensed tannins from grape seeds and quebracho had similar effects. Gallic acid, D-catechin and ellagic acid had little or no effect on nitrogen in faeces.

There was no difference in the recovery of $^{14}C$ in the faeces from
rats given 5% tannic acid or controls given casein labelled with $^{14}$C in the diet. The proteolytic enzyme activity of intestinal contents compared with that of control rats, was increased three-fold by 5% tannic acid and four-fold by 5% grape seed tannin. Tannic acid did not affect the size or proteolytic activity of the pancreas.

Mitjavila et al. (1970) studied the effect of tannic acid on intestinal absorption in mice and found that Gallotannic acid at a concentration of 1 mg. per liter significantly decreased the absorption of glucose and methionine—the absorption decreased by about 6 and 10% compared with control values. The absorption of butyric acid was not significantly decreased by concentrations under 10 mgs. per liter but 10 mgs. decreased it by about 7%.

Kausal et al. (1971) investigated the possibility of utilization of oak (Quercus incana) Kernels in poultry mash. Oak kernels were incorporated in chick starter mash at 0, 2, 5 and 10% levels replacing an equal quantity of maize. There was no significant difference in growth rate of chicks during the age of 10 to 50 days on 0, 2 and 5% levels of incorporation, though chickens on 10% level showed a significant growth depression. There was also a gradual decrease in feed efficiency and N-retention per gram of mash intake with increasing level of oak Kernels in the mash. The high tannin content in oak Kernels was suspected to play an incriminating role. On boiling and washing with water, the tannin content in oak kernels was reduced slightly from 92 to 42% on D.W. basis. The effect of incorporating treated Kernels in chick mash at 0, 5, 10 and 15% levels at the expense of maize was studied. During the age of 10 to 50 days, there was an increasing growth depression with increasing levels of treated kernels in mash. The feed efficiency also declined with increase in proportion of treated Kernels in the mash.
CHAPTER III

MATERIALS AND METHODS

About 4000 of the purchased specimens ready were boiled with
1:40 (w/v) for 1½ hr each time. Cool-
ated water was collected in 10-ml quantities in sterilized 15-ml vials
with cap. 10-ml volumes of a constant weight. From a bulk sample was

CHEMICAL METHODS.

The chemical analysis of the feedstuffs used in this experiment was

conducted by the standard official methods of analysis of

DOKEL (1934).
MATERIALS AND METHODS

COLLECTION AND ANALYSIS OF FOOD-STUFFS USED IN THIS WORK:

Green pipal leaves and decorticated Sal seeds were used in this work. The green pipal leaves were collected from different areas in the vicinity of the college and pooled for proximate analysis. Sal fruits were obtained from the Sal forests of Ranchi district of this State in the month of May, 1971 when the fruits matured. The fruits were dried in sun and decorticated. A photograph of matured Sal fruits is illustrated in Fig. I.

Both Pipal leaves and decorticated Sal seeds were oven-dried at 105° - 110°C for 8-10 hours to a constant weight and powdered finely in mortar and pestle. Thus, a bulk sample of the powdered material was obtained for use throughout this work.

PREPARATION OF THE EXTRACTED SAMPLE:

About 20 gms. of the powdered unextracted sample was boiled with distilled water in the ratio of 1 : 40 (w/v) for half an hour each time. Cooled and strained through a double folded fine muslin cloth and washed 2-3 times with about 100 ml. distilled water and collected in petridish for drying in an oven at 105° - 110°C for 8-10 hours to a constant weight. Thus, a bulk sample was obtained for use throughout this work.

PROXIMATE ANALYSIS:

The proximate analysis of the food-stuffs used in this experiment were done according to the appropriate standard official methods of analysis of A.C.A.C (1960).

TANNIN ESTIMATION:

Tannin estimation was done by the Lowenthal - Procter method of tannin estimation in tea leaves (A.C.A.C, 1960).
FIG. 1.

PHOTOGRAPH SHOWING MATURERED SAL FRUITS USED IN THE EXPERIMENT.
PROCEDURE

5 gms. feed sample was weighed and boiled for 30 minutes with 400 ml. distilled water in a reflux condenser. Cooled and filtered with glass-wool and the filtrate was transferred to a 500 ml. volumetric flask and diluted up to the mark by adding distilled water. 10 ml. of this infusion was taken in a 1000 ml. conical flask and 25 ml. Indigo-carmine solution and about 750 ml. distilled water was mixed to it. N/10 KMnO₄ solution was added from a burette a little at a time while stirring until the colour changed from blue to light-green, then dropwise until the colour changed to bright-yellow or a faint-pink at the rim. The number of ml. of N/10 KMnO₄ solution used was designated as 'a'.

Then 100 ml. of the infusion was mixed with 50 ml. gelatin solution 100 ml. Acid sodium chloride solution and 10 gms. of powdered kaolin and shaken thoroughly for several minutes in a stoppered bottle. It was allowed to settle and decanted through filter paper. 25 ml. of this filtrate was mixed with 25 ml. Indigo-carmine solution and about 750 ml. distilled water in a 1000 ml. conical flask and titrated against N/10 KMnO₄ solution as above. The number of ml. of N/10 KMnO₄ solution used was designated as 'b'.

Now, (a-b) gave the number of ml. of N/10 KMnO₄ solution used to oxidize the tannin.

CALCULATION

Since 500 ml. infusion was prepared from 5 gms. of feed sample, 10 ml. of the infusion represented \( \frac{5 \times 10}{500} = 0.1 \) gms. of the feed sample.

\[
1 \text{ ml. N/10 KMnO}_4 \equiv 1 \text{ ml. N/10 oxalic acid and 1 ml. N/10 oxalic acid} = 0.00416 \text{ gm. of tannin.}
\]

Percentage of tannin in feed sample = \((a-b) \times 0.00416 \times 10 \times 100\).
RUHEN LIQUOR USED IN INCUBATION:

The rumen contents used in the incubation throughout this work was brought from slaughtered goats from the Butcher's shop very near to the college, immediately after slaughter. The rumen wall was slit open by a sharp knife and about 1 kg rumen content was taken in a 1000 ml wide-mouth bottle with bakelite screw cap which was gassed previously in the laboratory with CO₂ to drive out the air within it. The rumen content was immediately brought to the laboratory and again CO₂ gas was passed for a few minutes and the bottle was immersed immediately in an electric water-bath maintained thermostatically at 39°-40°C till finally used. The rumen content was immediately strained through a double folded fine muslin cloth in 1000 ml conical flask which was previously gassed with CO₂ to remove air within it and kept in an electric water-bath maintained thermostatically at 39°-40°C, and used for the incubation work.

'IN VITRO' INCUBATION:

Two different methods of incubation were followed in this work:

First method of incubation: The all glass system outlined by Bentley et al. (1954) was followed using strained rumen liquor. No mineral mixture was added to the incubation medium during incubation.

Second method of incubation: Mineral mixture was added to the incubation medium during incubation but no buffer solution was added as the mineral mixture acted also as buffer solution.

FIRST METHOD OF INCUBATION:

In this method incubation was carried on in 100 ml conical flasks fitted with rubber corks (no.5A) provided with both inlet and outlet device of glass tubes. The inlet tube being well below the outlet one in the incubation flasks so as to expell air inside the flasks by CO₂. The outlet tube being only
up to the inner end of the cork. The food-stuffs used in incubations were finally powdered pipal leaves and decorticated sal seeds, both unextracted and extracted. The tannic acid used was pure gallo-tannic acid.

The different weighed amounts of feed sample and gallo-tannic acid were added in different experimental flasks and kept ready before hand. 20 ml. strained rumen liquor (SRL) and 2 ml. M-phosphate buffer of pH 6.5 was added into the experimental flasks. 3 ml. distilled water was also added in each flask to make the volume up to 25 ml. The flasks were connected in series with rubber tubes and immersed in an electric water-bath maintained thermostatically at 39°-40°C. The rubber corks were fitted firmly to the flasks with a drop or two of water to make it air tight. The incubation experiments were always done in duplicate. Two other flasks treated similarly but without the food-stuffs were always used as control in each incubation experiments.

The inlet tubes were connected to a Y-tube, the Y-tube was connected to an empty 600 ml. conical flask (To regulate the flow of CO₂) fitted with rubber cork having an inlet and outlet device and the inlet tube was connected to the CO₂ cylinder with pressure tube. All the flasks were gassed at the same time with CO₂ which was passed through the inlet tube from the CO₂ cylinder for 10 minutes to remove the air and thus an anaerobic condition was maintained inside the experimental flasks. This was tested by passing the outlet gas in Barium chloride solution which became turbid and also by a lighted taper which was extinguished. When it was sure that CO₂ was coming out at the outlet, both the outlets and inlets were closed with Khair's screws. Thus, the incubation of rumen content was carried on under anaerobic condition. The flasks were gently shaken every half-an-hour throughout the incubation period. Leakage in the flasks and the connecting tubes were tested by placing a drop of water at the junction of the flasks and cork, glass tube and rubber tube.
As the incubations were of longer duration (6 hours), it was presumed that methane, H₂S and CO₂ which were produced during incubation, if accumulated would prevent the normal thrive of the microbes so outlets were opened at hourly intervals and fresh CO₂ was passed in all the flasks.

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

In a rough check up on the effect of exposure of rumen content to air, it was noticed that neither the pH nor the fermentative activity was affected perceptibly on exposure to air for 15-30 minutes. It is, therefore, safe to infer that no deleterious effect takes place during exposure of the rumen content in air for at least 15-30 minutes.

Before each incubation, immediately after straining the rumen liquor, 20 ml of it was taken in a separate beaker and 2 ml K-phosphate buffer solution at pH 6.5 and 3 ml distilled water was added to it. It was acidified with 2 ml 10 N H₂SO₄ to stop fermentation. This was done for estimating the initial concentration of volatile fatty acids (VFA) in the strained rumen liquor (SRL).

After the specified incubation period (6 hours) all the flasks were taken out from the water-bath and the contents were immediately acidified with 2 ml 10 N H₂SO₄ to stop further fermentation.

SECOND METHOD OF INCUBATION

The second method of incubation was practically similar in detail to that adopted by Pant et al. (1962) with minor modifications. The details of the procedure for incubation was similar to that followed in the first method excepting that 5 ml mineral mixture (Burroughs et al., 1950 a) of the following composition was also used and no buffer solution was added as the mineral mixture
also acted as buffer solution.

**MINERAL MIXTURE:**

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Phosphate (Monobasic)</td>
<td>52.50 Gms</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>52.50</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>37.50</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>77.50</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>7.50</td>
</tr>
<tr>
<td>Magnesium Sulphate</td>
<td>2.25</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>0.75</td>
</tr>
<tr>
<td>Ferrous Sulphate</td>
<td>0.15</td>
</tr>
<tr>
<td>Manganese Sulphate</td>
<td>0.06</td>
</tr>
<tr>
<td>Zinc Sulphate</td>
<td>0.06</td>
</tr>
<tr>
<td>Copper Sulphate</td>
<td>0.04</td>
</tr>
<tr>
<td>Cobalt Chloride</td>
<td>0.02</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>2000 ml</td>
</tr>
</tbody>
</table>

**PLAN OF INDIVIDUAL INCUBATION EXPERIMENT:**

The arrangement of the incubation flasks in the incubation experiments with different food-stuffs investigated are narrated below:

**A. INCUBATION WITH UNEXTRACTED AND EXTRACTED PIPAL LEAVES:**

1. 20 ml. SRL + 2 ml. M-Phosphate buffer + 3 ml. distilled water.

2. 20 ml. SRL + 2 ml. M-Phosphate buffer + 6.25 mgs. Tannic acid + 3 ml. distilled water.

3. 20 ml. SRL + 2 ml. M-Phosphate buffer + 500.0 mgs. Unextracted Pipal leaves powder + 3 ml. distilled water.

4. 20 ml. SRL + 2 ml. M-Phosphate buffer + 539.2 mgs. Extracted Pipal leaves powder + 3 ml. distilled water.

5. 20 ml. SRL + 2 ml. M-Phosphate buffer + 539.2 mgs. Extracted Pipal leaves powder and 6.25 mgs. Tannic acid + 3 ml. distilled water.

An excess of 39.2 mgs. extracted Pipal leaves powder was taken to adjust the N.P.E. level same as in 500.0 mgs. of unextracted sample. Tannic acid was added in the same amount as it was present in 500.0 mgs. of unextracted sample - 6.25 mgs.
B. INCUBATION WITH UNEXTRACTED PIPAL LEAVES WITH HIGH LEVEL OF ADDED TANNIC ACID

1. 20 ml. SRL + 2 ml. M-Phosphate buffer + 3 ml. distilled water.
2. 20 ml. SRL + 2 ml. M-Phosphate buffer + 62.5 mgs. Tannic acid + 3 ml. distilled water.
3. 20 ml. SRL + 2 ml. M-Phosphate buffer + 500.0 mgs. unextracted Pipal leaves powder + 3 ml. distilled water.
4. 20 ml. SRL + 2 ml. M-Phosphate buffer + 500.0 mgs. unextracted Pipal leaves powder + 62.5 mgs. Tannic acid + 3 ml. distilled water.

As 6.25 mgs. Tannic acid was present in 500.0 mgs. of unextracted sample, so ten times higher - 62.5 mgs was added.

C. INCUBATION WITH UNEXTRACTED AND EXTRACTED DECORTICATED SAL SEEDS:

1. 20 ml. SRL + 5 ml. mineral mixture.
2. 20 ml. SRL + 5 ml. mineral mixture + 66.5 mgs. Tannic acid.
3. 20 ml. SRL + 5 ml. mineral mixture + 665.0 mgs. Tannic acid.
4. 20 ml. SRL + 5 ml. mineral mixture + 500.0 mgs. unextracted Sal seed powder.
5. 20 ml. SRL + 5 ml. mineral mixture + 537.8 mgs. Extracted Sal seed powder.
6. 20 ml. SRL + 5 ml. mineral mixture + 537.8 mgs. Extracted Salseed powder + 66.5 mgs. Tannic acid.
7. 20 ml. SRL + 5 ml. mineral mixture + 537.8 mgs. Extracted Sal seed powder + 665.0 mgs. Tannic acid.

An excess of 37.8 mgs. of Extracted Salseed powder was taken to adjust the N.F.E. level same as in 500.0 mgs. of unextracted sample.

As 66.5 mgs. of tannic acid was present in 500.0 mgs. unextracted Salseed sample so ten times higher - 665.0 mgs. was added.

ESTIMATION OF VOLATILE FATTY ACIDS (VFA) IN RUMEN LIQUOR:

The total Volatile fatty acids in rumen liquor and incubation medium was estimated by the method of Elsden et al. (1945).
The acidified content of the flask was transferred to a 50 ml. measuring cylinder having glass stopper. The flask was washed 2 - 3 times with 4N H₂ S O₄ saturated with Magnesium sulphate and poured to the cylinder and the volume was made up to 40 ml. After thorough mixing, 10 ml. of its aliquot was taken in 'Markham Still' and steam distilled. About 150 ml. distillate was collected and titrated against N/50 Na O H solution - prepared by diluting N/5 Na O H solution, using 0.05% Bromo-Thymol-blue as indicator having p H range of 6 to 7.6. Further 25 ml. distillate was also collected and titrated as a precautionary measure against complete distillation of Volatile fatty acids. The second 25 ml. of distillate invariably did not contain any acid.

Calculations:-

Let the amount of N/50 Na O H used to neutralise the total VFA be X ml.

•• 10 ml. of diluted SRL = X ml. of N/50 Na OH Solution,

•• 40 ml. of diluted SRL = X x 40 ml. of N/50 Na OH Solution,

or, 20 ml. of original SRL = X x 40 ml. of N/50 Na OH Solution,

•• 1000 ml. of undiluted SRL = X x 40 x 1000 ml. of N/50 Na OH Solution,

or, X x 40 x 1000 x \( \frac{1}{10} \) = X x 40 x 1000 x \( \frac{1}{10} \) x \( \frac{1}{20} \) = X x 40 x 1000 x \( \frac{1}{50} \) ml. of N (Normal) Na OH Solution.

•• 1 ml. of N (Normal) Na OH Solution = 1 ml. mole of VFA.

Hence, the readings of N/50 Na OH used when multiplied by the factor '4' gave the total VFA in milli moles per litre of rumen liquor.

REAGENTS AND CHEMICALS USED :-

1. N/10 Oxalic acid solution = 1.575 gms. Oxalic acid (Analar) was accurately weighed and dissolved in 250 ml. distilled water ( 1 ml. N/10 Oxalic acid = 0.00416 gms. of tannin ).

2. N/10 K Mn O₄ solution = 1.333 gms. K Mn O₄ (Analar) was dissolved in 1000 ml. distilled water and standardised with N/10 Oxalic acid solution.
FIG. 2.

PHOTOGRAPH SHOWING A SET OF INCUBATION AND DISTILLATION APPARATUS USED IN THE EXPERIMENT.
3. Indigo solution - 6.0 gms. Indigo - Carmine powder was dissolved in 500 ml. distilled water by heating. Cooled and 50 ml. concentrated \( \text{H}_2\text{SO}_4 \) was added slowly and then the volume was made up to a litre by adding distilled water and filtered through ordinary filter paper.

4. Gelatin solution - 25 gms. Gelatin was soaked for an hour in saturated sodium chloride solution and heated until the gelatin was dissolved. Cooled and diluted with saturated sodium chloride solution to a litre.

5. Acid Sodium Chloride solution - 975 ml. of saturated Sodium chloride solution was acidified with 25 ml. concentrated \( \text{H}_2\text{SO}_4 \) added slowly.


7. M - Phosphate buffer of pH 6.5 - 92.8 gms. \( \text{KH}_2\text{PO}_4 \) and 113.8 gms. \( \text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O} \) per litre of distilled water.

8. Concentrated \( \text{H}_2\text{SO}_4 \) (GR)

9. 10 N \( \text{H}_2\text{SO}_4 \)

10. 4 N \( \text{H}_2\text{SO}_4 \)

11. N/50 Na OH solution

12. 3 N/7 \( \text{H}_2\text{SO}_4 \) solution.

13. N/7 Na OH solution

14. N/10 Na OH solution

15. Magnesium sulphate (L.R.)

16. 0.05% Bromo - Thymol blue indicator.

17. Copper Sulphate

18. Sodium Sulphate

19. Petroleum ether (Analar)

20. Gallotannic acid.

**STATISTICAL METHODS EMPLOYED (SNEDECOR, 1967):**

**ANALYSIS OF VARIANCE:**

This analysis was done for the total VFA produced from different
treatments and also from different incubations. The idea of this analysis was to know whether the variations in the production of VFA between treatments and between incubations were significantly different or not. The following is the set up of the analysis:

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between treatments</td>
<td>t - 1</td>
<td>$X_1$</td>
<td>I/III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\frac{X_1}{t - 1}$ (I)</td>
<td></td>
</tr>
<tr>
<td>Between Samples</td>
<td>s - 1</td>
<td>$X_2$</td>
<td>II/III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\frac{X_2}{s - 1}$ (II)</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td>$X_3$</td>
<td>(III)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\frac{(N-1)-(t-1)+(s-1)}{(N-1)-(t-1)+(s-1)}$</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>N-1</td>
<td>(X_1 + X_2 + X_3)</td>
<td></td>
</tr>
</tbody>
</table>

Where, $s =$ Number of incubations,
$t =$ Number of treatments,
$N =$ Total number of observations,
$X_1 =$ Corrected sum of squares (s.s.) between treatments,
$X_2 =$ Corrected s.s. between incubations,
$X_3 =$ Corrected s.s. of error.

The analysis of variance table provides a ready means of testing the significance of the difference between treatments and between incubations at 5% and 1% level. A comparison of the mean squares (M.S.) between treatments and between incubations with the error mean square provides a test of significance of differences arising from treatments and incubations. The comparison is done by finding out the ratio of the mean square concerned to the error mean square. This ratio is the variance ratio and is denoted by 'F'. This is referred in the 'F' table at the particular degree of freedom (d.f.) for significance.
If the tabulated 'F' value exceeds the calculated value then it is 'non-significant'. But if the calculated 'F' value exceeds that of the tabulated one for the particular level of significance, its difference between the means is 'significant'.

**CRITICAL DIFFERENCES**

The differences between the means of individual treatments were tested for significance only after the overall significant differences between treatments were indicated by 'F' test in the analysis of variance.

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

\[
C.D. = t \left( \text{with error d.f.} \right) \sqrt{S^2 \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}
\]

Where,

- \( C.D. \) = Critical difference,
- \( t \) = Table value with error degree of freedom (d.f.)
- \( S^2 \) = Error mean square (M.S.)
- \( n_1 \) \& \( n_2 \) = Number of replicates in each treatment.

The difference of the means between two treatments were found to differ significantly from each other if this difference between means of treatments exceeded the calculated value of critical difference.

**STANDARD ERROR**

The following formula was used to calculate the standard error:

\[
S.E. = \sqrt{\frac{\sum \chi^2 - (\Sigma \chi)^2}{N (N - 1)}}
\]

Where, \( S.E. \) = Standard Error, \( N = \) Number of observations.

- \( \sum \chi^2 \) = Crude sum of squares,
- \( \frac{(\Sigma \chi)^2}{N} \) = Correction factor.
CHAPTER IV

ESTIMATION OF ERRORS IN VARIOUS EXPERIMENTAL PROCEDURES

It is evident from Table 4.1 that the distillation process
varied by the water essentially recovered nearly 100% of acetone acid. A wa
ced sample recovery also indicates the high degree of reproducibility. It is
found that the rear 50 ml. of the distillate was almost 100% pure, with only
small amount of water. In order to achieve a certain level of accuracy, it is
important to use a suitable method to ensure that the sample is correctly
recovered and analyzed. In this research, the acetone acid was

(Continued on next page)
ESTIMATION OF ERRORS IN VARIOUS EXPERIMENTAL PROCEDURES

1. ACCURACY OF VFA ESTIMATION

The 'Markham Still' designed by Markham (1942) was used for the distillation of total VFA in the rumen liquor. The efficiency of the recovery of total VFA in this process was tested by the recovery of a known amount of VFA (Acetic acid). 10 ml. of standard Acetic acid solution was steam distilled in the 'Markham Still'. 150 ml. of distillate was collected and titrated against N/10 Na OH solution and compared with the reading obtained by direct titration of the acid. Further 25 ml. distillate was also collected and titrated to ensure complete recovery. But the latter invariably did not contain any acid. The close agreement of duplicate recovery from the standard Acetic acid solution which was invariably obtained also testify the accuracy of the full process. The result of recovery test is shown in table 4.1.

Table 4.1

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of obs</th>
<th>Amount of acid used (ml. N/10 Na OH)</th>
<th>Amount of acid recovered (ml. N/10 NaOH)</th>
<th>Percentage recovery with S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/10 Acetic acid</td>
<td>3</td>
<td>10.00</td>
<td>9.90</td>
<td>99.00 ± 0.005</td>
</tr>
</tbody>
</table>

It is evident from table 4.1 that the distillation process adopted in this work invariably recovered nearly 100% of Acetic acid. A very small standard error also indicates the high degree of reproducibility. It is also noted that the next 25 ml. of the distillate after first 150 ml. as a rule did not contain any trace of acidity. An uniform rate of distillation for a period of 25-30 minutes was found to be optimum and was regularly followed in this work.

2. ACCURACY OF LOWENTHAL - PROCTER METHOD OF ESTIMATION OF TANNINS IN TEA LEAVES (A.O.A.C., 1960)
A 5% standard solution of tannic acid was prepared. 10 ml. of this standard solution of tannic acid was taken in a 500 ml. volumetric flask and the volume was made up to the mark by adding distilled water and thoroughly mixed by shaking. 10 ml. from this solution was taken for estimation of the known amount of tannic acid present in it. The close agreement of duplicate recovery from the known solution which was obtained invariably testify the accuracy of the full process. The result of the recovery test is shown in Table 4.2.

Table 4.2

<table>
<thead>
<tr>
<th>No. of obs.</th>
<th>Amount of tannic acid taken (mgs.)</th>
<th>Amount of tannic acid recovered (mgs.)</th>
<th>Percentage recovery with S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>500.0</td>
<td>498.53</td>
<td>99.71 ± 0.075</td>
</tr>
</tbody>
</table>

It is evident from Table 4.2 that the recovery of tannin by the process adopted in this work is nearly 100%. A high degree of reproducibility is also indicated by the small standard error.

3. EXPERIMENT TO DETERMINE THE EFFECT OF DISSOLVED CO₂ IN THE INCUBATION MEDIUM ON VFA ESTIMATION

As the incubations were carried on in an anaerobic environment created by passing CO₂ gas in the incubation flasks, it was thought proper to estimate as to how much error is being caused in VFA estimation due to the presence of dissolved CO₂ in the incubation medium.

PROCEDURE

Three 100 ml. conical flasks were arranged in series. 20 ml. boiled distilled water was taken in each flask and 2 ml. M⁻ Phosphate buffer
was added in each flask to simulate the 'in vitro' incubation medium and CO₂ was passed through them for half an hour as in case of incubation experiments. The contents of the flasks were acidified with 2 ml. 10N H₂SO₄ and the whole content was transferred to a 50 ml. measuring cylinder having glass stopper. The flasks were washed 2 – 3 times with 4N H₂SO₄ saturated with magnesium sulphate and poured into the measuring cylinder making the volume up to 40 ml. 10 ml. of this aliquot was taken in 'Markham Still' after thorough mixing and steam distilled. 150 ml. of the distillate was collected and titrated against N/50 Na OH solution. Next 25 ml. distillate was also collected and titrated to ensure complete recovery of the acid. But the latter invariably did not contain any trace of acid. The distillation and titration was done in triplicate to ascertain variability. The result is shown in table 4.3.

Table 4.3

Table showing the effect of dissolved CO₂ in the incubation medium.

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>No. of obs.</th>
<th>Initial N/50 Na OH solution</th>
<th>Final N/50 Na OH solution</th>
<th>Difference N/50 Na OH solution</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0.0 ml.</td>
<td>1.50 ml.</td>
<td>1.50 ml.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.0 ml.</td>
<td>1.25 ml.</td>
<td>1.25 ml.</td>
<td>1.32 ± 0.88</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.0 ml.</td>
<td>1.20 ml.</td>
<td>1.20 ml.</td>
<td></td>
</tr>
</tbody>
</table>

It appears from table 4.3 that the error caused in VFA estimation due to dissolved CO₂ in the incubation medium is very small and constant which is also indicated by the small standard error.
RESULTS AND DISCUSSION

Chapter V

RESULTS AND DISCUSSION

Our present knowledge about the nutrition of contaminants have
been reviewed as a result of investigations carried on by various workers. In vitro and 'In vivo'. The 'In vitro' technique has been universally adopted by the
majority of workers due to its simplicity and economy. In the present work also,
the 'In vitro' technique has been adopted to investigate the effects of known
contaminants and also the wound tissue self on VPA production on 8 hours incubation
with Smith's medium sterilized.

The results obtained in this experiment have been presented
here under the following headings:

1. Extracting analysis of Pipal leaves and Sal seeds.
2. Irrigation of seeds in fresh saline media.
3. Effect of high levels of added hormones on 'In vitro' production
   of VPA from Pipal leaves.
4. Effect of high levels of added hormones on 'In vitro' production
   of VPA from Pipal leaves.
5. Effect of added hormones and added hormone acid on 'In vitro' production
   of VPA from Sal seeds.

A representative sample of Pipal leaves and Sal seeds (Dried-
processed) under used in this work were analyzed for proximate principles according

The result of analysis of the materials is shown in Table 5a.
RESULT AND DISCUSSION

RESULT

Our present knowledge about the nutrition of ruminants have accumulated as a result of investigations carried on by various workers 'in vitro' and 'in vivo'. The 'in vitro' technique has been universally adopted by the majority of workers due to its simplicity and economy. In the present work also, the 'in vitro' technique has been adopted to investigate the effects of tannins in feeds and also the added tannic acid on VFA production on 8 hours incubation with Goat's rumen microorganisms.

The results obtained in this experiment have been presented here under the following headings:

I. (A) Proximate analysis of Pipal leaves and Sal seeds,
   (B) Estimation of tannin in Pipal leaves and Sal seeds.
II. Effect of natural tannin and added tannic acid on 'in vitro' production of VFA from Pipal leaves.
III. Effect of high level of added tannic acid on 'in vitro' production of VFA from Pipal leaves.
IV. Effect of natural tannin and added tannic acid on 'in vitro' production of VFA from Sal seeds.

I. (A) PROXIMATE ANALYSIS OF PIPAL LEAVES AND SAL SEEDS:

A representative sample of Pipal leaves and Sal seeds (Decorticated) powder used in this work was analysed for proximate principles according to the standard official methods of analysis (A. O. A. C., 1960).

The result of analysis of the materials is shown in table 3.1.


<table>
<thead>
<tr>
<th>Material</th>
<th>Basis</th>
<th>Moisture</th>
<th>Crude Protein</th>
<th>Crude fiber</th>
<th>N.F.E.</th>
<th>E.E.</th>
<th>Total Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipal leaves powder</td>
<td>R. M.</td>
<td>9.72</td>
<td>14.09</td>
<td>24.32</td>
<td>35.24</td>
<td>3.48</td>
<td>13.15</td>
</tr>
<tr>
<td>(Before extraction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. M.</td>
<td></td>
<td>15.61</td>
<td>26.94</td>
<td>39.03</td>
<td>3.65</td>
<td>14.57</td>
</tr>
<tr>
<td>Pipal leaves powder</td>
<td>R. M.</td>
<td>9.34</td>
<td>14.97</td>
<td>27.13</td>
<td>32.68</td>
<td>3.88</td>
<td>12.00</td>
</tr>
<tr>
<td>(After extraction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. M.</td>
<td></td>
<td>16.51</td>
<td>29.92</td>
<td>36.05</td>
<td>4.28</td>
<td>13.24</td>
</tr>
<tr>
<td>Sal seeds powder</td>
<td>R. M.</td>
<td>8.90</td>
<td>5.90</td>
<td>4.50</td>
<td>63.35</td>
<td>15.20</td>
<td>2.15</td>
</tr>
<tr>
<td>(Before extraction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. M.</td>
<td></td>
<td>6.48</td>
<td>4.94</td>
<td>69.54</td>
<td>16.68</td>
<td>2.36</td>
</tr>
<tr>
<td>Sal seeds powder</td>
<td>R. M.</td>
<td>8.75</td>
<td>10.21</td>
<td>4.07</td>
<td>57.88</td>
<td>17.57</td>
<td>1.52</td>
</tr>
<tr>
<td>(After extraction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D. M.</td>
<td></td>
<td>11.19</td>
<td>4.47</td>
<td>63.42</td>
<td>19.25</td>
<td>1.67</td>
</tr>
</tbody>
</table>

*R. M.* = Raw matter basis.  
*D. M.* = Dry matter basis.  
*N.F.E.* = Nitrogen - free extract.  
*E.E.* = Ether extract.
From the above table 5.1, it is evident that in case of Pipal leaves, there was slight increase in crude protein, crude fiber and Ether extract in the Extracted sample whereas the N.F.E. and total ash decreased from 39.03 and 14.57 in the unextracted sample to 36.05 and 13.24 in the Extracted sample respectively.

In case of Sal seeds there was an increase in crude protein and Ether extract in the Extracted sample whereas N.F.E., crude fiber and total ash decreased from 69.54, 4.94 and 2.36 in the unextracted sample to 63.42, 4.47 and 1.67 in the Extracted sample respectively. It is also evident that Sal seed is poor in mineral content (7.36%).

The proximate composition of Pipal leaves and Sal seeds obtained is in general agreement with those obtained by various workers as shown in table 5.2.

I. (B) ESTIMATION OF TANNIN IN PIPAL LEAVES AND SAL SEEDS:

Tannin percentage was estimated in the representative samples of Pipal leaves and Sal seeds before and after extraction according to the standard official method of tannin estimation in tea leaves (A.O.A.C., 1960). The result is shown in table 5.3.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed meal</td>
<td>1.70</td>
<td>12.80</td>
<td>1.70</td>
<td>10.80</td>
<td>8.00</td>
<td>9.20</td>
<td>10.00</td>
<td>11.40</td>
<td>7.80</td>
<td>9.00</td>
<td>9.20</td>
</tr>
<tr>
<td>Salt leaf meal</td>
<td>1.10</td>
<td>14.80</td>
<td>1.10</td>
<td>13.60</td>
<td>9.80</td>
<td>9.00</td>
<td>9.00</td>
<td>10.00</td>
<td>7.80</td>
<td>9.00</td>
<td>9.20</td>
</tr>
<tr>
<td>Salt leaf meal</td>
<td>1.10</td>
<td>14.80</td>
<td>1.10</td>
<td>13.60</td>
<td>9.80</td>
<td>9.00</td>
<td>9.00</td>
<td>10.00</td>
<td>7.80</td>
<td>9.00</td>
<td>9.20</td>
</tr>
<tr>
<td>Salt leaf meal</td>
<td>1.10</td>
<td>14.80</td>
<td>1.10</td>
<td>13.60</td>
<td>9.80</td>
<td>9.00</td>
<td>9.00</td>
<td>10.00</td>
<td>7.80</td>
<td>9.00</td>
<td>9.20</td>
</tr>
</tbody>
</table>

Table showing approximate composition of plant leaves and salt seeds obtained by various workers

**Table 5.2**
<table>
<thead>
<tr>
<th></th>
<th>Mean + S.E.</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After extraction</td>
<td>2.50 ± 0.12</td>
<td>2.70</td>
<td>2.79</td>
<td>2.49</td>
</tr>
<tr>
<td>(seed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before extraction</td>
<td>1.39 ± 0.23</td>
<td>1.30</td>
<td>1.31</td>
<td>1.39</td>
</tr>
<tr>
<td>(seed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(seed)</td>
<td>1.25 ± 0.07</td>
<td>1.37</td>
<td>1.25</td>
<td>1.37</td>
</tr>
<tr>
<td>(leaf)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Table 5.3.4: Seeds and Leaves before and after extraction (in percentage).)

Table showing amount of tannin present in seed leaves and seed leaves before and after extraction.
Panda et al. (1969) estimated an average of 3.5% tannin in Sal seed meal by soaking the sample in cold water (1:4) for 24 hours. They also estimated an average of 1.4% tannin in the processed sample. The lower percentage of tannin obtained by them may perhaps be due to the difference in the process of estimation and also due to difference in variety and stage of maturity of the Sal seeds used by them. No work is available on tannin percentage in Pipal leaves.

II. EFFECT OF NATURAL TANNIN AND ADDED TANNIC ACID ON 'IN VITRO' PRODUCTION OF VFA FROM PIPAL LEAVES:

The rumen samples were collected from slaughtered goats as described under the chapter on 'Materials and Methods'. The first method of incubation mentioned therein was followed. The Pipal leaves tried were Unextracted and Extracted and finely powdered. The plan of these incubation experiments have been described in detail under the same heading under the chapter on 'Materials and Methods'. Total VFA production on 8 hours incubation from each source and their rate of production was determined. The results are shown in table 5.4.
<table>
<thead>
<tr>
<th>Range</th>
<th>F.S.E</th>
<th>Average</th>
<th>Range</th>
<th>F.S.E</th>
<th>Average</th>
<th>Rate per Hour</th>
<th>Total VFA produced</th>
<th>No. Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.90 - 6.69</td>
<td>4</td>
<td>6.3 + 1.6</td>
<td>28.0 - 69.2</td>
<td>4</td>
<td>69.2 + 3.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.10 - 4.11</td>
<td>4</td>
<td>6.6 + 1.6</td>
<td>69.2 - 64.9</td>
<td>4</td>
<td>64.9 + 2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.45 - 11.29</td>
<td>4</td>
<td>7.39 + 1.15</td>
<td>51.6 - 90.3</td>
<td>4</td>
<td>90.3 + 2.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.694 - 4.23</td>
<td>4</td>
<td>2.7 + 1.1</td>
<td>3.38 - 2.7</td>
<td>4</td>
<td>2.7 + 1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.78 + 1.1</td>
<td>4</td>
<td>2.99 + 0.85</td>
<td>6.2 - 2.42</td>
<td>4</td>
<td>2.42 + 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** VFA = Volatile Fatty Acid.

Table showing VFA production from Papill Leaves on six hours incubation (in moles of VFA/100 g of Rumen Liquid).
From table 5.4 it is apparent that Rumen liquor and Rumen liquor + added tannic acid at a level of 0.25 mg./ml. of incubation media on 8 hours incubation produced almost the same amount of fresh VFA = 22.9 and 21.3 m. moles per liter of rumen liquor respectively. The Unextracted Pipal leaves produced an average of 73.9 m. moles of fresh VFA per liter of rumen liquor. This indicates that Pipal leaves are easily fermentable. The Extracted Pipal leaves and the Extracted + added tannic acid at a level of 0.25 mg./ml. of incubation media on 8 hours incubation produced almost the same amount of fresh VFA = 52.4 and 50.5 m. moles per liter of rumen liquor respectively.

The above results indicate that added tannic acid at a level of 0.25 mg./ml. of incubation media did not seem to affect VFA production on 8 hours incubation from Extracted Pipal leaves and also from Rumen liquor alone. On the other hand, the VFA produced from Extracted Pipal leaves is significantly lower (above 30.0%) than from Unextracted Pipal leaves. The probable reasons for this lower production of VFA from Extracted Pipal leaves have been discussed in under the same heading "Discussion". The results are also represented in the form of a 'Histogram' in fig.3.

RATE OF VFA PRODUCTION:

From table 5.4 it is apparent that Rumen liquor and Rumen liquor+ added tannic acid at a level of 0.25 mg./ml. of incubation media on 8 hours incubation produced fresh VFA almost at a similar rate of 2.9 and 2.7 m. moles per hour per liter of rumen liquor respectively. Extracted Pipal leaves and Extracted + added tannic acid at a level of 0.25 mg./ml. of incubation media on 8 hours incubation produced fresh VFA almost at a similar rate of 6.6 and 6.3 m. moles per hour per liter of rumen liquor respectively. The Unextracted Pipal leaves on 8 hours incubation produced fresh VFA at a highest rate of 9.2 m. moles per hour per liter of rumen liquor. The probable reasons
FIG. 3 - PHOTOGRAPH OF HISTOGRAM.

HISTOGRAM SHOWING TOTAL VFA PRODUCED FROM DIFFERENT TREATMENTS ON 8 HOURS INCUBATION OF PIPAL LEAVES 'INVITRO'.

→ 8 HOURS INCUBATION.
for lower rate of fresh VFA production on 8 hours incubation from Extracted Pipal leaves have been discussed under the same heading in 'Discussion'.

From the above results it appears that added tannic acid at a level of 0.25 mg./ml. of incubation media has no effect on the rate of fresh VFA production from Extracted Pipal leaves and also from Rumen liquor alone.

To know that the differences between various treatments described above are statistically significant or not, the data in table 5.4 were subjected to 'F' test on the basis of analysis of variance and the results are presented in table 5.5.

**TABLE 5.5**

Table showing analysis of variance of total VFA produced on 8 hours incubation under various treatments.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.</th>
<th>F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between treatments</td>
<td>4</td>
<td>7858.670</td>
<td>1964.668</td>
<td>69.069 **</td>
</tr>
<tr>
<td>Between samples</td>
<td>3</td>
<td>3367.996</td>
<td>1122.665</td>
<td>39.467 **</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>341.314</td>
<td>28.445</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>11567.980</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Denotes significance at 1% level.

The analysis of variance (table 5.5) indicates an overall difference between treatments to be highly significant. The samples also differ highly significantly as expected due to the fact that the goats from which rumen samples were obtained had been of different ages and fed on different diets and also fasted for different unknown periods before being slaughtered.
Another possible reason for this high significant difference between rumen samples may be the small number of samples used in this work.

Since variation between the treatments were found highly significant on the basis of analysis of variance, critical difference test was applied to know the significant difference between any two treatments at 1% and 5% level as shown in table 5.6.

**TABLE 5.6**

Comparison of VFA produced from different treatments on 8 hours incubation with added tannic acid at a level of 0.25 mg./ml. of incubation media.

<table>
<thead>
<tr>
<th>No. of obs.</th>
<th>Treatments</th>
<th>Differences between averages</th>
<th>Critical difference 1% level</th>
<th>Critical difference 5% level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SRL Vs. SRL+6.25 mg. Tannic acid</td>
<td>1.6 N.S.</td>
<td>11.106</td>
<td>7.923</td>
</tr>
<tr>
<td>2.</td>
<td>SRL Vs. Unextracted Papal leaves</td>
<td>51.0 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>SRL Vs. Extracted Papal leaves</td>
<td>29.5 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>SRL+6.25 mg. Tannic acid Vs. Unextracted Papal leaves</td>
<td>52.6 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>SRL+ 6.25 mg. Tannic acid Vs. Ext. Papal leaves+6.25 mg. Tannic acid</td>
<td>29.2 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Unextracted Papal leaves Vs. Extracted Papal leaves</td>
<td>21.5 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Unextracted Papal leaves Vs. Ext. Papal leaves+6.25 mg. Tannic acid</td>
<td>23.4 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Extracted Papal leaves Vs. Ext. Papal leaves+6.25 mg. Tannic acid</td>
<td>1.9 N.S.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Denotes significance at 1% level.
'N.S.' Denotes non-significance.
From the critical difference test as shown in table 5.6 it is apparent that there is highly significant difference between any two treatments except SRL Vs. SRL + 6.25 mg. tannic acid and Extracted Pipal leaves Vs. Extracted Pipal leaves + 6.25 mg. tannic acid on 8 hours incubation.

Since the difference between averages of Rumen liquor and Rumen liquor + 6.25 mg. tannic acid (0.25 mg./ml. of incubation media) does not differ significantly it is safe to infer that there is no significant effect of added tannic acid on fresh VFA production from rumen liquor on 8 hours incubation.

Similarly, since the difference between the averages of Extracted Pipal leaves and Extracted Pipal leaves + 6.25 mg. tannic acid does not differ significantly it has been concluded that there is no significant effect of added tannic acid on fresh VFA production from Extracted Pipal leaves on 8 hours incubation.

However, the significant difference has been found in fresh VFA production between Unextracted and Extracted Pipal leaves on 8 hours incubation. The possible reasons have been discussed under the same heading in 'Discussion'.

III. EFFECT OF HIGH LEVEL OF ADDED TANNIC ACID ON 'IN VITRO' PRODUCTION OF VFA FROM PIPAL LEAVES

Since the low level of tannins (1.25%) in Pipal leaves did not affect the rate of VFA production in the previous incubations, it was thought advisable to see the effect of addition of higher levels of tannic acid. For this purpose a level of 62.5 mg. of tannic acid, i.e. 2.5 mg./ml. of incubation media (Ten times more than it is present in Pipal leaves) was added and its effect was studied. The results are tabulated in table 5.7.

The rumen samples were obtained from slaughtered goats.
as described under the chapter on 'Materials and Methods'. The first method of incubation mentioned therein was followed. Total VFA produced from each source on 8 hours incubation and their rate of production was determined. The results are shown in table 5.7.

From table 5.7 it is apparent that Rumen liquor and Rumen liquor + Ten times added tannic acid at a level of 62.5 mgs. or 2.5 mg./ml. of incubation media on 8 hours incubation produced almost the same amount of fresh VFA = 20.2 and 19.8 m. moles per liter of rumen liquor respectively.

The Unextracted Pipal leaves and Unextracted Pipal leaves + Ten times added tannic acid at a level of 62.5 mg. or 2.5 mg./ml. of incubation media on 8 hours incubation produced almost the same amount of fresh VFA = 57.9 and 56.9 m. moles per liter of rumen liquor respectively.

The above results indicate that added tannic acid even at a level of 2.5 mg./ml. of incubation media did not appear to affect VFA production from Unextracted Pipal leaves and also from Rumen liquor alone on 8 hours incubation. The results have been represented also in the form of a 'Histogram' in Fig. 4.

**RANGE OF VFA PRODUCTION**

From table 5.7, it is evident that Rumen liquor and Rumen liquor + Ten times added tannic acid at a level of 62.5 mg. or 2.5 mg./ml. of incubation media, on 8 hours incubation produced fresh VFA at the same rate of 2.5 m. moles per hour per liter of rumen liquor.

The rate of fresh VFA production from Unextracted Pipal leaves and Unextracted Pipal leaves + Ten times added tannic acid at a level of 62.5 mg. or 2.5 mg./ml. of incubation media on 8 hours incubation was almost the same = 7.2 and 7.1 m. moles per hour per liter of rumen liquor respectively.
FIG. 4 - PHOTOGRAPH OF HISTOGRAM.

HISTOGRAM SHOWING TOTAL VFA PRODUCED FROM DIFFERENT TREATMENTS ON 8 HOURS INCUBATION OF PIPAL LEAVES 'INVITRO'.

- Rumen Liquor
- RL + Tannic Acid (Ten times more) 62.5 mg (2.5 mg/ml of incubation media)
- RL + Unext + Tannic Acid (Ten times more) 62.5 mg (2.5 mg/ml of incubation media)

M. MOLES VFA/LITER RUMEN LIQUOR

8 HOURS INCUBATION.
<table>
<thead>
<tr>
<th>Range</th>
<th>Average + S.E.</th>
<th>Range</th>
<th>Average + S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate per hour</td>
<td>Rate Total VFA produced</td>
<td>Rate Obs. of</td>
<td>Rate Samples</td>
</tr>
<tr>
<td>I</td>
<td>II</td>
<td>III</td>
<td>IV</td>
</tr>
<tr>
<td>1.06 - 3.78</td>
<td>2.0 + 1.0</td>
<td>2.5 + 0.9</td>
<td>2.0 + 0.9</td>
</tr>
<tr>
<td>3.13 - 3.79</td>
<td>2.5 + 0.9</td>
<td>2.5 + 0.9</td>
<td>2.5 + 0.9</td>
</tr>
<tr>
<td>5.00 - 8.99</td>
<td>7.1 + 1.5</td>
<td>7.1 + 1.5</td>
<td>7.1 + 1.5</td>
</tr>
<tr>
<td>8.00 - 11.8</td>
<td>4.0 - 7.16</td>
<td>4.0 - 7.16</td>
<td>4.0 - 7.16</td>
</tr>
<tr>
<td>11.8 - 45.9</td>
<td>6.9 + 11.8</td>
<td>6.9 + 11.8</td>
<td>6.9 + 11.8</td>
</tr>
<tr>
<td>P.L + 500.0 mg. 40.0 mg. 7.0.6 45.4 7.0.9 4 7.0.9 4 7.0.9 4</td>
<td>P.L + 500.0 mg. 40.0 mg. 7.0.6 45.4 7.0.9 4 7.0.9 4 7.0.9 4</td>
<td>P.L + 500.0 mg. 40.0 mg. 7.0.6 45.4 7.0.9 4 7.0.9 4 7.0.9 4</td>
<td>P.L + 500.0 mg. 40.0 mg. 7.0.6 45.4 7.0.9 4 7.0.9 4 7.0.9 4</td>
</tr>
</tbody>
</table>

Table showing VFA production from PPL leaves with high level of added tannic acid on 8 hours.

TABLE 5.7
From the above results, it appears that added tannic acid even at a level of 2.5 mg./ml. of incubation media did not affect the rate of fresh VFA production either from Pipal leaves or Rumen liquor on 8 hours incubation.

To know the difference between the extents of VFA production from different treatments and also the samples of rumen liquor, the data in table 5.7 was subjected to 'F' test on the basis of analysis of variance as shown in table 5.8.

**TABLE 5.8**

Table showing analysis of variance of total VFA produced on 8 hours incubation under various treatments.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.</th>
<th>F*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between treatments</td>
<td>3</td>
<td>5603.34</td>
<td>1869.447</td>
<td>90.913 **</td>
</tr>
<tr>
<td>Between samples</td>
<td>3</td>
<td>2323.02</td>
<td>775.007</td>
<td>37.689 **</td>
</tr>
<tr>
<td>Error</td>
<td>9</td>
<td>185.07</td>
<td>20.563</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>8118.43</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Denotes significance at 1% level.

The analysis of variance (table 5.8) shows that the variation between different treatments is highly significant. The variation between rumen samples is also highly significant which is quite natural as the samples were obtained from different goats of variable age and without any control on feeding and also the unknown varying periods of fasting before slaughter. The small number of samples used in this work is also a factor in causing high significant variation between rumen samples.

Since variations between treatments were found highly significant
on the basis of analysis of variance, critical difference test was applied to know the significant difference between the averages of any two treatments both at 1% and 5% level as shown in table 5.9.

**TABLE 5.9**

Comparison of VFA produced from different treatments on 8 hours incubation with high level of added tannic acid (2.5 mg./ml. of incubation media).

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatments</th>
<th>Difference between averages</th>
<th>Critical Difference 1% level</th>
<th>Critical Difference 5% level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SRL Vs. SRL + Ten times tannic acid (62.5 mg.)</td>
<td>0.4 N.S.</td>
<td>10.419</td>
<td>7.252</td>
</tr>
<tr>
<td>2.</td>
<td>SRL Vs. Unextracted Pipal leaves</td>
<td>37.7 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>SRL + Ten times tannic acid (62.5 mg.) Vs. Unext. Pipal leaves + Ten times tannic acid (62.5 mg.)</td>
<td>37.1 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Unextracted Pipal leaves Vs. Unext. Pipal leaves + Ten times tannic acid (62.5 mg.)</td>
<td>1.0 N.S.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Denotes significance at 1% level.**

'N.S.' Denotes non-significance.

From the critical difference test as shown in table 5.9, it is evident that there is highly significant difference between any two treatments except Rumen liquor Vs. Rumen liquor + Ten times added tannic acid (62.5 mg. or 2.5 mg./ml. of incubation media) and Unextracted Pipal leaves Vs. Unextracted Pipal leaves + Ten times added tannic acid (62.5 mg. or 2.5 mg./ml. of incubation media).
media) on 8 hours incubation.

Since Rumen liquor and Rumen liquor + Ten times added tannic acid does not differ significantly, it is safe to conclude that there is no significant effect of added tannic acid even at a level of 62.5 mg. or 2.5 mg./ml. of incubation media on VFA production from Rumen liquor on 8 hours incubation.

Similarly, since Unextracted Pipal leaves and Unextracted Pipal leaves + Ten times added tannic acid does not differ significantly it has been concluded that there is no significant effect of added tannic acid even at a level of 62.5 mg. (2.5 mg./ml. of incubation media) on VFA production from Unextracted Pipal leaves on 8 hours incubation. The probable reason for this has been discussed under the same heading in 'Discussion'.

IV. EFFECT OF NATURAL TANNIN AND ADDED TANNIC ACID ON 'IN VITRO' PRODUCTION OF VFA FROM SAL SEEDS.

The rumen samples were obtained from slaughtered goats as described under the chapter on 'Materials and Methods'. The second method of incubation mentioned therein was followed, since the addition of mineral mixture of Burroughs et al. (1950 a) was thought to be essential due to the low concentration of minerals in the Sal seeds (2.36%). The Sal seeds tried were Unextracted and Extracted and finely powdered. The plan of these incubation experiments have been described in detail under the same heading under the chapter on 'Materials and Methods'. The total VFA production on 8 hours incubation from each source and their rate of production was determined. The results are shown in table 5.10.

Table 5.10 shows that Rumen liquor, Rumen liquor + 66.5 mg. (2.66 mg./ml. of incubation media) added tannic acid, Rumen liquor + 665.0 mg. (26.6 mg./ml. of incubation media) added tannic acid and Extracted Sal seeds + 665.0 mg. (26.6 mg./ml. of incubation media) added tannic acid on 8 hours incubation produced almost the same amount of fresh VFA - 22.8, 22.9, 22.6 and 22.8 m. moles per liter of rumen liquor respectively.
<table>
<thead>
<tr>
<th>Range</th>
<th>Average + S.E.*</th>
<th>Range</th>
<th>Average + S.E.*</th>
<th>Average</th>
<th>IV obsa</th>
<th>I II III</th>
<th>Hr per hour</th>
<th>Total VFA produced</th>
<th>No. of rumen samples</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table showing VFA production from 50 seeds on 8 hours incubation in moles of VFA / liter of rumen liquor

TABLE 5.10
It is also evident from table 5.10 that Unextracted Sal seeds, Extracted Sal seeds and Extracted Sal seeds + 66.5 mg. (2.66 mg./ml. of incubation media) added tannic acid on 8 hours incubation produced almost the same amount of fresh VFA = 56.6, 57.0 and 56.5 m moles per liter of rumen liquor respectively. The results are also represented in the form of a 'Histogram' in fig. 5.

The above results indicate that added tannic acid even at a level as high as 26.6 mg./ml. of incubation media (Ten times more than it is present in Unextracted Sal seeds) did not appear to affect fresh VFA production from rumen liquor alone on 8 hours incubation.

It is also evident from the above results that added tannic acid and also the natural tannin at a level of 2.66 mg./ml. of incubation media (the level present in Unextracted Sal seeds) did not have any effect on fresh VFA production on 8 hours incubation from Rumen liquor and also from Unextracted and Extracted Sal seeds.

On the other hand, added tannic acid at a high level of 26.6 mg./ml. of incubation media completely inhibited fresh VFA production from Extracted Sal seeds on 8 hours incubation. The probable reasons for this have been discussed under the same heading in 'Discussion'.

RATE OF VFA PRODUCTION:

From table 5.10 it is evident that Rumen liquor, Rumen liquor + 66.5 mg. added tannic acid, Rumen liquor + 665.0 mg. added tannic acid and Extracted Sal seeds + 665.0 mg. added tannic acid produced fresh VFA almost at the same rate of 2.9, 2.9, 2.8 and 2.9 m moles per hour per liter of rumen liquor respectively on 8 hours incubation.

On the other hand, Unextracted and Extracted Sal seeds and Extracted Sal seeds + 66.5 mg. added tannic acid produced fresh VFA at the same rate of 7.1 m moles per hour per liter of rumen liquor on 8 hours incubation.

From the above results it appears that added tannic acid even at
Fig. 5 - Photograph of histogram.

Histogram showing total VFA produced from different treatments on 8 hours incubation of Sal seeds 'in vitro'.

- Rumen liquor
- RL+Tannic Acid, 56.5 mg (2.66 mg/ml of incubation media)
- RL+Tannic Acid (ten times more), 565.0 mg (26.6 mg/ml of incubation media)
- RL+ Ext. Sal seeds + Tannic Acid (ten times more), 565.0 mg (26.6 mg/ml of incubation media)

M. Moles VFA/Liter Rumen Liquor

8 hours incubation.
is highly significant. The variation between rumen samples is also significant at 5% level which is natural as the samples were obtained from different goats of variable age and without any control on feeding and also the unknown varying periods of fasting before slaughter. Another reason is the smaller number of samples used in this experiment.

Since variation between different treatments were found highly significant on the basis of analysis of variance, critical difference test was adopted to know the significant difference between any two treatments at 1% and 5% levels as shown in table 5.12.

From the critical difference test as shown in table 5.12 it is apparent that the differences between Rumen liquor Vs. Rumen liquor + 66.5 mg. added tannic acid, Rumen liquor Vs. Rumen liquor + 665.0 mg. added tannic acid did not differ significantly. Hence, it has been concluded that there is no significant inhibitory effect of added tannic acid even at a level as high as 26.6 mg./ml. of incubation media on fresh VFA production from Rumen liquor on 8 hours incubation.

Since the difference between Unextracted Sal seeds Vs. Extracted Sal seeds and Unextracted Sal seeds Vs. Extracted Sal seeds + 66.5 mg. added tannic acid did not differ significantly, it can be safely concluded that there is no significant inhibitory effect of either natural tannin or added tannic acid at a level of 26.6 mg./ml. of incubation media on fresh VFA production from Unextracted and Extracted Sal seeds on 8 hours incubation.

However, the difference between Extracted Sal seeds Vs. Extracted Sal seeds + 665.0 mg. added tannic acid differs highly significantly. So, it is safe to conclude that added tannic acid at a high level of 26.6 mg./ml. of incubation media completely inhibited fresh VFA production from Extracted Sal seeds. The probable reasons for this have been discussed under the same heading in 'Discussion'.
a level as high as 26.6 mg./ml. of incubation media did not have any depressing effect on the rate of fresh VFA production from Rumen liquor alone whereas the same level of added tannic acid completely inhibited fresh VFA production from the Extracted Sal seeds. The probable reasons for this have been discussed under the same heading in 'Discussion'.

It is also evident from the above results that either natural tannin or added tannic acid at a level of 2.66 mg./ml. of incubation media did not seem to have any depressing effect on the rate of fresh VFA production from Unextracted and Extracted Sal seeds on 8 hours incubation.

To know the difference between the extent of fresh VFA production from different treatments and also from the different samples of rumen liquor, the data in table 5.10 was subjected to 'F' test on the basis of analysis of variance as shown in table 5.11.

**TABLE 5.11**

Table showing analysis of variance of total VFA produced on 8 hours incubation under various treatments.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>d.f.</th>
<th>S.S.</th>
<th>M.S.</th>
<th>F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between treatments</td>
<td>6</td>
<td>7883.79</td>
<td>1313.965</td>
<td>8.807 **</td>
</tr>
<tr>
<td>Between samples</td>
<td>3</td>
<td>1775.97</td>
<td>591.990</td>
<td>3.968 *</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>2685.60</td>
<td>149.200</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>12345.36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Denotes significance at 1% level.
* Denotes significance at 5% level.

Table 5.11 shows that the variation between different treatments...
TABLE 5.12

Comparison of VFA produced from different treatments on 8 hours incubation with added tannic acid at different levels.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatments</th>
<th>Differences between averages</th>
<th>Critical difference 1% level</th>
<th>Critical difference 5% level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SRL vs. SRL + Tannic acid (66.5 mg.)</td>
<td>0.1 N.S.</td>
<td>24.857</td>
<td>18.146</td>
</tr>
<tr>
<td>2.</td>
<td>SRL vs. SRL + Ten times Tannic acid (665.0 mg.)</td>
<td>0.2 N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>SRL vs. Extracted Sal seeds</td>
<td></td>
<td></td>
<td>34.2 **</td>
</tr>
<tr>
<td>4.</td>
<td>SRL + Tannic acid (66.5 mg.) vs. SRL + Ten times Tannic acid (665.0 mg.)</td>
<td>0.3 N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>SRL + Tannic acid (66.5 mg.) vs. Unextracted Sal seeds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>SRL + Tannic acid (66.5 mg.) vs. Ext. Sal seeds + Tannic acid (66.5 mg.)</td>
<td>33.6 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>SRL + Ten times Tannic acid (665.0 mg.) vs. Ext. Sal seeds + Ten times tannic acid (665.0 mg.)</td>
<td>0.2 N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Unextracted Sal seeds vs. Extracted Sal seeds</td>
<td></td>
<td></td>
<td>0.4 N.S.</td>
</tr>
<tr>
<td>9.</td>
<td>Unex. Sal seeds vs. Ext. Sal seeds + Tannic acid (66.5 mg.)</td>
<td>0.1 N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Extracted Sal seeds vs. Ext. Sal seeds + Tannic acid (66.5 mg.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Ext. Sal seeds vs. Ext. Sal seeds + Ten times Tannic acid (665.0 mg.)</td>
<td>34.2 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Ext. Sal seeds + Tannic acid (66.5 mg.) vs. Ext. Sal seeds + Ten times Tannic acid (665.0 mg.)</td>
<td>33.7 **</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** Denotes significance at 1% level. 'N. S.' Denotes Non-significance.


**DISCUSSION**

The various important aspects of the results obtained in the present study have been discussed here under the following headings:

I. *In vitro* incubation technique,

II. Estimation of tannins in feeds,

III. Effect of natural tannin and added tannic acid on *in vitro* production of VFA from Pipal leaves,

IV. Effect of high level of added tannic acid on *in vitro* production of VFA from Pipal leaves,

V. Effect of natural and added tannic acid on *in vitro* production of VFA from Sal seeds.

I. *IN VITRO* INCUBATION TECHNIQUE:

*In vitro* studies are usually adopted due to convenience, economy and simplicity with easy control over wide range of variations. It is one of the most promising laboratory methods for studying metabolism in the rumen and for estimating the forage nutritive value. Good correlations have been shown between *in vivo* digestibility and *in vitro* estimates of forage cellulose digestibility (Hershberger *et al.*, 1959; Quicke *et al.*, 1959; Donefer *et al.*, 1960; Baumgardt *et al.*, 1962).

The basic components for all the *in vitro* systems are essentially the same, but various Laboratories make specific modifications for the adaptation of the procedure to their conditions. El-Shazly *et al.* (1960) compared the all-glass, semipermeable membrane and continuous flow types of apparatus for *in vitro* rumen fermentations and found no major differences between types of apparatus. They concluded that the all-glass system appeared advantageous because of its simplicity.
Since digestion in the rumen entirely depends upon the presence of microbes in the rumen, the results obtained from artificial rumen can only be comparable to the actual 'in vivo' phenomenon only when the apparatus is capable of maintaining true rumen microbes during the whole incubation period, simulating as far as possible the natural conditions prevailing in the intact rumen. Artificial rumen provides greater facility and control for extensive study under a wide variety of experimental conditions than does 'in vivo' method. But it is imperative that environmental conditions set up in an artificial rumen should be identical as far as practicable to that in the intact rumen. The limitation is primarily the real problem of transferring results obtained in the laboratory to the intact animal. The results obtained from the 'artificial rumen' do not necessarily duplicate similar functions in the ruminants. However, the 'artificial rumen technique' was used in this work since it permitted a quantitative approach to the study of the Volatile fatty acids without the usual 'in vivo' difficulties of absorption or passage of these metabolites from the rumen.

In the present investigation two 'in vitro' methods were adopted (Bentley et al., 1954; Pant et al., 1962) to carry on incubations for 8 hours to estimate the effect of natural tannin and added tannic acid on total VFA production from Pipal leaves and Sal seeds using goat's rumen inoculum. The first method adopted has already been described in detail under the same heading under the chapter on 'Materials and Methods'. There is nothing specific to be discussed. In the second method mineral mixture of Burroughs et al. (1950 a) was used keeping in view the low mineral content in Sal seeds (2.36%). Burroughs et al. (1950 a,b,c & 1951 a,b) have made extensive investigations using artificial rumen technique on the mineral requirements of rumen microorganisms and found that cellulose digestion could be stimulated by addition of small quantities of salts to the artificial rumen.
II. **ESTIMATION OF TANNINS IN FEEDS**

There are various methods of estimating tannins in different substances. But in the present work, the standard method of estimation of tannins in tea leaves (A.O.A.C., 1960) has been adopted as it was thought to be more suitable, accurate and reliable for tannin estimation in leaves and seeds, for in an efficiency test 99.7% recovery was obtained with a small standard error of + 0.075 indicating the reproducibility of the full process. Further more, variance in reports of different investigators may result from the method by which tannin was determined. According to Wilkins *et al.* (1955) the formaldehyde precipitate method of determining tannin gave values approximately 25% higher than the official methods. Herman *et al.* (1953) employed the hide powder method of the American Leather Chemists Association. The different methods used in the analysis may explain the marked differences in the values reported. Smith *et al.* (1955) compared the Lowenthal and Pro-methods (methods depending on permanganate titration and colorimetric phosphomolybdic - tungstate reduction) and concluded that both are highly empirical. Panda *et al.* (1966) estimated 3.5% tannin in Sal seed meal by official method of analysis (A.O.A.C., 1960). But they did not specify which of the several A.O.A.C. methods was adopted by them for tannin estimation. They did not mention anything about the physical nature of the Sal seeds used by them. They soaked the material for 24 hours in cold water (1 : 4) and used the filtrate for tannin estimation. However, they standardised their process with that in which boiling of the material for 30 minutes was required, and found to be satisfactory. The lower value obtained by them may be due to difference in the method of estimation adopted by them and due to the differences in variety, physical state and stage of maturity of the Sal seeds used by them. They also estimated 1.4% tannin in the processed Sal seed meal. In the present work tannin was found to be completely extracted from Pipal leaves probably due to its very low percentage (1.25%) or perhaps due to its unknown free state of presence in
the Pipal leaves. But in case of Sal seeds, about 2.3% tannin was left behind in the Extracted sample probably due to its presence in higher concentration (13.3%) or perhaps, due to formation of pasty mass of the Extracted material from which Extracted tannin may not have been washed out completely even after repeated washings with water.

III. EFFECT OF NATURAL TANNIN AND ADDED TANNIC ACID ON 'IN VITRO' PRODUCTION OF VFA FROM PIPAL LEAVES:

It is apparent from the results obtained in the table 5.4 that either the natural tannin present in Pipal leaves (1.25%) or the added tannic acid at the same level (1.25% or 0.25 mg./ml. of incubation media) had no significant effect on VFA production from Pipal leaves on 8 hours incubation in an artificial rumen.

On the other hand, the VFA production from the Extracted Pipal leaves was significantly (Above 30%) lower than from Unextracted Pipal leaves. This fact may perhaps be explained on the basis of slightly lower content of more soluble portion of N.F.E. and total ash in the Extracted sample caused by the removal of the more soluble portion of carbohydrates and minerals during the process of tannin extraction or by unknown alteration in the nature of both protein and N.F.E. during this processing (Table 5.1).

The lack of vitamins, specially the heat-labile vitamins which might have been destroyed during the process of tannin extraction may also be a causative factor in the lower VFA production from the Extracted Pipal leaves. That an isolated culture of rumen microorganisms has complex vitamin and other growth factor requirements was indicated in a report by Wasserman et al. (1953). Bentley et al. (1954) demonstrated the importance of certain B-vitamins, for rumen microorganisms activity and provide additional evidence for the existence of undetermined nutritional factors which are required by rumen microorganisms growth 'in vitro'.
Added tannic acid at a level of 0.25 mg./ml. of incubation media did not also inhibit fresh VFA production from rumen liquor. The low rate of production from rumen liquor in both the cases is as expected due to its lower substrate concentration. Amongst the four samples of rumen liquor the rate of VFA production also varied considerably (0.78 to 4.28), perhaps due to the combined effect of the concentration of the substrate in them and the number and types of microorganisms initially presented in them (Quin, 1943; Quin et al., 1951). It has been established that the variation in the number or the proportion of various species of the microorganisms is due to the variation in the amount, composition and nutritive value of the diet (Gall et al., 1949; Moore, 1951; Pounden and Hibbs, 1948; Masson, 1950; Blaxter, 1954).

Another interesting fact apparent from table 5.4 is that the rate of production of fresh VFA both from Unextracted and Extracted Pipal leaves is generally proportional to the rate obtained from incubation of rumen liquor alone. Thus while a total of 51.6 and 29.6 m. moles of VFA was produced from Unextracted and Extracted Pipal leaves respectively from a sample of rumen liquor showing the lowest VFA production of 6.2 m. moles, the same figures from the sample of rumen liquor producing the highest amount (34.2 m. moles) were as high as 78.8 and 69.8 m. moles respectively.

Apparently this relation is due to the number and nature of the microorganisms initially present in the different samples of rumen liquor (Carroll and Hungate, 1954). The variation in the microbial population of the rumen has been observed either by direct count and isolation of different species of rumen microorganisms in pure culture (Bryant & Burke, 1953; Hamlin & Hungate, 1956; Hungate, 1957); or indirectly by (i) estimating in the rumen liquor and/or venous blood coming out of the rumen the end-products of microbial fermentation at different intervals following feed (Gray & Pilgrim, 1951; Mc Donald, 1952; Kiddle et al., 1951) and/or (ii) ‘in vitro’ techniques (Carroll & Hungate, 1954).
Pant et al. (1962) observed in sheep, goat and buffalo calf that starvation for 24 hours was found to decrease the cellulolytic activity of rumen liquor as well as the concentration of total VFA and ammonia.

IV. EFFECT OF HIGH LEVEL OF ADDED TANNIC ACID ON 'IN VITRO' PRODUCTION ON VFA FROM PIPAL LEAVES:

Since the low level of 1.25% or 0.25 mg./ml. of natural tannin and also the added tannic acid did not affect the rate of fresh VFA production from Pipal leaves in the previous incubations, it was thought advisable to see the effect of addition of higher levels of tannic acid. For this purpose the effect of addition of tannic acid at a level of 2.5 mg./ml. (Ten times higher) was studied and the results are tabulated in Table 5.7.

It is evident from the results shown in Table 5.7 that added tannic acid even at a higher level of 2.5 mg./ml. of incubation media did not affect significantly fresh VFA production either from rumen liquor or Unextracted Pipal leaves on 8 hours incubation. However, the detrimental effects of natural tannins and added tannic acid on protein degradation and cellulolysis in the rumen have been reported by various workers (Becker et al., 1955; Leroy et al., 1964; Tagari et al., 1965; Murty & Khan, 1969; Delort & Zelter, 1969; Zelter et al., 1970).

A relation between the VFA produced from rumen liquor and from Unextracted Pipal leaves (with and without added tannic acid), similar to that obtained in the previous incubation was also observed in these cases.

V. EFFECT OF NATURAL TANNIN AND ADDED TANNIC ACID ON 'IN VITRO' PRODUCTION OF VFA FROM SAL SEEDS:

From the results obtained in Table 5.10 it is apparent that either natural tannin or added tannic acid at a level of its presence in Sal seeds (13.3% or 2.66 mg./ml. of incubation media) did not affect significantly
the fresh VFA production either from rumen liquor alone or Extracted Sal seeds on 8 hours incubation.

It is also evident that added tannic acid even at a level as high as 26.6 mg./ml. (Ten times higher than it is present in Sal seeds) had no significant effect on fresh VFA production from rumen liquor alone on 8 hours incubation.

However, added tannic acid at a level of 26.6 mg./ml. almost completely inhibited fresh VFA production from Extracted Sal seeds on 8 hours incubation. This total inhibition of fresh VFA production from Extracted Sal seeds may perhaps be due to the precipitation of all the proteins in the form of insoluble tannates and also formation of a protective layer around the starch granules. This inhibition may also be possibly due to specific inhibition of the activity of saprophytic cellulolytic bacteria specially the 'cell vibrio' (Henis et al., 1964). Tannins of various origin have different effects on the decomposition of organic matter (Basaraba, 1960).

On comparison of the results obtained in table 5.4, 5.7 and 5.10 we find that the fresh VFA production from Extracted Sal seeds was almost completely arrested by the addition of gallo-tannic acid at a concentration of 26.6 mg./ml. on 8 hours incubation whereas gallo-tannic acid or the natural tannins of either Pipal leaves or Sal seeds at a concentration of 0.25 mg./ml. and 2.66 mg./ml. did not inhibit fresh VFA production from Pipal leaves and Sal seeds respectively on 8 hours incubation. The addition of tannic acid at the above concentrations did not affect fresh VFA production from rumen liquor alone on 8 hours incubation. Mention may be made here of a similar type of work by Tagari et al. (1965) who concluded on the basis of their 48 hours incubation results that cellulose decomposition was almost completely arrested by the addition of carob extract to a final tannin concentration of 1.5 mg./ml.

However, whereas the addition of gallo-tannic acid resulted in a similar effect,
the tannin fraction of the carob extract alone did not inhibit cellulolysis significantly. They compared the effect of carob extract on cellulolysis with that of its sugar fraction and tannin and found that all the three fractions have a similar effect on cellulolysis, with a 12 mg./ml. concentration being almost completely inhibitory.

In the present work, it was observed that the total VFA production from Pipal leaves and Sal seeds on 8 hours incubation was not inhibited by either natural tannins or gallotannic acid at a concentration of their presence in Pipal leaves (1.25% or 0.25 mg./ml.) and Sal seeds (13.3% or 2.66 mg./ml.).

However, gallotannic acid at a concentration of 2.5 mg./ml. did not inhibit total VFA production from Pipal leaves on 8 hours incubation. On the other hand, gallotannic acid at the high concentration of 26.6 mg./ml. completely inhibited fresh VFA production from Extracted Sal seeds. Although this inhibitory effect was totally absent with rumen liquor alone, indicating that the high concentration of tannic acid did not affect the microorganisms but perhaps prevented the substrate from Sal seeds being acted upon by the rumen microorganisms.

Volcani et al. (1963) concluded that carob pod meal and its various extracts inhibited cellulolysis and deamination 'in vitro' and it has also been observed 'in vivo' after prolonged feeding of carob meal by Y. Rabi (cited by Tagari et al., 1965).

Mc Ginty (1969) conducted 'in vitro' and 'in vivo' experiments in cattle with sorghum grains and concluded that tannic acid had inhibitory effect on fermentation process and also precipitated proteins significantly.

Zelter et al. (1970) reported on the basis of their 'in vitro' studies that the minimum dose of each aldehyde (Tannin containing substance) which would completely protect the protein degradation, decreased cellulolytic activity of an inoculum of rumen contents on a wheat straw substrate by
13 to 20%, while doses which gave 90% protection reduced cellulolytic activity by 3%.

There is no reference available on 'in vitro' experiments with substances chosen in the present work. However, indirect evidence on the effects of tannins of feeds on carbohydrate utilization have been reported by many workers.

Bondi and Meyer (1944) reported about low digestibility and nutritive value of high tannin containing carobs for ruminants. On the other hand, Herman et al. (1953) observed no significant effect of natural tannins present up to about 12% and of added tannic acid up to 5% level respectively on the growth, food consumption and milk yield in lactating cows and also there was no digestive disturbances. They thought that tannic acid was apparently converted to tannates in the mouth and part of digestive tract and these are further broken down to non-astringent gallic and pyrogallic acids.

Bissel and Weir (1957) found poor digestibility for crude protein and crude fiber both in sheep and deer by feeding oak and alfalfa hay. Hawkins and Autrey (1957) observed by feeding experiments in lactating cows that tannin containing sericea lespedeza contained a milk depressing substance.

Bejovic et al. (1957) reported poisoning of cattle by tannic acid after exclusive feeding in early spring on buds and newly opened oak leaves. The natural tannins and tannin containing substances have long been known to inhibit decomposition process of organic matter (Siu, 1951; Oslage & Becker, 1958; Basaraba, 1960).

Oslage & Becker (1958) also reported that tannins of various origin have different effect on cellulolysis and proteolysis.

From a comparison of tables 5.4 and 5.10, it appears that the fresh VFA produced from Extracted Pipal leaves is significantly lower (Above 30%) than the Unextracted Pipal leaves whereas the fresh VFA produced from the Extracted and Unextracted Sal seeds is almost equal. The probable reasons for
this difference in case of Pipal leaves and Sal seeds may be that in case of Pipal leaves, the more soluble portion of N.F.E. and total ash quite possibly had been washed away during the process of tannin extraction by boiling and washing and mostly cellulose and crude fiber had remained in the Extracted sample, which are known to be not so easily acted upon by the rumen microorganisms. On the other hand, in case of Sal seeds which is known to be rich in starch, most probably a larger amount of the soluble portion of N.F.E. had been left behind in the Extracted sample even after the processing in comparison to Pipal leaves. Although the percentage reduction of N.F.E. in the Extracted sample is almost the same (About 7-8%, c.f. table 5.1) both in Pipal leaves and Sal seeds. Besides this, the addition of mineral mixture in the case of Sal seeds compensates for the lower mineral content in it whereas in case of Pipal leaves no mineral mixture was added as it was rich in mineral content (12%, c.f. table 5.1).

Mention may be made here about the reproducibility of the different incubation experiments. It is evident from the results obtained in table 5.10 that the variation in fresh VFA produced from different rumen liquor samples ranged from 14.2 to 29.0 m. moles per liter of rumen liquor on 8 hours incubation. Similarity in the trend of the results of all the four incubations with different substrates give much confidence in the results obtained, though in biological experiments like this, wide quantitative variations may occur due to variation in the type of microflora in different samples of rumen liquor. The regular high agreement between the duplicate incubation flasks gave further confidence in the findings.
CONCLUSION

On the basis of the results obtained during the investigation of the two feeds under study, it can be concluded that natural tannins present in these feeds and added gallocatechin acid at the same levels have no inhibitory effect on fresh VFA production from Pipal leaves and Sal seeds by the rumen microorganisms in an artificial rumen on 8 hours incubation. However, at artificially higher levels of addition of gallocatechin acid (26.6 mg./ml.), the fresh VFA production from Extracted Sal seeds by the rumen microbes was completely inhibited on 8 hours of incubation 'in vitro'. This effect was absent in case of rumen liquor alone.

From the results obtained it is clear that no advantage can be gained by extraction of tannin from these two feeds as far as their VFA production is concerned and presumably therefore, on their actual feeding values as far as their carbohydrate content is concerned.
CHAPTER VI

SUMMARY

The "artificial green technique" was adopted in this study for the production of filamentous and gel seeds with and without added llama milk. The methods of inoculation were followed using a slightly buffered and mannitol (200 g/L) solution, with different amounts of local WV24 and WV24M, respectively.

The production of filamentous and gel seeds with and without added llama milk was observed at two different stages of inoculation, using WV24 and WV24M. Viable WV24M were produced from extracted and inoculated WV24M at concentrations of 4.2% by weight, respectively, on the same period of time.

The growth of the seedlings at 3 hours in vitro with and without added llama milk was observed. WV24M extended a clear area, while WV24M extended a clear area at a concentration of 4.2% by weight. WV24M extended a clear area at a concentration of 3.5% by weight.

Tannin acid added at the highest level of its presence caused the reduction of the WV24M production from filamentous while it reduced significantly the WV24M production from gel seeds.

The content of WV24M production from the different substrates for each stage was similar between the different inoculations, indicating a low degree of reliability of the results obtained.

The tannin contents were found to be 1.35% and 1.57% respectively.

Conclusions:

- Filamentous and gel seeds were successfully produced from filamentous and gel seeds.
- Tannin acid addition was effective in reducing WV24M production.
- The reliability of the results was low due to the similarity between the inoculations.
- Further studies are needed to improve the reliability of the results.
SUMMARY

The 'artificial rumen technique' was adopted in this study for fermentation of Pipal leaves and Sal seeds with and without added tannic acid. Two methods of incubation were followed using M-Phosphate buffer and Mineral Mixutre of Burroughs et al. (1950 a) respectively.

On fermentation of Pipal leaves with and without added tannic acid 'in vitro', higher amount of total VFA was reproduced from the Unextracted Pipal leaves. The Unextracted leaves produced an average of 73.9 m. moles of fresh VFA/liter of rumen liquor on 8 hours of incubation whereas 52.4 and 50.5 m. moles of fresh VFA were produced from Extracted and Extracted + added tannic acid at a concentration of 0.25 mg./ml. respectively on the same period of incubation.

The incubation of Sal seeds for 8 hours 'in vitro' with and without added tannic acid produced almost similar amounts of total VFA from Unextracted, Extracted and Extracted + added tannic acid at a concentration of 2.66 mg./ml. - 56.6, 57.0 and 56.5 m. moles/liter of rumen liquor respectively.

Tannic acid added at ten times higher level of its presence in the feeds, did not affect the VFA production from Pipal leaves while it inhibited completely the VFA production from Sal seeds.

The trend of VFA production from all the different substrates used, were very much similar between the different incubations, indicating a high degree of reliability of the results obtained.

The tannin contents were found to be 1.25% and 13.3% respectively in Pipal leaves and Sal seeds. Complete extraction of tannin from Pipal leaves was achieved by extraction with boiling water for 30 minutes, which process also removed about 81% of the tannins of Sal seeds.
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