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IN VITRO EVALUATION OF FORAGE NUTRITIVE VALUE.

THE USE OF AN *IN VITRO* RUMEN FERMENTATION PROCEDURE TO PREDICT THE NUTRITIVE VALUE OF FORAGES

by

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I. INTRODUCTION

The family of animals - Bovidae (ruminants), characterized by their ability to consume large quantities of fibrous feeds, have evolved through many millions of years into the domesticated species which today constitute one of the most important components of animal agriculture. Their specialization as forage consumers is dependent on the capacious nature of a portion of their digestive tract (rumen), and the micro-organisms living in a symbiotic relationship within this organ. It is these microflora and microfauna which are the actual converters of the cellulosic forage components into nutrients which can be absorbed and utilized by the host animal.

It is hypothesized that the ruminants of the Eocene period evolved and survived due to their ability to hurriedly ingest large quantities of grassy materials, rapidly retreat from carnivorous predators, and regurgitate, remasticate and reinsalivate the foodstuffs contained in their rumen at their leisure. Although these traits no longer have any survival value to the domesticated ruminants of today, these same characteristics have in part enabled these species to play a dominant role in the production of human food. Modern-day commercialization of animal husbandry has resulted in an intensive effort to increase the efficiency of meat, milk, and wool production, an important manifestation of which is the feeding of rations which most economically can result in high levels of production.

Although ruminants may not be as efficient as other classes of livestock when efficiency is defined in terms of units of feed needed to produce a given unit of product, they compare more favorably when the rations used to feed the various classes of livestock are examined

qualitatively. For while species such as swine and poultry require high energy concentrate feeds to maintain levels of efficient production, ruminants have the ability to utilize relatively low energy and low cost fibrous feeds. In addition, because of the high human population densities in relation to available food supplies in many parts of the world, people in these areas cannot always afford the luxury of raising livestock on concentrate feeds more efficiently used directly as human food sources. On the other hand, forage crops fed to ruminants are for the most part not utilizable by humans, and are generally grown on land which cannot be adapted to more intensive crop production. Even in those more privileged areas where agriculturists can justifiably use concentrate feeds in animal rations, correct agronomic practice dictates the exclusive use of forage crops on certain types of land as a conservation measure and the inclusion of forage in crop rotation systems as an aid in maintaining soil fertility.

The recognition that forages may vary widely in quality necessitates the study of the cause and effect of these differences on the efficient use of forage crops in all phases of animal production. Insight into the complexity of studying qualitative aspects of forage utilization can be realized when we examine the numerous types of forages (pasture, silage, and hay - among the most important) from grass, legume, or mixed sources, which are fed to the various domesticated ruminants (cattle, sheep, goats, and water buffalo - among the most common) for a variety of purposes (reproduction, lactation, growth, maintenance, and work). In evaluating forage crops for their specific roles in animal nutrition, accurate and concise criteria of nutritive quality which can be determined with speed and precision must be chosen.

Since a cardinal prerequisite in measuring forage quality is the ability to apply experimental results directly to problems of animal production, it is customary to use as primary experimental subjects the same types of animals to which the information obtained will be eventually applied. The soundness of this reasoning is affirmed by the multitude of nutritional experiments successfully carried out over the past several decades with growing and fattening cattle and sheep as well as lactating cows. The criterion of forage quality in these tests usually becomes some measurement of actual production in reference to the diet consumed.

Another type of nutritional study also makes use of farm animals as experimental subjects, although in this case they are usually in a non-productive (maintenance) state. This type of study is illustrated by the determination of coefficients of digestibility of various diet fractions. These experiments usually have the advantage of more intensive study with a limited number of animals, but their success is dependent on the establishment of a definite relationship between the criteria being determined and some aspect of actual animal productivity.

Although feeding trials constitute an essential tool in the study of animal nutrition, their use in the evaluation of feedstuffs has definite limitations. The principles of experimental and statistical design dictate that adequate numbers of animals must be used with appropriate control treatments included and that replication of results is necessary. Therefore a properly designed experiment testing the quality of a forage material with cattle, for example, might cover a period of several months with several tons of feed material required. Although less demanding, forage evaluation tests with sheep (as conducted at Macdonald College) are approximately 3-4 months in length and require

a minimum of 500 pounds of each of the forages to be tested. These situations serve to indicate the need for accurate methods of forage evaluation which can be rapidly performed utilizing small samples of the test materials.

Many "laboratory type" systems for evaluating forages have been suggested including the chemical and physical analysis of various plant components, the use of "laboratory size" animals such as rabbits, and the use of cellulolytic microorganisms to determine in vitro cellulose digestion. For any of these systems to be useful assays of forage quality, a high correlation must be established between the observed data and certain criteria of forage quality, previously defined in animal trials. Of equal importance, the criteria of forage quality which are chosen must be adequate in completely describing nutritive value, since a "laboratory method" highly correlated to some inadequate nutritive measure is thus limited in practical application.

The purpose of the research to be described in this thesis was to develop an in vitro rumen fermentation system which, with a high degree of accuracy and reproducibility, could be used as a tool in forage evaluation. A successful in vitro method would have the ability to predict forage nutritive value, based on actual samples obtained from in vivo trials. The measure or index of forage nutritive value to which the in vitro data were correlated was based on sheep forage experiments conducted by the Department of Nutrition at Macdonald College over a period of several years.

A corollary of the above study, also described in this thesis, was the examination of various factors affecting the accuracy and reproducibility of the proposed in vitro system in the prediction of forage quality.

II. REVIEW OF LITERATURE

A. NUTRITIVE VALUE OF FORAGES FOR RUMINANTS.

1. Function of rumen.

a) Early views.

Although records have dated the existence of domesticated cattle to the ancient civilizations of Mesopotamia, Egypt, and India (approximately 5000 B.C.), it is only within the past hundred years that knowledge as to the exact mechanism of ruminant digestion has been elucidated. Until the middle of the nineteenth century the popular belief concerning the rumen, the largest compartment of the ruminant stomach, was that it functioned as a storage organ. It was thought that this structure enabled these animals to consume large quantities of fibrous feeds thus being able to make up in quantity what was lacking in quality of feed. Varlo (1785) describes the "maw" [rumen] as "a place by nature designed for a repository." Comstock (1836) designated the rumen as the "large sac, or store room." The concept was clearly one that ruminants utilized forages only because they had adequate capacity to store this bulky material in their rumen.

The study which perhaps inaugurated the science of ruminology was reported by Haubner in 1855 (cited by Sijpesteijn, 1948), in which by analyzing the hay fed to an ox as well as the resultant feces for crude fiber, he found that 60% of this constituent had been digested. Armsby (1896) comments on this discovery as follows: "Cellulose was long thought to be indigestible. Haubner was the first to show ... this ... was erroneous, and that ruminants were capable of digesting large quantities of this substance." Haubner's observation was quickly confirmed by other workers, laying the groundwork for future revelations concerning ruminant digestion.

b) Role of microorganisms.

Sijpesteijn (1948) has reviewed reports published in the latter half of the nineteenth century in which the site of cellulose digestion was identified as the rumen, and rumen liquid shown to possess cellulolytic activity. The agent responsible for this activity was a matter of controversy among many of the workers of this period, with Tappeiner suggesting in 1884 that microorganisms present in the rumen were responsible for cellulose degradation. This latter point was firmly established by several workers in the succeeding decades resulting in extensive studies of rumen microorganisms. However much information still remains to be elucidated on this subject.

Bryant (1959), in a recent review of bacterial species of the rumen, summarizes the rumen processes which have been shown to be due to microbial activity as follows: "Degradation of carbohydrates such as cellulose that cannot be utilized unless digested by microorganisms and those such as starch and certain sugars that can be utilized by the animal without microbial action. Proteins, organic acids, and many other feed constituents are also attacked."

The situation in regard to the large number of protozoa present in the rumen is not as clear, in that their possible significance to the nutrition of the host has not been fully substantiated. Oxford (1955) has reviewed the literature concerning the rumen ciliate protozoa.

c) Nutritive value of cellulose.

Armsby (1896) stated that the digested portion of crude fiber had been shown "to consist of cellulose only, which has exactly the composition of starch ... and therefore is assumed to have the same nutritive value as the latter." Kellner (1913) substantiated this

hypothesis in studies comparing the ability of crude fiber ("straw pulp") to store body fat when fed to oxen in comparison to digestible starch, finding "it [crude fiber] has about the same effect as pure starch." On the basis of observations by several workers, Armsby (1917) suggested that the products of the "destructive fermentation" of cellulose were "carbon dioxid [sic] and methane and small amounts of hydrogen, which are excreted, and various organic acids of the aliphatic series which combine with the alkalies of the saliva ... . The salts thus formed are resorbed and constitute the sole contribution which cellulose makes to the nutrition of the body. The principal acids formed appear to be acetic and butyric, although others are present."

The actual significance of the products of cellulose digestion - the volatile fatty acids, to the nutrition of the ruminant was not fully appreciated until experiments reported by British workers in the 1940's as to the actual metabolism of these fatty acids. Phillipson (1947) reviewing the literature on this subject, suggested that an important prerequisite to the knowledge of fatty acid metabolism was the development by Elsden of a chromatographic method of separating a mixture of volatile fatty acids. "By this technique he [Elsden] proved that the principal acid present is acetic, and that two higher acids also are formed, namely, propionic and butyric." Phillipson estimated that the lower fatty acids are produced in the rumen in sufficient quantity to supply at least 40 per cent. of the fasting energy requirements.

Of recent interest, Shaw et al. (1960) have shown that when a diet of steamed corn and ground hay was fed to steers there was a marked decrease in the molar proportion of rumen acetate and an equally marked increase in the molar proportion of rumen propionate, as compared

to steers fed an unaltered ration. With the increase in propionate it was noted that the efficiency of feed utilization increased 15.3% and body weight gain increased 22%. They suggested that "both rate and efficiency of body weight gain in beef cattle may be controlled to a remarkable degree by controlling rumen microbial metabolism."

The preceding reports serve to summarize the nutritional contribution of the cellulose component of forages in the diet of ruminants as mediated through the cellulolytic rumen microorganisms. These microorganisms degrade cellulose to form as end-products the volatile fatty acids, which upon absorption through the rumen wall enter into various pathways of intermediate metabolism to serve as a major energy supply for the host.

2. Criteria used to evaluate forages.

a) Available energy.

"It is obvious that in the feeding of the individual animal a primary consideration must be the adequacy of the energy supply. Shortages of dietary energy are usually far more important causes of low productivity in farm livestock than are dietary deficiencies of vitamins, minerals, or amino acids (Blaxter, 1956)."

"Available energy, rather than some specific nutrient, is the fundamental limiting factor in the nutritive value of forage. ... If a forage is consumed in amounts to meet energy needs, it will normally also meet the needs with respect to protein, calcium, and phosphorus (Crampton, 1957)."

"Much more nutriment is required to maintain normal energy metabolism than all other purposes combined. There is a high degree of likelihood that if this need is satisfied that all other essential

requirements will be incidentally covered.(Swift, 1957)."

"The main purpose served by forages in the diet of ruminants is the provision of energy (Reid et al., 1959)."

As indicated by the above statements, there appears to be unanimous agreement that the most important criterion of the nutritive value of a forage is the amount of available energy[1] it supplies to the animal. Acceptance of this premise necessitates that all proposed criteria of forage nutritive value be examined in terms of their relationship either directly or indirectly, to the measurement of available energy.

i. Net energy.

From a theoretical standpoint, the ideal method of measuring the nutritive value of a forage in terms of available energy is by calorimetry techniques in which all "expenses" of feed utilization are subtracted from the gross energy of a feed, leaving that portion of the energy available solely for productive purposes. This measure, termed net energy, was suggested by both Kellner and Armsby at the beginning of this century, and today is the basis of essentially all European methods of assessing nutritive value of feedstuffs.

Practical criticisms of the net energy system have evolved because of the difficulties encountered in its determination. These include the elaborate and expensive equipment required, the lengthy process of accumulating data (Blaxter, 1956), and the many factors affecting its determination unrelated to the nutritive composition of the

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[1] Available energy might be defined as that portion of the gross energy of a feedstuff which is available to the animal for productive purposes.

feed (Swift, 1957). Although it would be technically impossible to use this system to rapidly measure forage crop quality, its maximum usefulness may be in elaborating general principles in regard to comparative feed quality.

ii. Metabolizable energy.

This measure corrects the gross energy of feeds for energy losses in the feces, urine, and methane produced by the animal. The difficulty in its determination involves the measurement of methane production, either directly (requiring elaborate equipment) or indirectly (involving computations based on digestible carbohydrate).

When forages alone are considered, the relationship between metabolizable and the much easier determined digestible energy are relatively constant, as suggested by Swift (1957) who found a correlation coefficient of 0.98 between the two measures. He suggested that this high correlation "lends further support for the use of digestible energy as a simple and meaningful measure of nutritive value."

iii. Digestible energy.

Although arrived at by different methods, the following criteria are all essentially measures of energy value in which the gross energy of a feed is corrected for fecal energy losses: total digestible nutrients (TDN)[1], digestible energy (DE), digestible calories, digestible dry matter, and digestible organic matter.

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[1] TDN differs from the other measures of digestible energy in that in addition to accounting for fecal energy losses, the energy equivalent of protein is also corrected for urinary nitrogen energy losses (Crampton, 1957).

The TDN system evolved and is still used extensively in North America, although questions as to its accuracy in measuring the energy content of feeds, particularly forages, have often been raised. One objection is the observation that TDN values for forages are not "as valuable for productive purposes as the TDN in concentrates (Moore et al., 1953)." This is based on the fact that energy losses through combustible gas formation and heat loss are relatively larger for roughages than for concentrates (Maynard and Loosli, 1956).

Perhaps a more serious objection to the use of TDN in forage evaluation is the observation that "poor quality" forages tend to be overevaluated in comparison to so-called "good quality" forages. Blaxter (1956) compared the TDN system and several European systems based on net energy in evaluating two representative forages, one being an artificially dried grass (19% crude protein) and the other a grass hay with a 6% crude protein content . The TDN values suggested that the former hay was superior to the latter only by a factor of 29%, while the systems based on net energy estimated the artificially dehydrated grass to be 46 to 86% superior, depending on the system used. Morrison (1956) compared the TDN values for average alfalfa hay, average grass hay, and oat straw, and suggested that "no experienced stockman believes that oat straw is really worth over four-fifths as much as good alfalfa hay, or nearly as much as average grass hay, for stock being fed for production."

The fact that the actual determination of TDN is a lengthy and complicated procedure has motivated many workers to suggest similar methods of measuring feed-fecal energy differences. Crampton (1955)

proposed the use of digestible calories, since "the procedure for the energy measurement by calorimetry is straightforward, involves no assumptions as to relative importance of energy-yielding components, is rapid, and can be adapted to routine manipulation by technicians." Swift (1956) likewise suggested that digestible energy is "obviously more direct and accurate and free from empirical procedures and assumptions [than TDN]."

b) Voluntary intake.

Huffman (1959) noted that "palatability is frequently used to denote appetite" but that appetite measured in terms of total feed consumed is influenced by many factors including palatability, environmental temperature, inheritance, and health of the animal. Voluntary intake may be defined as total feed consumption under ad libitum feeding conditions.

Early workers recognized that forage quality, intake level, and production were closely related. Armsby (1896) suggested that "in rapid fattening it is especially important to induce the animal to eat as large a quantity [of forage] as possible." Kellner (1913) defined the best hay as "distinguished ... by its tenderness, aroma, and exceptional palatableness."

It is only within recent years that it has been suggested that voluntary intake may serve as a quantitative measure of forage quality. According to a classification scheme for forages proposed by Crampton (1956), average daily voluntary intake was directly proportional to the available energy content of a forage. He further elaborated (Crampton, 1957), that "the feeding value of a forage depends primarily on the magnitude of its contribution toward the daily energy need of an

animal," and that differences between forages, in this respect, "are almost completely a consequence of the relative amounts in which they are voluntarily consumed." Sheep feeding trials over a period of several years at Macdonald College have confirmed this hypothesis. Lister (1957), and Smith (1958), found significant differences in voluntary intake between 5 species of hay. Smith (1958), and Beacom (1959), found significant correlations between voluntary intake and liveweight gains. Jeffers (1960) demonstrated that voluntary intake of timothy hay decreased with advancing maturity.

Crampton (1957) proposed that "a practical numerical rating of feeding value might be given to a forage by expressing its voluntary daily consumption as a percentage of a 'normal' or 'expected' value of 3.0 lb. (dry weight) per 100 lb. of live weight of animal." Reid et al. (1959) stated that the common denominators of forage quality include intake of forage dry matter and the concentration of energy in forage, with the product of these two factors equal to forage energy intake. Crampton et al. (1960) formalized this relationship into a "Nutritive Value Index" (N.V.I.) for evaluating forage quality. The N.V.I. of a forage was arrived at by multiplying its percent energy digestibility by its "Relative Intake", as determined in in vivo trials. Relative Intake of a forage is an expression of the voluntary intake of a forage computed per unit of metabolic size of the test animal ($Weight_{kg}^{0.75}$) in relation to a standard forage.

B. IN VITRO RUMEN FERMENTATION METHODS.

1. Early in vitro studies.

Because of the many difficulties encountered in studying digestive processes in the intact ruminant, early emphasis was placed on the development of appropriate *in vitro* techniques which could make significant contributions to the science of ruminology. A precedent in the use of *in vitro* procedures had been set in the classical studies of human digestion by Beaumont (1833), who, obtaining gastric juice from the stomach of an accidentally fistulated subject, mixed it with various foodstuffs in small vials which were placed in a sand bath "being kept as near as practicable at the natural temperature, 100° Fahrenheit, with frequent agitation." Beaumont was thus able to determine the time required to digest the foodstuffs *in vitro* and to compare these data with the digestion time as determined by inserting the same foodstuffs directly into the intact stomach through the fistula.

Probably the first application of *in vitro* techniques in the study of ruminant nutrition was made by Tappeiner in 1884 (cited by Sijpesteijn, 1948). In an attempt to determine the site of fiber (cellulose) digestion, he incubated undiluted samples of rumen, small intestine, and a mixture of caecum and colon contents in bottles maintained at body temperatures. After an incubation period of several days he noted that the fiber was digested in the rumen, and in the mixed caecum and colon contents, but not in those of the small intestine. He therefore concluded that the former were the sites of fiber digestion in the intact animal. Tappeiner also established in *in vitro* studies that the microorganisms normally present in the rumen

contents were responsible for the cellulolytic activity observed. He demonstrated this by adding a microbicide (chloroform) to rumen contents which was then found to lose its ability to degrade cellulose.

Many other workers following Tappeiner continued to use various aspects of cellulose utilization by rumen microorganisms, and a series of reports by Woodman and co-workers were outstanding in their contribution to the development of *in vitro* methods. In their earlier studies (Woodman and Stewart, 1928), they cultured a thermophilic cellulolytic bacterium isolated from well-rotted horse manure. Optimum growth of this organism was obtained under aerobic conditions (at a temperature of 65°C), with filter paper providing a purified cellulose substrate. In addition to fermentations conducted in all-glass systems[1], they attempted to demonstrate the end-products of cellulose digestion by placing the cellulose substrate and bacterial inoculum in collodion sacs which were immersed in distilled water. Using this technique they could not positively demonstrate that glucose, their postulated cellulose fermentation end-product, dialyzed into the distilled water, unless toluene (a microbicide) was added to the fermentation mixture after

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[1] An all-glass *in vitro* rumen fermentation system might be defined as one in which the microbial inoculum, nutrient medium, and (cellulose) substrate are combined in a glass container, with no provision generally for the removal of fermentation end-products. This is in contrast to a semipermeable system in which the inoculum and substrate are contained in a closed sac constructed of a material possessing semipermeable properties, which allow fermentation end-products to dialyze out of the fermentation area into a liquid contained in an outer chamber.

active fermentation had started. In a later report, an all-glass *in vitro* system using subcultures of the previously isolated thermophilic organism was used in the study of the digestion of both native and isolated forage crude fiber (Woodman and Stewart, 1932). The long incubation period of 14 days used in this study illustrates the relatively low cellulolytic activity obtained in their system. The final paper in this series (Woodman and Evans, 1938) is perhaps the greatest contribution of this group to improved *in vitro* methodology, as attested to by the fact that many of the techniques described in this study are still used in *in vitro* rumen procedures. In contrast to their use of a thermophilic organism isolated from horse manure in previous studies, they were able to obtain bacterial inoculum from the rumen contents of sheep. This inoculum was added to a simple nutrient medium consisting of several inorganic salts and a "pinch of casein", and using ground filter paper as a purified cellulose substrate, the fermentation mixture was incubated at 37°C. In this study they found no essential difference in the nature of the volatile fatty acids produced whether aerobic or anaerobic conditions were maintained. The shortcomings in their *in vitro* and/or chemical procedures are illustrated by the fact that they could not detect propionic acid, a major fermentation product of normal rumen bacteria, in their fermentation mixtures.

2. Development of modern in vitro systems.

With increasing information becoming available concerning the biochemical, physiological, and microbiological aspects of ruminology, attempts were made to utilize this knowledge in the improvement of *in vitro* techniques. The concept developed that an *in vitro* rumen fermentation system should duplicate as closely as possible the

conditions found within the intact rumen, an "experimental imitation of the milieu" (Marston, 1943), if results obtained with these in vitro systems were to be applied to an in vivo situation.

Pearson and Smith (1943a) found that because of the heterogeneous nature of rumen contents and lack of experimental control in the intact animal, in vivo experiments could not be regarded as "supplying evidence either for or against the theory that urea is converted to protein in the rumen." As a result of this observation, they developed an in vitro technique (Pearson and Smith, 1943b) by which they would be able to study urea utilization by rumen microorganisms. Using an all-glass system with "rumen liquor" inoculum obtained by pressing rumen ingesta through muslin, they studied the conversion of an urea substrate to ammonia during an 8-day incubation period at 39°C. In these experiments they studied the influence of various factors on the urea-splitting power of rumen contents in vitro. These factors included; gas phase, with CO_2 being "slightly more efficient than nitrogen or air"; temperature, with urea conversion reaching the maximum at 43°C; and pH, the optimum falling between 7 and 8. In further studies (Pearson and Smith, 1943c), a microbiological examination of the fermentation mixture was made by F.Baker, who stated, "that even after 1 day's incubation great changes had taken place in the microflora and fauna, so that the 'microbiological picture' at the end of one day bore little resemblance to that in the initial sample." As a result of these observations, they adopted a short incubation period of 2- to 4-hours in all subsequent work, and were able to demonstrate during this time the microbiological synthesis of protein from non-protein nitrogen sources.

Further improvements in *in vitro* techniques were introduced by Marston (1948), who constructed an elaborate all-glass *in vitro* system which contained 3 liters of a mixture of a complex inorganic nutrient medium and bacterial inoculum, using purified cellulose obtained from birch wood or filter paper as a substrate. He was possibly the first to use separated bacterial cells as inoculum in *in vitro* studies. The rumen liquid which he obtained from newly slaughtered sheep was centrifuged at high speed in a Sharples super-centrifuge, with the sedimented cells resuspended and concentrated in phosphate buffer (pH 5.5) constituting the inoculum. Anaerobic conditions were maintained during fermentation periods of 24 to 48 hours by bubbling nitrogen through the fermentation mixture. Using this *in vitro* system, in which 34 to 69% of the cellulose substrate was digested, he was able to quantitatively determine gaseous and other products of microbial fermentation.

Because of the increasing complexity of the *in vitro* rumen fermentation studies that chronologically followed those of Marston, the succeeding discussion will be subdivided according to the major aspects of the *in vitro* systems; with a discussion of the various methods of preparing microbial inoculum followed by a description of the major types of *in vitro* systems that have been evolved.

a) Microbial inoculum.

1. Rumen liquid.

Although rumen liquid inoculum, prepared by straining rumen ingesta obtained either from fistulated or newly slaughtered animals or by means of a stomach tube, was used in some of the earliest *in vitro* studies, it is still used in many systems because of the

relative simplicity of its preparation, its characteristically high microbial activity, and close microbial similarity to actual rumen material due to the minimal "processing" which takes place in its preparation. Its use is advantageous when in vitro experiments do not involve detailed study of the effect of various levels of nutrients or other substances on microbial activity, since in this case the addition of substances found in the rumen, other than micro-organisms, may lead to erroneous results and lack of experimental control.

Workers using strained rumen liquid in recently reported in vitro studies include: Barnett and Reid (1957); Adler et al. (1958); Hershberger et al. (1959); Reid et al. (1959); and Stewart and Warner (1959).

Johnson et al. (1958) have proposed a modified procedure for obtaining rumen inoculum which may have definite advantages over the method of obtaining rumen liquid by straining rumen ingesta. In their procedure, they discard the original rumen liquid expressed from the ingesta, and add a definite amount of phosphate buffer (pH 7) to a weighed quantity of the solid ingesta residue. After a thorough mixing of the ingesta and buffer, the liquid is expressed and is designated as the "phosphate buffer extract." Suggested advantages of this modification include; higher cellulolytic activity of the extract as compared to rumen liquid because of the close association of rumen cellulolytic bacteria with the solid feed particles, and closer standardization of cellulolytic activity between fermentation runs since a given weight of solid ingesta which is extracted with a given volume of buffer is less subject to fluctuations and dilution

errors than the liquid phase of the rumen.

ii. Washed cells.

The recognition of the fact that rumen liquid inoculum could not be successfully employed in vitro when nutrient requirements of the rumen microorganisms were being studied, or when experiments were being conducted to isolate "unidentified factors" stimulatory to cellulose digestion, led to the development of more suitable types of inoculum for studies of this nature.

One of the earliest attempts to solve problems of inoculum preparation was made by Burroughs and co-workers, who after studying the effect of various additives on the nutritive value of corncobs in steer feeding trials, devised an in vitro system ("artificial rumen") in order to study under controlled laboratory conditions problems related to the feeding of farm animals (ruminants). Since Burroughs et al. (1950a) recognized that the use of rumen liquid as inoculum led to "the introduction of large quantities of unknown constituents aside from the microorganisms" to the in vitro system, they proposed a method by which the original rumen liquid inoculum added to their all-glass system was periodically diluted throughout their fermentation runs. This was accomplished by removing 50% of the fermentation mixture (175 ml.) every 36-hours for analysis of residual cellulose with the remaining half used to inoculate another flask for the next fermentation period after the addition of new cellulose substrate (ground filter paper) and mineral mix to bring the mixture back to its original volume. This meant that the original material taken from the rumen was "progressively diluted" during the course of eight 36-hour fermentation

periods. In a later study (Burroughs et al.,1950b) results were summarized by averaging cellulose digestion occurring during the last four fermentation periods, representing the greatest dilution of the original rumen material (other than the proliferating rumen microorganisms). The 50% dilution technique was modified in a study of urea utilization (Arias et al.,1951) by increasing the volume of fermentation mixture to provide larger samples for chemical analysis, and reducing the total time for each series by using 24-hour periods.

Perhaps not satisfied with the precision of the 50% dilution technique, Burroughs and co-workers later developed a new in vitro system, as described by Cheng, Hall and Burroughs (1955), the most outstanding feature of which was the use of "washed suspensions of rumen microorganisms" as inoculum. The washed cell suspension technique had been used by Sijpsteijn and Eldsen (1952) and Doetsch et al. (1953), in studies of rumen microbial reactions. Cheng and Burroughs prepared their washed cell inoculum by suspending in distilled water the sedimented bacterial cells obtained by high speed centrifugation, and recentrifuging the resultant mixture - this process being repeated twice, with the final yield of sedimented cells suspended in nutrient medium for use as inoculum. This technique served to physically separate the rumen microorganisms from their original environment, with the washing procedure removing any adhering materials from the cells, resulting in a suspension of mixed cells free of contaminating substances and particularly suited for studies involving the chemical composition of the medium and metabolic studies of the organisms. Disadvantages of this type of inoculum include the loss of cellulolytic activity which is generally related to "handling" of the fastidious

rumen microorganisms, and the loss of rumen protozoa due to the large gravitational forces used in centrifugation, although the significance of this latter point has not yet been resolved.

Other workers who have used washed rumen cell suspensions as inoculum *in vitro* include McLeod and Murray (1956) and Asplund *et al.* (1958).

iii. Resuspended cells.

Another type of "purified" inoculum, which is essentially a simplification of the washed cell technique, was described by Bentley *et al.* (1954_a). Instead of washing the cells obtained by high speed centrifugation as in the previous technique, they were directly suspended in phosphate buffer (pH 7) for use as inoculum. This modification represents less stress to the rumen microorganisms in that "handling" time is reduced, although a high degree of separation of cells and rumen liquid is obtained with contamination being minimal as illustrated in the studies of Bentley and co-workers. The modification of this method by Johnson *et al.* (1958) by using a phosphate buffer extract of the rumen ingesta as a source of bacterial cells, as described previously, further enhanced the precision of this type of inoculum in *in vitro* studies.

b. In vitro systems.

i. All-glass.

The all-glass *in vitro* system, representing the simplest type used, essentially consists of a glass flask, bottle, or tube, in which all the constituents of the fermentation mixture (inoculum, medium, and substrate) are contained, with various provisions made, either simple or elaborate, for maintenance of proper incubation

temperature and anaerobiosis. The tendency in recent years has been to a reduction of unit size, so that many units can be studied simultaneously in an in vitro system.

The system of Burroughs et al. (1950a) consisting of 1-liter flasks, placed in a water bath to maintain proper temperature and with CO_2 flushed through the system in order to obtain anaerobic conditions, was later modified (Cheng, Hall and Burroughs, 1955) so that each unit consisted of a 75 ml. centrifuge tube containing a total volume of 20 ml. of fermentation mixture and a 0.5% level of purified cellulose substrate (Solka Floc). Using this system approximately 60% of the cellulose was digested in a 24-hour fermentation period.

A similar evolution to smaller size units characterizes the in vitro systems described by Bentley and co-workers, who originally used 1-liter flasks as fermentation units (Bentley et al., 1954a), but later changed to the more convenient 100 ml. flask, (Bentley et al., 1955). In both cases, their studies were concerned with identifying factors which caused an increase in cellulolytic activity when added to the fermentation mixture. This same in vitro system was modified in studies of the digestibility of native forage cellulose (Quicke et al., 1959a) so that 75 ml. centrifuge tubes containing a total volume of 50 ml. of fermentation mixture constituted the in vitro unit.

Not all in vitro units were diminishing in size, as illustrated by the novel study of Hershberger and Harstook (1960), where an extremely large in vitro system was used. This consisted of 360 liters of nutrient medium and ovine rumen inoculum, with 10.8 kg. of

alfalfa hay substrate being incubated up to 32-hours in the Armsby Respiration Calorimeter at Pennsylvania State University. The purpose of this study was to determine the heat of rumen fermentation, and measure the amounts of carbon dioxide and methane produced by the rumen microorganisms.

ii. Semipermeable membrane.

Louw et al. (1949) sought to improve Marston's (1948) in vitro procedure by making provisions for the removal of nongaseous end-products which he stated "might be expected to slow the rate [of fermentation] and eventually inhibit digestion." For their new method, Louw and co-workers used a semipermeable membrane sac in which to contain the fermentation mixture. This provided for the removal of fermentation end-products from the fermentation area by dialyzis. Actually this same type of system had been used by Woodman and Stewart (1928), as previously described. Louw and co-workers comparing their semipermeable membrane system to an all-glass system, obtained slightly higher cellulose digestion with the former. Since their unit was relatively large, with 700 ml. of strained rumen liquid inoculum, 37.5 ml. of an inorganic element mixture, and 12 or 20 grams of cellulose substrate contained within the membrane sac, it was not practical where it was desired to study many factors in simultaneous fermentation runs.

Wasserman et al. (1952) used a slightly smaller semipermeable system, the sac containing 200 ml. of rumen liquid inoculum, in studying the effect of various antibiotics on in vitro cellulose digestion.

Huhtanen et al. (1954) described a "simplified adaptation of Louw's apparatus," which they termed the "miniature artificial rumen." This system consisted of a small cellophane sac, holding 10 ml. of rumen liquid inoculum and 500 mg. of alfalfa leaf meal substrate, suspended in a mineral solution contained in a 4-oz. glass screw-cap jar. Fiber digestion was determined in many such units (20 reported in one test) studied simultaneously by placing the jars in a 38°C incubator for a 16- to 24-hour fermentation period. Fiber was defined as water insoluble carbohydrate material, which was analyzed by the anthrone colorimetric method, Many workers who have adopted Huhtanen's miniature artificial rumen system, or modified versions of it include: Baumgardt and Hill (1956); Salsbury et al. (1956); Hanold et al. (1957); Asplund et al. (1958); Gaunt (1960); and Clark and Mott (1960).

Warner (1956) described an in vitro system utilizing a semi-permeable membrane sac containing 50 ml. of fermentation mixture. His system although of a similar size to Huhtanen's, was more complex in that it provided for the constant bubbling of nitrogen through both the fermentation mixture and outside dialyzing mixture, as well as containing inlet tubes to enable the addition or removal of material from either the sac or outside solution. El-Shazly et al. (1960) have recently modified Warner's system to provide for constant flow of the dialyzing solution by means of a siphon arrangement in the outer chamber.

iii. Manometric.

A manometric in vitro system, employing a Warburg respirometer,

for studying the utilization by rumen microorganisms of several substrates was described by McBee (1953). The main part of the Warburg vessel contained 1 ml. of rumen fluid and 1 ml. of sodium bicarbonate buffer, with 0.5 ml. of buffered substrate in the side arm prior to mixing and the initiation of fermentation. Hungate et al. (1955) used a modified manometric technique with an increased volume of rumen inoculum (10 to 40 g. of rumen contents). Hobson and Lloyd (1960) have developed a manometric system using 100 ml. of rumen liquid, 100 ml. of buffer solution, and 80 gm. of substrate, in which measurement of rate of gas production as well as quantitative analysis of gases produced can be accomplished during a 90-minute incubation period. The manometric in vitro system would appear to warrant further investigation because of the relative simplicity of its operation and speed and accuracy of its determinations.

iv. Continuous flow systems.

Perhaps the most complex of any of the in vitro systems developed are those of the continuous flow type. Adler et al. (1958) described a system in which, after inoculating an incubation chamber (1-liter 4-necked flask) containing nutrient medium with strained rumen liquid, automatic controls were used to regulate pH, and the continuous addition of new nutrient medium caused an overflow and removal of the fermentation mixture. In trials of 10-hour duration, data were collected which enabled the calculation of the growth rate of microorganisms present in the system.

Stewart and Warner (1959) used a continuous culture system containing 5.5 liters of fermentation mixture in which substrate and medium were added continuously, with total volume kept constant

by a float controlled overflow. During 24-hour fermentation periods the production of volatile fatty acids, and bacterial counts were determined for different substrates.

The semipermeable membrane system developed by Davey et al.(1960), contained 850 ml. of rumen content inoculum and had a constant flow of liquid in the outer chamber as well as provision for removal of gas samples for analysis.

Compared to the above described systems, the continuous flow system described by El-Shazly et al. (1960) is relatively simple. It consisted of a semipermeable sac containing 40 ml. of rumen liquid and 2 g. of substrate material, suspended in a glass tube containing 80 ml. of basal medium. Continuous flow was achieved by dripping nutrient medium through a small opening in the outer chamber, with a "Soxhlet" type siphon arrangement maintaining a constant level of medium in this chamber.

3, Validity and standardization of in vitro systems.

The extreme divergence of the in vitro systems described in the preceding sections, involving many different sources and methods of preparing microbial inoculum, nutrient media for varied composition, and numerous differences in the construction of the systems, illustrate the difficulty in duplicating and confirming in vitro results obtained by different workers. These factors perhaps serve to explain why certain results obtained in some laboratories have been followed by contradictory observations in others. In response to this chaotic situation, several workers have attempted to study the validity of various in vitro systems in order to determine if in vitro observations are true reflections of in vivo conditions

and not just artifacts of the in vitro procedure. Attempts have also been made to study the conditions necessary for standardization of in vitro systems both within and between laboratories.

Baker's observation on the effect of increasing incubation time (in the in vitro system of Pearson and Smith, 1943c) on the dissimilarity of the microflora and fauna as compared to the initial sample, resulted in these workers reducing their fermentation period from 8-days to between 2- and 4-hours.

Warner (1956) suggested several criteria of normal rumen function which could be applied in establishing the validity of in vitro studies. These are:

I.) The maintenance of numbers and normal appearance of microorganisms of the rumen.

II.) The maintenance of normal rates of digestion of cellulose, starch, and protein; and of normal interactions between these.

III.) The ability to predict quantitative results in vivo.

Certainly any in vitro system which could meet all of these conditions would constitute an accurate and valuable research tool.

The in vitro systems developed by Bentley and co-workers have recently been studied in reference to their precision and limitations. Johnson et al. (1958) studied the effect of various factors on in vitro cellulose digestion, reporting that a single "washing" of the sedimented bacterial cells reduced cellulolytic activity one-third, that after 15-minutes aeration of inoculum - 40% of the activity was lost, and that levels of fatty acids higher than those generally found in in vitro systems were necessary to cause partial inhibition of cellulolytic activity. The last result suggests that perhaps the use

of semipermeable membranes to dialyze fermentation end-products is not necessary for maximum cellulose digestion. El-Shazly et al. (1959), in biochemical and microscopic comparisons between in vivo (sheep) and in vitro rumen fermentation, concluded that the in vitro system used for cellulose digestion was "representative of conditions in vivo over a period of 24-30-hours." Further work by El-Shazly et al. (1960), compared all-glass, semipermeable, and continuous flow in vitro rumen fermentation systems, using cellulose digestion and volatile fatty acid production as criteria. They concluded that "there was little to prefer from one type of apparatus to another," and also that end-products such as volatile fatty acids are not inhibitory for cellulose digestion (in levels accumulated in the in vitro system) since the systems in which end-products were removed "did not give better cellulose digestion than the all-glass system."

Church and Petersen (1960) using an all-glass in vitro system, have studied the effect of several factors such as level of substrate, level of inoculum (rumen liquid), source of inoculum, pH adjustment, and substrate particle size on several in vitro criteria (cellulose and dry matter digestibility, and volatile fatty acid production).

The continuance of the preceding types of studies, is necessary for the maximum potential usefulness of in vitro rumen fermentation methods in research attempting to parallel or duplicate situations existing in the intact rumen.

4. Nutrient requirements of rumen microorganisms.

Accurate in vitro studies of the nutrient requirements of rumen microorganisms were not possible until advanced techniques had been developed to enable the preparation of microbial inocula which were

free of the heterogeneous and ill-defined substances present in the original rumen liquid. These "purified" inocula were made possible by the development of "washed" or "suspended" cell techniques, as described in a previous section. It was probably the recognition by several workers that rumen liquid contained certain "unidentified factors" necessary for maximum cellulolytic activity in vitro, that stimulated the development of the more precise techniques in an attempt to identify these substances.

a) Fatty acids and amino acids.

Burroughs et al. (1950a), using his "50% dilution" in vitro technique to reduce the influence of materials added in the original rumen fluid inoculum, found that several additions to the basal medium such as autoclaved rumen liquid, or autoclaved water extract of manure, "proved helpful to cellulose digestion." In another in vitro test, Burroughs et al. (1950c), studying the effect of adding various feedstuffs to the fermentation mixture, found that "many feeds influence rumen microorganisms favorably in cellulose digestion," such as dried distillers solubles, soybean oil meal, and linseed oil meal. A further report by these workers (Ruf et al., 1953) characterized an unidentified factor stimulatory to cellulose digestion as being "fairly widespread in common feeds" with yeast and manure extract being "particularly rich sources." Fractionation of the latter materials indicated the factor to be heat stable, water soluble, and destroyed by ashing. However, vitamins and casein hydrolysate did not exhibit any apparent stimulation. Since certain vitamins and casein hydrolysate were later shown to be stimulatory, the possibility of toxic levels in this study might explain the lack of effect.

Doetsch et al. (1952) using bacteriological techniques in culturing rumen bacteria, concluded that "there are factors in rumen fluid necessary for optimal growth of rumen bacteria" and that "these substances are not provided by a commercial medium devised for nutritionally fastidious bacteria." Bryant and Doetsch (1954), studying the growth requirements of a pure culture of Bacteriodes succinogenes - an actively cellulolytic rumen bacteria, observed that rumen fluid contained an unknown heat, acid, and alkali-stable factor which was not a common B-vitamin, amino acid, or mineral.

MacLeod and Brumwell (1954), using an in vitro system characterized by rumen liquid inoculum and vegetable parchment (cellulose) substrate, demonstrated that several fishery by-products (whale or herring solubles, herring stickwater, or halibut hydrolysate) each strongly stimulated cellulose digestion when added to the fermentation mixture. When a mixture of 18 amino acids was added, it was found to be "even more active" than the fishery by-products studied. Whale soluble ash, a mixture of 9 water soluble vitamins, cysteine and methionine, did not have stimulatory properties

Bentley et al. (1954a), using suspended cell inoculum and a highly purified medium, demonstrated a high level of cellulose digestion when either rumen liquid, rumen liquid supernatant (from centrifugation of cells), a water extract of alfalfa, yeast or molasses was added to the basal medium. During a series of fractionation studies of rumen liquid, they found the active factor was related to certain short-chain fatty acids (Bentley et al., 1954b), and finally valeric acid was shown to be primarily responsible for this activity (Bentley et al., 1955). Caproic, iso-butyric, and

iso-valeric were also found to markedly increase cellulose digestion, but not to the same extent as valeric acid.

Huhtanen and Elliott (1956) observed no effect on cellulose digestion when valeric acid or iso-valeric acid were added to the fermentation mixture. This lack of response was probably due to the fact that these workers used "whole unaltered rumen fluid" as inoculum, a material which would itself have contributed a substantial amount of the 5-carbon fatty acid to the medium.

That valeric acid was not the only active factor was suggested (Bentley et al. 1955) since a non-volatile substance present in yeast extract and rumen liquid demonstrated cellulolytic activity.

Hall et al. (1954), using washed cell inoculum, showed that mild hydrolysates of purified casein and other protein-rich materials "exhibited a highly favorable influence... in the digestion of cellulose." Since severe hydrolysis, reducing the protein to the amino acid state, resulted in loss of cellulolytic factor activity, they suggested that perhaps the active principles were water-soluble peptides. In a later study (Hall et al., 1955), it was shown that the active substance in the protein hydrolysate was not identical to any of the short-chain fatty acids.

MacLeod and Murray (1956), having adopted a washed cell inoculum in their in vitro system, demonstrated that a combination of three amino acids (valine, leucine, and isoleucine) were "primarily responsible for the strong stimulation of cellulose digestion previously shown to be produced by a mixture of 18 amino acids." They also produced inhibition of cellulose digestion when too high a level of nitrogen occurred in the medium.

Dehority et al. (1957), using ion exchange and large scale paper chromatography techniques, fractionated and isolated the cellulolytic factors present in autolyzed yeast and casein hydrolysate, and identified them as the amino acids valine, leucine, isoleucine and proline.

A possible relationship between the amino acids and fatty acids shown to be required by cellulolytic rumen microorganisms, as suggested by the work of El-Shazly (1959), is that the former may be converted by the microorganisms to 4- and 5-carbon volatile fatty acids. This claim is supported by the observation of Dehority et al. (1957) that valeric acid and the amino acids when tested in various combinations "did not show any appreciable additive effects."

b) B-vitamins.

The use of washed or suspended cell inocula has also made it possible to demonstrate in vitro, rumen microbial requirements for nutrients other than the amino and fatty acids discussed in the previous section. Hall et al. (1953) reported that the B-vitamins riboflavin, pyridoxine, biotin, para-amino benzoic acid (PABA), folic acid, and B_{12}, all stimulated cellulose digestion, with the combination of B_{12} and biotin more stimulatory than any single vitamin or vitamin combination. Bentley et al. (1955) showed that biotin and PABA are required by rumen microorganisms for maximum cellulose digestion, and that valeric acid had no stimulatory effect unless the 2 B-vitamins were also added to the basal medium. When valeric acid and the B-vitamins were added in combination they exhibited an additive effect. MacLeod and Murray (1956) found pyridoxine "consistently effective in stimulating cellulose digestion" with

consistent stimulation with thiamine, niacin, folic acid, and PABA.

Although limited reports on B-vitamin requirements are not entirely in agreement with each other, there appears to be a definite requirement for several of these vitamins.

c) Inorganic elements.

Most of the mineral solutions used in recent in vitro nutrient media are related to the "synthetic saliva" formula as suggested by McDougall (1948), who based the composition of this solution on results obtained by actual chemical analysis of sheep saliva. Additions to and modifications of McDougall's formula have been based on observations made by several workers, particularly Burroughs and associates, on the in vitro response of rumen microorganisms for different inorganic elements, as related to their source and availability.

Using an in vitro phosphorus availability test (as described by Anderson et al., 1956), Raun et al. (1956) demonstrated that phytate phosphorus was almost 100% available to rumen microorganisms, and suggested that this was due to the substantial phytase activity of viable rumen microorganisms. Hubbert et al. (1958a) have determined the optimum and toxic concentrations of 9 inorganic elements. In another report (Hubbert et al., 1958b), they described the effect of 5 other inorganic elements on cellulose digestion as well as the interrelationships existing between certain of the elements when added to the medium at various levels in factorial experimental designs. As a result of this study, sodium was shown to have no effect when added in levels from 50 to 4,000 mcg./ml., although a significant additive effect with sodium was observed when

potassium levels were increased from 50 mcg./ml. to 100-400 mcg./ml. Trenkle et al. (1958) studied the necessary level of sulfur, and the availability of several sulfur sources. Chamberlain and Burroughs (1960) have reported that when magnesium or manganese were omitted from the medium, cellulose digestion was 50-60% of normal.

Although rumen microbial nutrient requirements may be consistently demonstrated in any one in vitro system, it is perhaps premature to accept these qualitative or quantitative requirements as absolute, unless they can be repeatably confirmed using other systems. Since this later consideration seems to be the exception rather than the rule, there appears to be a need for standardization of procedures so that all in vitro studies can be carried out under similar conditions with adequate controls.

C. FORAGE STUDIES USING IN VITRO RUMEN FERMENTATION METHODS.

1. Studies of the nutritive value of forages.

Since cellulolytic activity, as related to substrates of either purified or native forage cellulose, has been the major criterion of *in vitro* rumen fermentation systems, the use of these systems to study factors affecting the nutritive value of forages as well as the more concise "indexing" of this nutritive value, is not illogical.

a) Effect of lignification.

Perhaps the first *in vitro* study of forage quality was made by Woodman and Stewart (1932), who stated - "the primary object was to ascertain whether such measurements could form the basis of a quick *in vitro* method for estimating the digestibility of the fibrous constituents of feeding stuffs." Even though their *in vitro* system, as previously described, was advanced for their time it left much to be desired by present-day standards although this did not seem to affect the quality of their observations. In their study, they compared *in vivo* (sheep) and *in vitro* fiber digestibility, using as substrate in the *in vitro* tests the intact feedstuff as fed, as well as fiber isolated from these feedstuffs. They demonstrated that the isolated fiber was digested to a greater degree *in vitro* than the corresponding intact feedstuff in animal trials, and suggested that the "formation of a small amount of lignocellulose may be responsible for a distinct lowering of the digestibility of the fibre in a crop." They further stated that "it is not necessarily the amount of lignocellulose which determines the running off in digestibility, but rather the manner of its deposition within the [plant] cell walls."

They also studied the effect of stage of maturity of ryegrass on the character of its fiber content, demonstrating increasing lignification with maturity, and reduced digestibility which was associated with the production of relatively small amounts of lignocellulose. Certainly this study set the stage for future experiments on forage quality.

In order to demonstrate the possible mode of action of lignin, Stallcup (1957) added commercially purified lignin[1] to in vitro flasks at four different levels, with no significant difference in cellulose digestion observed in any of the flasks. He suggested that the data "lend credence to the idea that the reduction in digestibility usually associated with increasing amounts of lignin appears to be due to its role in the physical structure of the plant, rather than chemical action as toxicity to the microorganisms of the rumen."

Salsbury et al. (1958) determined cellulose digestion after 3, 6, 9, 12 and 24 hours of incubation, using native and purified cellulose substrates as well as halo-, and alpha-cellulose fractions of these materials. Native alfalfa cellulose showed most rapid early digestion, while the delignified cellulose fractions prepared from forages were more rapidly and completely digested than the original plant material. Kamstra et al. (1958) also compared the in vitro digestion of native cellulose as well as the cellulose containing fractions prepared from the same forages. These workers studied an extensive range of forage species, each harvested at several stages of maturity, and demonstrated that "separating cellulose from lignin greatly improved its digestibility in vitro with the effect of maturity lignification diminished greatly when the cellulose was isolated

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[1] "Indulin A", 99.5% lignin.

from the plant."

Quicke and Bentley (1959) in a study of the effect of lignin and methoxyl groups as related to decreased digestibility of mature forages, stated that lower digestibility *in vitro* could not be fully explained in terms of proximate composition or lignin content when brome and orchard grass hays were compared, but that in the case of 4 stages of maturity of a single species (timothy hay), decreasing digestibility did appear related to lignin content.

Dehority *et al.* (1960) further demonstrated that the effect of lignin in decreasing cellulose digestion was most probably due to its "incrusting" effect rather than its total concentration, by ball-milling several forage samples causing physical rupture of the cell wall structures. Ball-milling was shown to substantially increase cellulose digestion, particularly in the case of the more mature forages.

The preceding *in vitro* studies serve to emphasize the importance of lignin as a factor in reducing forage utilization, its detrimental action most likely related to its ability to isolate forage nutrients from bacterial as well as other forms of enzymatic degradation.

b) *Cellulose structure*.

Baker *et al.* (1959) studied certain physical properties of 4 purified cellulose materials for which *in vitro* cellulose digestion had been determined. Although data from X-ray diffraction studies, as expressed in a "crystallinity index", were inversely related to cellulose digestion for the purified celluloses, only small "index" differences were noted between forages with widely

differing digestibilities. Tomlin and Davis (1959) also reported no relation between the crystalline index of the cellulose and *in vitro* cellulose digestion of various forages.

In order to determine if pelleting conditions affect cellulose availability, Jahn and Kamstra (1960) studied the effect of pelleting hay of early and late maturity at various temperatures and pressures on the *in vitro* cellulose digestion of these forages. They concluded that improvement in digestibility was more pronounced with low quality or more mature forages at all temperature and pressure combinations.

c) Undigested cellulose as related to rate of passage.

Although lignin has been shown to inhibit digestion of cellulose and other nutrients to a significant extent, another factor decreasing forage digestion is the amount of time the forage remains in those parts of the animal's digestive tract where it can be enzymatically attacked. Johnson *et al.* (1959) determined the *in vitro* cellulose digestibility of "undigested" cellulose isolated from the feces of sheep fed different forages. They showed that 77% of the "undigested" cellulose of soybean hulls was digested in the *in vitro* system, while 16% of alfalfa cellulose and only 5% of bromegrass or orchard grass hay cellulose was further digested. These workers suggested the observed differences were a result of both rate of digestion and rate of passage in the intact animal. This explanation was substantiated by Quicke *et al.* (1959), who obtained 96% digestion of soybean hull cellulose both *in vitro* and when samples of the same material contained in a dacron bag were placed in the rumen of a fistulated steer. This was compared to 54% observed in *in vivo* digestion trials with sheep. The soybean hulls fed as the sole feed to the sheep resulted in very

soft feces possibly caused by "too rapid passage." A further observation on the relationship between rate of digestion and passage, was made by Phillips et al. (1960), who showed that in vitro fermentation rates were significantly negatively correlated with rumen retention times as determined in vivo with Zebu and European type cattle.

The preceding reports indicate that the potential digestibility of a lignified forage is further limited or enhanced by the length of time the feed is retained in the digestive system.

d) Effect of nutrient supplementation.

Early in vitro studies indicated that supplementation of poor quality forages with a variety of nutrients would increase the digestibility of such forages. These increases were accomplished by either supplementing the feed of the animal serving as inoculum donor or by direct additions to the in vitro nutrient medium. However, many of these claims have yet to be substantiated in large scale animal feeding trials.

Burroughs et al. (1950b) determined the cellulose digestion of good and poor quality forages in one of their early in vitro studies. It was found that a supplement of nitrogen, complex mineral solution, or autoclaved water extract of cow manure, each increased and maintained cellulose digestion throughout the fermentation periods when added to the poor quality forages (corn stover, wheat straw, corncobs, mature grass hay), while the good quality forages (legume hays) maintained a high level of digestion independent of supplementation.

Bentley et al. (1951) reported that after changing the feed of a fistulated steer from good quality hay (alfalfa) to poor quality

hay (late cut grass), in vitro cellulose digestion, using rumen liquid inoculum obtained from the steer, decreased 90% during the first 4-weeks of poor hay feeding. They also reported that supplementation of the poor quality hay with bone meal, limestone, and salt "resulted in in vitro microorganism activity similar to that observed when the alfalfa hay was fed." Removal of the supplement resulted in lowered activity. They noted that the low phosphorus content of the poor quality hay was a possible "limiting factor in cellulose digestion." Since no details of the feeding regime were given in this report, it is not possible to ascertain the possible contribution of protein present in the bone meal to the observed stimulatory effects. Hunt et al. (1954), using the same in vitro system, presented data obtained with inoculum prepared from the rumen contents of a steer fed either a good or poor quality hay. Their results showed that in vitro riboflavin and vitamin B_{12} synthesis, cellulose digestion, and ammonia utilization were all depressed on the "poor hay inoculum." The addition of starch to the medium resulted in a much larger depression of cellulolytic activity with the poor hay inoculum than with the inoculum prepared from the steer fed good quality hay.

Several workers have recently studied in vitro the effect of nitrogen fertilization of forage crops. Hall et al. (1958) noted marked negative effects of nitrogen fertilization on cellulose digestion of certain forages, with no significant effects on others. In a later study (Hall et al., 1960), additions of sodium nitrate and sodium nitrite to fermentation tubes caused inhibition of in vitro cellulose digestion and it was suggested that "poorer utilization of a heavily nitrated young Sudan forage ... may be due to the

presence of nitrate and/or nitrites in the forage." Hopkins et al. (1960) demonstrated that in vitro gas production decreased as the level of nitrogen fertilization of several forages increased. Ethanol extraction of the nitrogen fertilized hay removed inhibitory factors, which were isolated and identified as nitrate. Perez et al. (1960) using the same in vitro system, further noted that when an amount of nitrite corresponding to that present in a given fertilized hay was added to a non-fertilized hay, gas production was depressed to the level noted in the fertilized hay. Reduced gains of calves fed nitrogen fertilized hay compared to non-fertilized hay were also noted with the suggestion that the "level of nitrite may account in part for the significantly lower gains."

These studies illustrate the use of in vitro systems to demonstrate the presence of inhibitory material, related of course to the fact that these materials must be in some manner inhibitory to the rumen microorganisms themselves.

e) Volatile fatty acid production.

Although it has been demonstrated that the main products of cellulose degradation in the rumen are volatile fatty acids, in vitro studies of the effect of forage quality on volatile fatty acid production and ratio are limited.

Barnett and Reid (1957a) studied in vitro volatile fatty acid production from both fresh and dried grass samples representing various stages of maturity and harvested in two different years. They observed that acetic acid was the main acid produced during early growth stages (with fresh grass) but that propionic acid production increased with advancing maturity, finally becoming the

major acid produced. Dried samples which corresponded to the fresh grasses invariably yielded acetic acid in greater proportion than propionic acid, and it was suggested that variations in results between fresh and dried samples were "due to changes in carbohydrate content resultant upon storage of the latter." In another study (Barnett and Reid, 1957b), they showed that water extracted dried grass gave increased amounts of propionic acid while the water extract itself resulted in consistently higher acetic acid levels, revealing itself "as the chief source of acetic acid in the whole dried grass." When crude fiber and cellulose extracted from the dried grass samples were tested (Barnett and Reid, 1957c), the "proportions of different volatile fatty acids resembled those obtained from purified cellulose, propionic acid being produced in greatest relative yield."

Asplund et al. (1958) found a significant correlation between (in vitro) total fatty acid production and dry matter digestibility (in vivo - sheep) using 11 forages of varying species and stages of maturity.

Rice et al. (1960) observed that in vitro rate of volatile fatty acid production was significantly greater when alfalfa hay was used as a substrate as compared to the use of oat straw. These workers also found a significantly lower acetic to propionic acid ratio for the alfalfa substrate during the first 4-hours of fermentation, but no significant differences for the balance of the 24-hour fermentation period.

Since the volatile fatty acids produced by rumen microorganisms represent one of the major energy sources of the ruminant, detailed

in vitro studies on this subject hold promise as important sources of information, particularly because of the high degree of accuracy made possible through the use of purified nutrient media.

2. *Use of in vitro techniques to predict nutritive value of forages.*

The indexing or ranking of forages according to some criterion related to their nutritive value (*in vivo*), constitutes one of the more practical uses of *in vitro* rumen fermentation methods. The validity of such an index system must depend on the establishment of a high correlation between the *in vitro* observations and some *in vivo* measure of forage nutritive quality, with the identical forages studied in each case.

The *in vivo* criteria of forage nutritive value which have been used are related, almost without exception, to the digestibility of some component of the forage, this component usually being some measure of energy content. To illustrate, digestibility coefficients most often used as *in vivo* measures of forage nutritive value are: dry matter, organic matter, energy (calories), crude fiber, cellulose, and TDN. It is obvious that an *in vitro* observation which is found to be highly correlated to the digestibility of one of these components, is only as precise in describing the nutritive value of a forage as the *in vivo* criterion to which it is compared.

Although previous discussion emphasized the necessity of *in vitro* systems paralleling or duplicating as closely as possible *in vivo* rumen conditions, this consideration is not an important one when the objectives of the *in vitro* method can be well defined in terms of some predetermined criterion of forage nutritive value, as established *in vivo*. In other words, the actual *in vitro* method used

to obtain data on forages is of secondary importance to the establishment of a high and consistent correlation between the in vivo and in vitro observations. It will be noted, however, that most in vitro methods proposed for forage evaluation are identical or slightly modified versions of methods used in other ruminology studies.

The previously noted studies of Woodman and Stewart (1932), were perhaps the first in which in vivo and in vitro digestibility coefficients (crude fiber), obtained using the same forages in each case, were compared. The results of this study did not show any consistent relationship between the data observed, most probably due to defects in the in vitro methods used.

Muller (1947) devised an in vitro system to determine the organic matter digestibility of forages, which utilized as inoculum pure cultures of aerobic cellulolytic bacteria isolated from soil samples. His 18 forage samples for which in vivo coefficients of organic matter digestibility had been determined with sheep, consisted mainly of chemically treated straw with a few untreated samples, and only 6 samples of hay or dried grass. As a result of this "heterogeneous collection of materials," Muller stated that therewas "no general parallelism for the two methods" (in vivo and in vitro). When the untreated straw and hay samples were examined there was a closer relationship, but Muller observed that, "the number of samples investigated of these two groups is far too small to warrant any definite conclusion." The lack of a statistical analysis of the experimental data makes it difficult to comment on the accuracy of this method. However, graphical presentation of the data indicates

that this type of *in vitro* system, simplified by the use of a pure culture of an aerobic cellulolytic bacterium, holds promise as an accurate and easily standardized method for studying forage quality.

Pigden and Bell (1955), perhaps the first workers to obtain positive results, reported a highly significant correlation between organic matter digestibility of 11 forages as determined with sheep, and carbohydrate fermented *in vitro* as measured by the anthrone method.

Barnett (1957), studying 27 samples of dried ground silages *in vitro*, found "a reasonable degree of correlation with the corresponding figures for the digestibility coefficients of crude fiber obtained by feeding trial" (sheep). Although no statistical measure of this relationship was reported, visual observation of the graph presented indicates that the correlation between the *in vivo* and *in vitro* observations was very high.

Asplund *et al.* (1958) studied the effect of various modifications of their *in vitro* technique on the applicability of the *in vitro* system as an assay method in determining the nutritive value of forages. They used a total of 17 forage samples for which dry matter digestibility coefficients had been determined in sheep trials. The *in vitro* observations made were: percent dry matter loss, total fatty acid production, and ratio of percent acetic acid to percent propionic acid. The semipermeable membrane system of Huhtanen was used, with variables studied including rumen liquid vs. washed cell inoculum, source of inoculum, and length of fermentation period. With a group of 11 forages harvested in one season, the highest correlation ($r = 0.87$) was found between *in vivo* dry matter digestibility and

in vitro dry matter loss after 48 hours incubation using rumen liquid inoculum obtained from a fistulated sheep fed solely on straw. Other combinations of fermentation time, type, and source of inoculum also yielded highly significant correlations with *in vivo* dry matter digestibility. None of the *in vitro* observations was as related to *in vivo* dry matter digestibility as was the chemically determined crude protein content of the forages ($r = 0.91$).

Baumgardt *et al.* (1958) compared the ability of *in vitro* and chemical techniques to estimate forage nutritive value defined in terms of various *in vivo* digestibility coefficients obtained with 11 forages. Of the methods tested, *in vitro* "cellulose digestion appeared to be the most closely related to the *in vivo* digestibility data," in particular dry matter ($r = 0.81$) and energy ($r = 0.80$). In a later report Baumgardt *et al.* (1959), proposed a simplified *in vitro* procedure, "applicable to routine [forage] testing," with which a highly significant correlation ($r = 0.85$) was found between *in vitro* cellulose digestion and *in vivo* digestible energy, using 31 hay samples.

Quicke *et al.* (1959) described an *in vitro* method which was used in studies comparing *in vivo* and *in vitro* cellulose digestion. Inoculum prepared from either strained rumen liquid or resuspended cells did not appear to result in differences in cellulose digestion. In comparing *in vivo* and *in vitro* cellulose digestion, a forage species effect seemed evident in that no significant difference was observed between results with grass hays, but in some of the legume hays, cellulose digestibility coefficients were significantly different.

Hershberger *et al.* (1959) using rumen liquid inoculum obtained from sheep, compared the *in vivo* and *in vitro* cellulose digestibility

of 35 forages, observing a correlation coefficient of 0.97, perhaps the closest relationship of this type reported in the literature. The relationship between *in vitro* cellulose digestion and *in vivo* digestible energy (r = 0.92) was also highly significant.

Reid *et al.* (1959) extended *in vitro* forage studies to pasture evaluation. Methods of preparing the pasture samples for *in vitro* assay were compared, with oven drying giving a more accurate prediction of *in vivo* digestibility than either fresh or freeze dried samples. In a later study (Reid *et al.*, 1960) utilizing 124 forage samples obtained from seven different research stations, a highly significant correlation was found between *in vitro* cellulose digestion at 36-hours and *in vivo* dry matter or energy digestibility.

Clark and Mott (1960) using an *in vitro* system similar to Huhtanen's with a washed cell inoculum, determined the dry matter digestion of 11 forage samples for which *in vivo* dry matter digestibility had been determined. These forages were identical to a group tested by Asplund *et al.* (1958), having been obtained from the same source[1]. Clark and Mott observed a seasonal effect, depending on whether rumen inpculum was obtained in spring or fall, on the relationship between *in vivo* and *in vitro* dry matter digestibility. A highly significant correlation coefficient (r = 0.77) was obtained in the spring, with a non-significant correlation (r = 0.49) observed in the fall. Information regarding the feeding regime of the steers used as inoculum donors, the most probable cause of the variation observed

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[1] W.J.Pigden, Animal Research Institute, Canada Department of Agriculture, Ottawa.

was not given. These same workers also studied *in vitro* digestibility of 12 varieties of timothy samples, each harvested on the same day at 10-day intervals, representing all maturity stages. After early leaf stage, significant differences were found between some of the varieties at any of the harvesting dates. This latter study, for which *in vivo* data were not available, illustrates the application of *in vitro* rumen fermentation techniques to agronomic selection of plant varieties on the basis of nutritive value.

Gaunt (1960) has used an *in vitro* system to determine dry matter digestibility of samples in a state-wide (Massachusetts) forage evaluation program, with observations of 567 samples of hay, corn and grass silage reported. Since no studies were mentioned in which the validity of the particular *in vitro* system used was examined in relation to forage samples of known nutritive value (determined *in vivo*), the accuracy of this procedure is unknown.

Shelton and Reid (1960) have recently reviewed the use of *in vitro* rumen techniques to measure the nutritive value of forages, with a concluding statement that "no other single factor would stimulate forage research and production to a greater extent than the development of a simple procedure for determining the nutritive value of a forage to the ruminant."

The preceding discussion of the various *in vitro* methods which have been proposed as a test for forage nutritive value can perhaps be summarized with the statement that it appears possible to predict with a high degree of precision the *in vivo* digestibility coefficient of the main energy-yielding components of a forage using a wide variety of *in vitro* techniques, providing each is used under

standardized conditions as experimentally determined for each system. In this particular application of *in vitro* rumen fermentation systems,- i.e. that of predicting the nutritive value of forages,- the need for standardization of techniques between different laboratories cannot be overemphasized. Otherwise *in vitro* observations as determined by any one experimenter are only valid under the local conditions where they have been standardized.

III. OBJECT OF RESEARCH.

The description of the nutritive value of forages by conventional *in vivo* feeding trials is a lengthy and expensive process, requiring large amounts of forage sample material to be fed to experimental animals maintained in special housing facilities. Because of these difficulties, information in regard to the comparative nutritive value of forages is not readily available to the nutritionist, the plant breeder, or the farmer.

The object of this research was:

a) To develop an *in vitro* rumen fermentation method which could be used to predict the nutritive value of a forage, the efficacy of this method to be measured by a correlation between the *in vitro* data and some concise *in vivo* criterion of nutritive value.

b) To examine various factors affecting the accuracy and reproducibility of the proposed *in vitro* method in the prediction of forage nutritive value.

IV. PRELIMINARY STUDIES ON THE ESTABLISHMENT OF AN IN VITRO RUMEN FERMENTATION SYSTEM.

A. GENERAL INTRODUCTION.

The *in vitro* rumen fermentation techniques used in these studies were based on those developed by Bentley and co-workers at the Ohio Agricultural Experiment Station, as described by Bentley *et al.* (1955) and later modified by Quicke *et al.* (1959a). This particular system was chosen because of the success achieved with it by Bentley and co-workers in demonstrating the quantitative requirements for certain fatty and amino acids by rumen micro-organisms.

The studies to be described in this section were designed to confirm and possibly extend results obtained by other workers, as well as to study factors which might affect *in vitro* results. One phase of these studies was the examination of the adequacy of the nutrient medium suggested by Bentley and co-workers to determine if certain additives would result in increased cellulolytic activity.

Successful completion of these preliminary studies was considered a prerequisite to the development of a standardized *in vitro* technique which could be used in forage evaluation studies.

B. GENERAL EXPERIMENTAL PROCEDURE.

1. Preparation of bacterial inoculum.

a) Source of rumen ingesta.

Two rumen fistulated Holstein steers, fed ad libitum a diet consisting exclusively of good quality hay (unless otherwise noted) with cobaltized-iodized salt and water available at all times, served as the source of rumen ingesta throughout all studies. The surgical technique used in the fistulation operations[1] was as described by Dougherty (1955), with the cannulae consisting of threaded tubing, internal and external flanges, and cap, all constructed of acrylic ("Lucite") plastic. Inside cannula diameters of 4" or of 5" enabled insertion of the hand into the rumen and rapid collection of sample.

b) Phosphate buffer extract.

A polyethylene bucket lined with 2 layers of cheesecloth was used to collect a sample of approximately 6 liters of rumen ingesta (Figure 1). The sample contained in the cheesecloth was placed in a small press and the rumen liquid expressed and discarded. A sample of 4 lb. of the resultant solid rumen ingesta (Figure 2) was mixed with 1500 ml. of phosphate buffer solution (pH 7)[2] according to the method described by Johnson et al. (1958). After moderate agitation the ingesta-phosphate buffer mixture was re-pressed and the resultant liquid, designated as phosphate buffer extract (P.B.E.), was filtered through 4 layers of cheesecloth into a preheated thermos container for transportation to the laboratory (Figure 3).

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[1] Performed by Dr. D.G. Dale (D.V M.) Department of Animal Science, Macdonald College.

[2] 1.059 g. Na_2HPO_4 and 0.436 g. KH_2PO_4 per liter.

Prior to making the extract, the phosphate buffer solution was preheated to 45-48°C (to compensate for drop of temperature to approximately 40°C during extracting procedure), saturated with CO_2 by rapidly bubbling gas through the solution for 5-10 minutes (in order to lower oxidation-reduction potential of solution), and readjusted to pH 7 with Na_2CO_3 (to compensate for pH drop due to CO_2 saturation). In actual practice, these steps were accomplished by adding 60 ml. of 20% Na_2CO_3 solution to 1500 ml. of the buffer solution (heated by placing flask in a container of hot water) and bubbling CO_2 through this solution until the pH returned to 7, as measured by a pH meter (Beckman Zeromatic).

c) Resuspended bacterial cells.

A sample of 1250 ml. of the P.B.E. was passed through a Sharples supercentrifuge (steam powered) operating at a speed between 25,000 and 30,000 r.p.m. (Figure 4). Rate of flow through the supercentrifuge was adjusted so that 3-5 minutes was required for the entire solution to pass through. The bacterial sediment, collected on a celluloid liner placed inside the centrifuge bowl (Figure 5), was resuspended by means of a loose-fitting tissue homogenizer (Pyrex No.7725) in 250 ml. of phosphate buffer (composition and preparation as previously described), This suspension was filtered through 4 layers of cheesecloth and constituted the bacterial inoculum. Prior to resuspension, the bottom inch of sediment on the celluloid liner, consisting of plant and protozoan debris, was discarded.

2. Nutrient medium.

The composition of the nutrient medium (Table 1) was that reported by Quicke et al. (1959a), with several modifications,

including an increase in the amount of Na_2CO_3 and the addition of phosphate buffer. Preliminary trials showed that these modifications resulted in a pH of 6.8-7.0 of the fermentation tube contents, which was maintained even after extended fermentation periods. The addition of casein hydrolysate to the medium was based on observations in preliminary trials to be reported.

Various deviations in the makeup of the nutrient medium (as listed in Table 1) will be discussed in reference to the particular trials in which they occurred. These deviations, in general, were associated with trials completed previous to the publication of the modified "Ohio medium" by Quicke and co-workers, and in studies in which requirements for specific nutrients were determined by removing them from the medium and adding them in graded amounts to specific fermentation tubes.

3. Cellulose substrates.

The purified cellulose substrate used was Solka Floc SW 40A[1], prepared commercially from delignified soft wood. This material, which analyzed 93.9% cellulose, was used at a level of either 500 or 230 mg. per fermentation tube.

Dried forage substrates were prepared for in vitro studies by grinding in a Raymond hammer mill fitted with a screen having 0.024" diameter round holes (equivalent approximately to U.S.B.S. sieve No.30), and stored at room temperature in amber-colored glass jars with tight-fitting caps. Forages containing between 26% and 34% cellulose were used at a substrate level of 700 mg. per tube,

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[1] Supplied by Brown Forest Products Company, Montreal, P.Q.

TABLE 1

COMPOSITION OF NUTRIENT MEDIUM AND BACTERIAL INOCULUM MIXTURE.

Solution	Concentration mg./ml.	Used per tube ml.	Used per tube mg.
(1) Mineral mixture:		10	
Na_2HPO_4	5.65		56.5
$NaH_2PO_4.H_2O$	6.27		62.7
KCl	2.15		21.5
NaCl	2.15		21.5
$MgSO_4.7H_2O$	0.582		5.82
Na_2SO_4	0.750		7.50
(2) Glucose[1]	20	2.5	50
(3) Urea[1]	25.2	2.5	63
(4) Casein hydrolysate (enzymatic)[1,2]	20	2.5	50
(5) n-Valeric acid[1]	3	5.0	15
(6) PABA	0.5	0.05	0.025
(7) Biotin[3]	0.2	0.05	0.010
(8) Na_2CO_3	200	1.5	300
(9) Phosphate buffer (pH 7):		10	
Na_2HPO_4	1.059		10.59
KH_2PO_4	0.436		4.36
(10) Iron and calcium:		0.5	
$FeCl_3.6H_2O$	4.40		2.200
$CaCl_2.2H_2O$	5.29		2.645
(11) Bacterial inoculum	-	5	-
(12) Distilled water, to make		50	

[1] Prepared on day previous to fermentation run, and refrigerated until used.

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] 50% ethanol solution.

thus supplying a cellulose level of approximately 200 mg. For those forages analyzing below 26% or above 34% cellulose, a substrate level of 800 mg. or 600 mg., respectively, was used per tube so as to maintain the same appreximate level of cellulose (200 mg.) in all tubes. In relation to substrate level, Quicke et al. (1959a) had demonstrated that varying forage levels from 0.6 to 1.3 gm. per tube had no effect on percent cellulose digestibility.

4. In vitro system.

The in vitro system (Figures 7, 8) consisted of 32 fermentation tubes (90 ml., Pyrex No.8260), each fitted with a 1-hole rubber stopper (No.6) through which was inserted a glass delivery tube (Fisher No.13-711) attached by means of rubber tubing to a gas manifold with 32 outlets each fitted with a needle valve. The rate of gas flow of approximately 160 bubbles per minute was adjusted by means of an individual needle valve for each fermentation tube. The gas manifold was connected to a large (50 lb. capacity) tank of "medical quality" CO_2, fitted with a gas pressure regulator. The delivery tube was adjusted so that its tip was approximately 40 mm. from the bottom of the fermentation tube, and this together with the relatively slow gassing rate enabled the substrate to settle to the bottom of the tube during fermentation. Gas was exhausted by way of the clearance between the pouring lip of the tube and the rubber stopper.

Fermentation tubes were maintained at a temperature of 40°C ± 0.5 by immersion in a water bath (Fisher No.15-470) for which a stainless steel tube rack had been specially constructed to accommodate the 32 tubes plus one extra tube filled with buffer solution to contain the

pH meter electrodes. The miniature electrodes (Beckman No.39166) inserted in a rubber stopper (Figure 8) enabled pH measurements to be taken during fermentation runs with a minimum of disturbance to the tube contents.

5. Setting-up procedure.

During the course of these in vitro studies a series of procedures were developed to minimize potential sources of stress to the microorganisms, as well as to reduce the total setting-up time. These procedures will be outlined briefly (with approximate times given) in the belief that close adherence to these steps resulted in the high level of cellulolytic activity and good reproducibility in the in vitro system.

a) Day previous to fermentation run.

Appropriate substrates are weighed into the fermentation tubes. Labile nutrient solutions (as noted in Table 1) are prepared and refrigerated.

(4:00 P.M.) Fistulated steer is fed a quantity of hay which would be consumed in approximately 3 hours.

b) Day of initiation of fermentation run,

(7:00 A.M.) Solutions (1) to (8) (Table 1) are mixed in a 2-liter Erlenmeyer flask in quantities necessary for the inoculation of 40 fermentation tubes. The resultant mixture is placed in a water bath (40°C) and CO_2 bubbled through the flask contents. Phosphate buffer solution (2.5 liters) is conditioned as previously described, with 1.5 liters prepared in a preheated thermos container for transportation to barn.

(8:30 A.M.) Fistulated steer is sampled (approximately 15 hours since last meal) and P.B.E. of solid ingesta prepared.

(8:00 A.M.) P.B.E. is centrifuged in Sharples supercentrifuge and bacterial sediment resuspended in phosphate buffer to constitute inoculum. Inoculum and solutions (9) and (10)[1] are added to flask containing nutrient medium. Distilled water is added to bring flask contents to 2 liters (pre-calibrated line etched on neck of flask).

(8:15 A.M.) Nutrient medium and inoculum mixture are placed on magnetic stirrer (teflon-coated magnet placed in flask) and attached by means of a delivery tube to an automatic pipeting machine (Brewer) previously adjusted to dispense 25 ml.

(8:25 A.M.) Nutrient medium and inoculum dispensed into fermentation tubes by pipeting mixture into all tubes in sequence, with this step repeated to bring total tube volume to 50 ml. (Figure 6).

In those trials in which the effect of various supplements on cellulose digestion are bring determined, the particular nutrient(s) under study are not included in the nutrient medium and inoculum mixture. The total amount of mixture is adjusted to either 1600 or 1800 ml. and the automatic pipet adjusted to deliver 40 or 45 ml. per tube, respectively. After dispensing the basal medium, the supplements or distilled water are then hand-pipeted into the appropriate tubes so that the final volume of all tubes is 50 ml.

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[1] Iron and calcium solution (10) is added to the mixture last because of the tendency of these elements to precipitate in the more concentrated solution.

(8:30 A.M.) Two drops of mineral oil are added to each tube to prevent foaming. The fermentation tubes are fitted with delivery tubes connected to CO_2 source, contents well mixed by swirling, and placed in rack in water bath. Fermentation period timing is initiated with the completion of this step.

During the first 8 hours of the fermentation period, the fermentation tube contents are mixed hourly by swirling the tube gently. This is repeated at the 12th and 24th hour of the fermentation period. Unless otherwise noted, all fermentation periods are terminated after 30 hours.

6. Termination of fermentation.

At the conclusion of the fermentation period, the tubes are removed from the water bath, with any adhering material washed from the CO_2 delivery tube and fermentation tube sides. The fermentation tubes are then immediately centrifuged at 2200 r.p.m. for 6 minutes, after which the supernatant liquid is discarded and the residue contained in the bottom of the tubes (Figure 9) either analyzed immediately for cellulose or refrigerated for subsequent analysis.

7. Cellulose analysis.

The cellulose content of the fermentation residue (and original substrate as well) are determined according to a slight modification of the method described by Crampton and Maynard (1938). Because of the importance of this analysis in the determination of *in vitro* results, a brief outline of the modified method is as follows:

a) Acid digestion.

The acid digestion mixture is prepared by mixing 650 ml. of

acetic acid, 150 ml. of distilled water, and 80 ml. of concentrated nitric acid. Using an automatic pipet (Machlett), 25 ml. of the mixture is dispensed into each fermentation tube (tubes analyzed in series of 8). A glass stirring rod is inserted in each tube and the contents are well mixed, with the stirring rods left in the fermentation tubes during the entire digestion period.

Eight tubes, placed in a stainless steel wire basket are immersed in a boiling water bath for a 30-minute period (Figure 10). Contents of the tubes are mixed every 10 minutes. At the end of the digestion period the tubes are removed from the boiling water bath and allowed to cool for 5 minutes.

b) Filtration.

After the addition of 25 ml. of 95% ethanol to each tube, they are immediately transferred quantitatively to a filtering crucible (Selas - extremely coarse porosity), using a polyethylene wash bottle containing 95% ethanol to wash down the sides of the tubes (Figure 10).

The precipitate in the crucible is then washed with approximately 10 ml. each of acetone and ethyl ether, in succession.

c) Drying and ashing.

The crucibles were next dried in a vacuum oven at 95°C for approximately 4 hours, after which they are cooled in a desiccator and weighed. They are then ashed overnight in a muffle furnace (600°C), cooled in a desiccator and reweighed.

8. Calculations.

a) Cellulose content.

The cellulose content of either the initial unfermented substrate or of the fermentation residue is calculated as the loss on ashing in the cellulose determination, as follows:

Cellulose (g.) = Wt. (g.) dry crucible and contents
- Wt. (g.) ashed crucible and contents

$$\text{Cellulose (\%)} = \frac{\text{Wt. (g.) of cellulose}}{\text{Wt. (g.) of substrate}} \times 100$$

b) Cellulose digestibility.

Cellulose digestibility (%) =

$$\frac{\text{Wt. (g.) initial cellulose - Wt. (g.) cellulose residue}}{\text{Wt. (g.) initial cellulose}} \times 100$$

9. Statistical analysis.

Analysis of variance procedures used are as described by Crampton (1959). Where required, treatment means are compared using the Multiple Range Test (L.S.R.) developed by Duncan (1955).

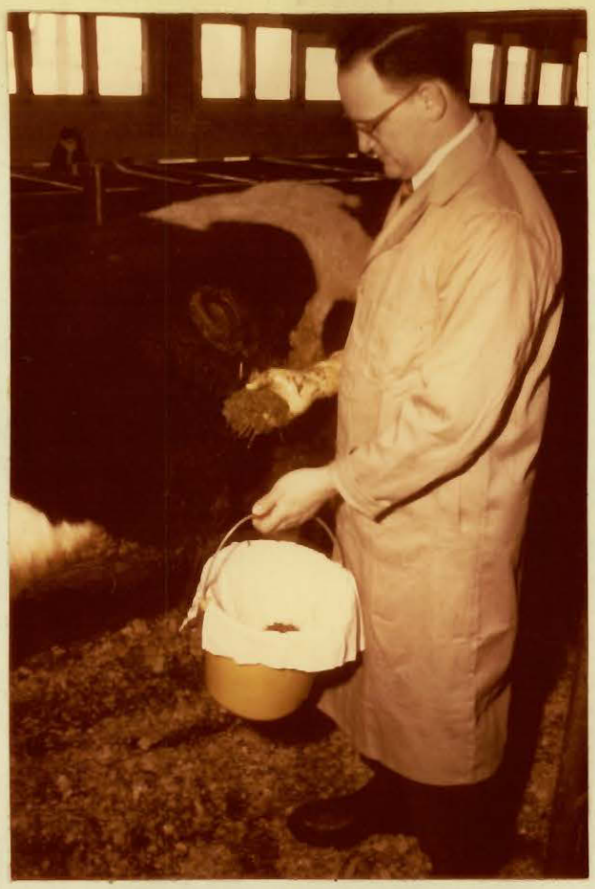

Figure 1. Removing sample of rumen ingesta from fistulated steer.

Figure 2. Solid rumen ingesta used in preparation of "phosphate buffer extract".

Figure 3. Phosphate buffer extract pressed and filtered into thermos container for transportation to laboratory.

Figure 4. Phosphate buffer extract passing through super-centrifuge with cell-free supernatant discharging into bucket.

Figure 5. Removal of celluloid liner (containing bacterial sediment) from inside of supercentrifuge bowl.

Figure 6. Use of automatic pipeting machine to dispense nutrient medium-inoculum mixture to fermentation tubes.

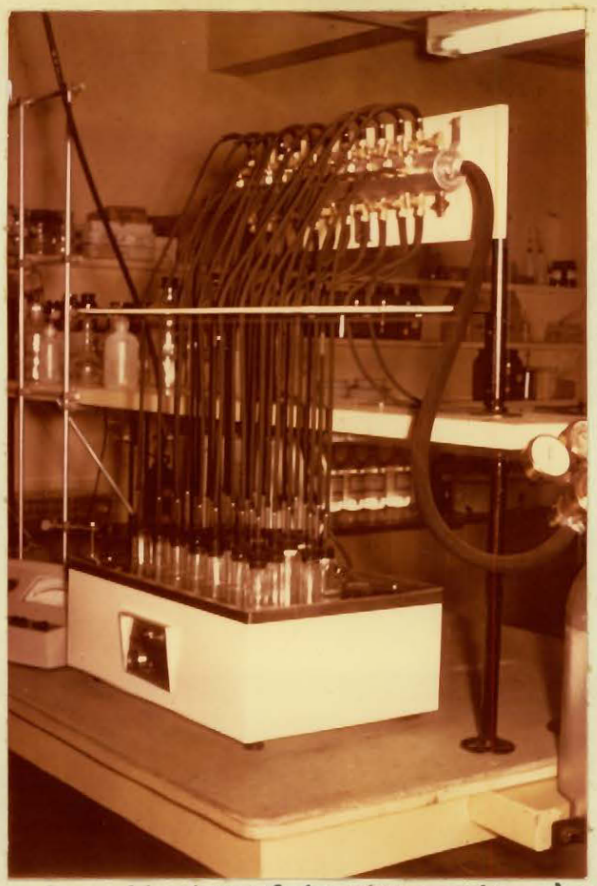

Figure 7. Overall view of *in vitro* unit, showing gas manifold and distribution tubes, fermentation tubes, and water bath.

Figure 8. Close-up view of several fermentation tubes fitted with gas delivery tubes, and tube containing miniature pH electrodes.

Figure 9. Undigested cellulose residue sedimented by centrifugation following termination of fermentation period.

Figure 10. Cellulose analysis: Eight tubes (containing acid digestion mixture) immersed in boiling water bath (foreground).
Filtration of acid digestion residue (background).

C. TRIAL 1. THE EFFECT OF VARIOUS SUPPLEMENTS ON THE IN VITRO DIGESTION OF PURIFIED CELLULOSE.

1. Introduction.

Bentley et al. (1955) observed a low level of in vitro cellulose digestion when a purified nutrient medium and resuspended cell inoculum was used, due to the absence of "rumen liquid factors" necessary for maximum cellulolytic activity. The addition of valeric acid to the medium resulted in a level of cellulose digestion equal to that observed when cell-free rumen liquid was used. Dehority et al. (1957) demonstrated that a mixture of several amino acids could substitute for valeric acid with a similar stimulatory effect. Hall et al. (1954, 1955) reported that casein hydrolysate added to the in vitro medium resulted in a "considerable increase in cellulose digestion."

The purpose of Trial 1 was to ascertain the relative and possible additive effects of these supplements on the digestion of purified cellulose in the in vitro system as previously described.

2. Experimental procedure.

This trial deviated from the general procedures described in IV, B in the following respects:

a) Nutrient medium.

Solutions (4) casein hydrolysate, and (5) valeric acid, were not included in the medium but added separately to certain fermentation tubes.

All of the substances in solutions (1), (2), (3), (6), (7), and (10), were added in half the amounts per tube indicated in Table 1.

No phosphate buffer (9) was added to the nutrient medium, and

only 200 mg. of sodium carbonate (8) was added per tube.

b) Substrate and inoculum.

Bacterial inoculum was used at a level of 5 ml. per tube, with Solka Floc at 500 mg. to supply 470 mg. of cellulose substrate. Total tube volume was 35 ml.

c) Supplements.

The amino acid mixture, based on that reported by Dehority et al. (1957), was as follows (Table 2):

TABLE 2

COMPOSITION OF AMINO ACID MIXTURE.

Amino acid	mg./ml.
L Leucine	2
L Isoleucine	2
L Valine	4
L Proline	4
Total	12 mg./ml.

The levels of the various supplements used are indicated in Table 3.

The supernatant, used as a supplement, was the cell-free liquid obtained from the centrifugation of the bacterial cells, and thus served as a source of "rumen liquid factors."

d) Fermentation runs.

This trial consisted of 3 fermentation runs, with 4 tubes per treatment in each run.

3. Results and discussion.

The results of this trial are summarized in Table 3, and graphically illustrated in Figure 11. Individual cellulose digestibility determinations appear in Appendix Table 1a, with the results of the statistical analysis of the data presented in Appendix Table 2a.

TABLE 3

EFFECT OF VARIOUS SUPPLEMENTS ON THE IN VITRO DIGESTION OF PURIFIED CELLULOSE (TRIAL 1).

Supplements:		Amount added per tube							
valeric acid	(mg.)	0	0	2.5	5	2.5	0	2.5	0
casein hydrolysate	(mg.)	0	0	0	0	0	0	50	50
amino acid mixture	(mg.)	0	0	0	0	0	12	12	0
supernatant	(ml.)	0	5	0	0	5	0	0	0
Cellulose digestion[1],[2] (%)		27	56	70	76	78	78	79	82

[1] 12 observations per treatment.

[2] Any two means not underscored by the same line are significantly different (P = .01)

Although all supplements resulted in a highly significant increase in cellulose digestion when compared to the basal medium, the most striking feature of this trial was the magnitude of these increases ranging from 100 to 200%. The ability to compare the relative stimulatory activity of the various supplements is limited by their similar effect as well as the highly significant interaction observed between supplementation and fermentation runs. Of interest is the observation that np other supplement or combination of supplements, at the levels used, could consistently account for the activity of the casein hydrolysate.

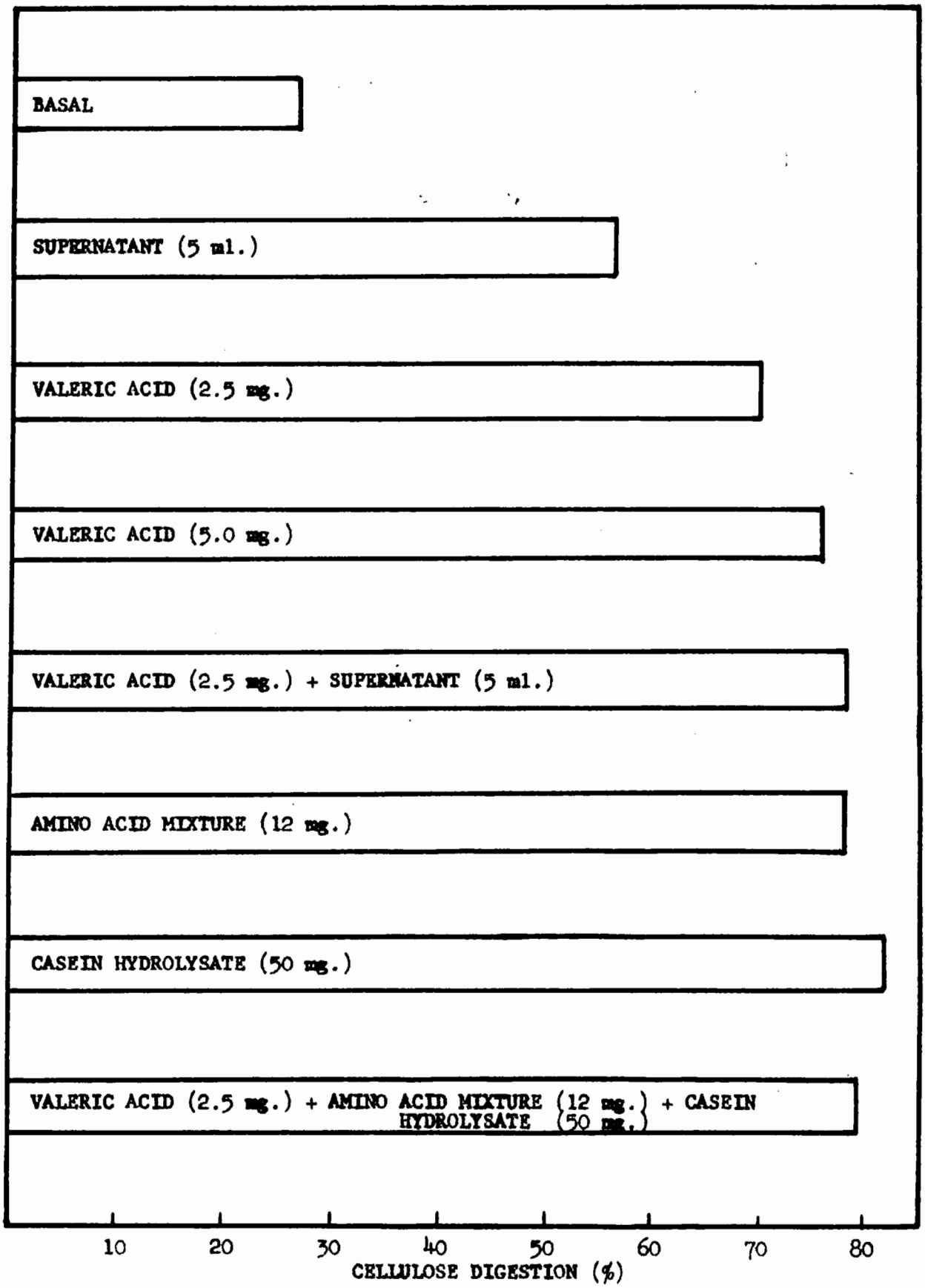

Figure 11. The effect of various supplements on the in vitro digestion of purified cellulose (TRIAL 1).

The relatively low potency of the supernatant is probably a manifestation of the 7-fold dilution of this material by the fermentation mixture, as well as the possibility that this material prepared from a phosphate buffer extract of rumen ingesta did not have as high a nutrient concentration as the original rumen liquid. The supernatant used by Bentley at al. (1955) was a product of the original rumen liquid.

D. TRIAL 2. THE EFFECT OF VARIOUS SUPPLEMENTS AND FORAGE FED TO INOCULUM DONOR STEER ON THE IN VITRO DIGESTION OF PURIFIED CELLULOSE.

1. Introduction.

With the modification by Quicke et al. (1959a) of the in vitro procedure used at the Ohio Agricultural Experiment Station, the suggested changes were adopted in the system being used in these studies.

This trial was a continuation of the study of the effect of supplementation of the basal medium as a test of its nutritional adequacy in supporting maximum cellulose digestion. Another factor studied was the possible effect on in vitro cellulose digestion of the forage fed to the inoculum donor steer. This factor was introduced since the original forage used was not available during the latter two replicates of this trial.

2. Experimental procedure.

This trial followed the general procedure as described in IV, B., with the following additions and modifications:

a) Substrate.

Purified cellulose (Solka Floc) was used at a level of 500 mg. per tube to supply 470 mg. of cellulose substrate.

b) Supplements.

The levels of the various supplements studied are indicated in Table 4. The composition of the amino acid mixture was as listed in Table 2.

c) Fermentation runs.

This trial consisted of 4 fermentation runs, 2 conducted in the summer of 1958, and 2 in the summer of 1959. Each fermentation run contained 4 tubes for each of the treatments.

d) Forage fed to inoculum donor steer.

The chemical analysis of several components of the alfalfa hays fed the inoculum donor steers during the summers of 1958 and 1959 is listed in Appendix Table 3a. The hay fed in 1958 (No.2) was characterized by a lower protein content than that used in 1959 (No.3). Since this was the most obvious difference between these hays, they are referred to as "low-protein" and "high-protein" alfalfa hay, respectively. This terminology is used only in a relative sense.

3. Results and discussion.

The results of this trial are summarized in Table 4, and graphically illustrated in Figure 12. Individual cellulose determinations appear in Appendix Table 1b, with the results of the statistical analysis presented in Appendix Table 2b.

TABLE 4

EFFECT OF VARIOUS SUPPLEMENTS AND FORAGE FED TO INOCULUM DONOR STEER ON THE IN VITRO DIGESTION OF PURIFIED CELLULOSE (TRIAL 2).

Supplements:	Amount used per tube						
valeric acid (mg.)	0	0	0	20	15	0	0
casein hydrolysate (mg.)	0	0	0	0	0	50	100
amino acid mixture (mg.)	0	24	12	0	0	0	0
Forage fed inoculum donor:							
Low protein (No.2)	16	67	71	84	84	90	92
High protein (No.3)	16	67	73	82	84	91	92
Average [1,2]	16	67	72	83	84	90	92

[1] 16 observations per treatment.

[2] Any two means not underscored by the same line are significantly different (P = 0.01).

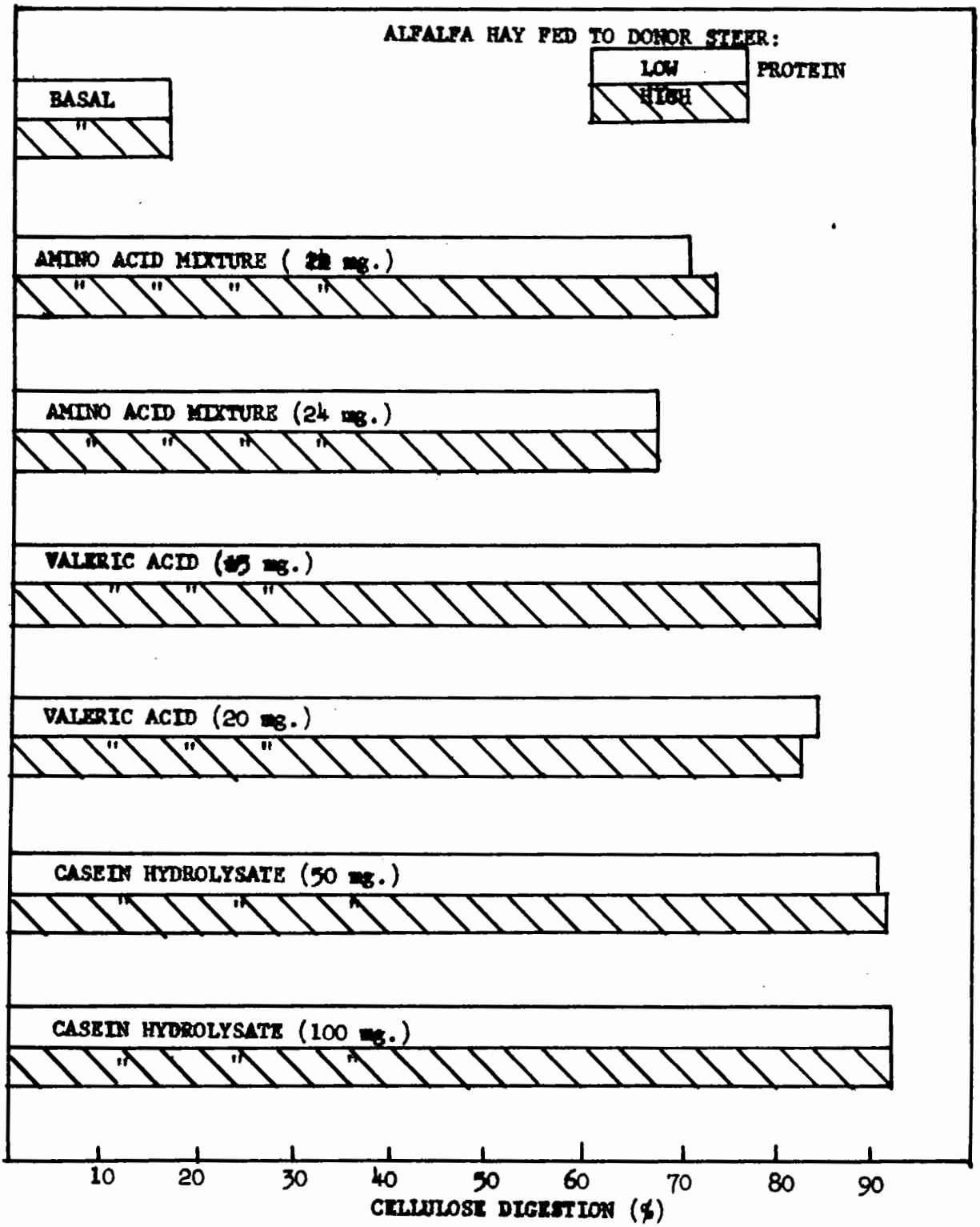

Figure 12. The effect of various supplements and forage fed to inoculum donor steer on <u>in vitro</u> cellulose digestion.

Although there were many marked similarities between this trial and the previous one (Trial 1), no attempt will be made to compound the data because of quantitative differences in the in vitro procedures used in the two trials. These differences included the total liquid tube volume (35 ml. vs. 50 ml.) resulting in dissimilar levels when concentration of the supplements are expressed on a mg./ml. basis. Other differences between the two trials were the level of nutrients in the nitrient medium and level of some of the supplements.

As observed in Trial 1, the most obvious result was the stimulatory effect of any of the supplements when compared to the basal medium. Each supplement was highly significantly different ($P = 0.01$) from the other, with the amino acid mixture the least effective, the casein hydrolysate the most effective, and valeric acid in an intermediary position.

Among the supplements, doubling the level of the amino acid mixture had a highly significant ($P = 0.01$) depressing effect on cellulose digestion. In order to determine if inhibition, in fact, was also occurring at the lower level (12 mg./tube), a total of 8 fermentation tubes containing 6 mg. of the amino acid mixture were tested in two of the fermentation runs (not included in the statistical analysis because of incomplete replication). With this level of supplementation, average cellulose digestion was 70%, suggesting that the optimum amino acid mixture level was between 6 and 12 mg., under the conditions of this experiment. Inhibition of in vitro cellulose digestion has been noted by Dehority et al. (1957) as a result of high levels of casein hydrolysate and certain

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amino acids, and by McLeod and Murray (1956) when the concentration of the amino acids valine, leucine and isoleucine was doubled in their fermentation mixture.

No significant difference was observed between the results obtained from runs in which the inoculum donor steers were fed different forages, although this observation was also confounded with time.

Of interest, is the extremely low experimental error observed in this trial, as shown by the standard deviation of ± 1.9. This contributed, in part, to the highly significant interaction between treatments and fermentation runs. This interaction was considered to have no practical meaning.

E. TRIAL 3. THE EFFECT OF VARIOUS COMBINATIONS OF VALERIC ACID AND CASEIN HYDROLYSATE ON THE IN VITRO DIGESTION OF PURIFIED CELLULOSE:

1. Introduction.

Bentley et al. (1955) reported that valeric acid added to the nutrient medium could account for all of the cellulolytic activity present in cell-free rumen liquid (centrifuge supernatant). In contrast, the results of Trials 1 and 2, as reported here, indicated that casein hydrolysate resulted in slightly greater cellulose digestion than valeric acid.

In Trial 3, a series of observations were made on the effect of valeric acid and combinations of valeric acid and casein hydrolysate, in order to define a nutrient medium which supported maximum in vitro cellulose digestion. The level of cellulose substrate used in this trial, and all subsequent trials, was reduced to the level suggested by Quicke et al. (1959a).

2. Experimental procedure.

As described in IV, B., with the following additions and modifications:

a) Substrate.

Purified cellulose (Solka Floc) was used at a level of 210 mg. to supply a cellulose substrate level of 200 mg. per fermentation tube.

b) Fermentation runs.

The observations made in this trial did not constitute an entire fermentation run, but were a small part of 3 fermentation runs, with the remainder of the data presented elsewhere. The effect of treatments (supplementation) studied in this trial was determined in duplicate tubes in each of the fermentation runs.

3. Results and discussion.

The results of this trial are summarized in Table 5, with the individual observations appearing in Appendix Table 1c. Statistical analysis of the data is presented in Appendix Table 2 c.

TABLE 5

THE EFFECT OF VARIOUS COMBINATIONS OF VALERIC ACID AND CASEIN HYDROLYSATE ON THE IN VITRO DIGESTION OF PURIFIED CELLULOSE (TRIAL 3).

Supplements:	Amount added per tube			
valeric acid (mg.)	15	30	15	15
casein hydrolysate (mg.)	0	0	100	50
Cellulose digestion[1,2] (%)	93	94	95	96

[1] 6 observations per treatment.

[2] Any two means not underscored by the same line are significantly different. (P = 0.01).

These observations were not intended to encompass all possible levels and combinations of valeric acid and casein hydrolysate, but rather to determine if the nutrient medium used by Quicke et al. (1959a), containing 15 mg. of valeric acid per tube, could be improved on by the addition of casein hydrolysate, as indicated in previous trials. The most useful medium would be one that could be defined in terms of its actual chemical composition. Any additions of casein hydrolysate (an undefined mixture of nitrogenous compounds), would thus have to be justified by its positive contribution to the medium in supporting maximum cellulolytic activity.

As shown in Table 5, the addition of 50 mg. of casein hydrolysate to the basal level of 15 mg. valeric acid per tube, did result

in a slight but highly significant ($P = 0.01$) increase in cellulose digestion. Based on this observation, these levels of casein hydrolysate and valeric acid were adopted in the nutrient medium (as indicated by Table 1), in those studies where information other than the effect of nutrient supplementation was required.

F. TRIAL 4. THE EFFECT OF CASEIN HYDROLYSATE SUPPLEMENTATION AND FORAGE FED TO INOCULUM DONOR STEER ON THE IN VITRO DIGESTION OF NATIVE FORAGE CELLULOSE.

1. Introduction.

The preceding trials were intended to test the adequacy of the nutrient medium to support maximum digestion of purified cellulose. Trial 4 was an extension of these studies, utilizing as substrate native cellulose of 5 species of dried forages.

Major factors studied in this trial were the necessity of adding casein hydrolysate to the nutrient medium containing valeric acid, and the effect on cellulose digestion of different forages fed the inoculum donor steer.

2. Experimental procedure.

As described in IV, B., with the following additions and modifications:

a) Substrates.

The five forage substrates used in this trial (alfalfa, red clover, birdsfoot trefoil, bromegrass, and timothy) are described in Appendix Table 3b (samples No.1-5).

b) Nutrient medium.

The nutrient medium designated as the control contained all the substances listed in Table 1 except casein hydrolysate, which was added separately to half the fermentation tubes.

c) Fermentation runs.

This trial consisted of 4 fermentation runs, 2 conducted in the summer of 1958, and 2 in 1959. Each fermentation run contained 2 tubes for each treatment.

d) Forage fed to inoculum donor steer.

The chemical analysis of several components of the alfalfa hays fed the inoculum donor steers during this trial is listed in Appendix Table 3a. As previously described in Trial 2, these forages are designated as low and high protein, these terms being used in a relative sense only.

3. Results and discussion.

The results of this trial are summarized in Table 6, with the individual observations appearing in Appendix Table 1d. Statistical analysis of the data is presented in Appendix Table 2d.

TABLE 6

EFFECT OF CASEIN HYDROLYSATE SUPPLEMENTATION AND FORAGE FED TO INOCULUM DONOR STEER ON THE IN VITRO DIGESTION OF NATIVE FORAGE CELLULOSE (TRIAL 4).

Forage fed donor steer	Nutrient medium	Forage Cellulose Digestion (%)				
		Alfalfa (1)	R.Clover (2)	B.Trefoil (3)	Brome (4)	Timothy (5)
Low protein alfalfa	Control	56[1]	57	56	54	53
	+ Casein hydrolysate	56	57	61**	61**	61**
High protein alfalfa	Control	57	63	63	62	60
	+ Casein hydrolysate	57	62	61	62	62

[1] 4 observations per treatment.

** Highly significant increase in cellulose digestion due to casein hydrolysate ($P = 0.01$).

Perhaps the most important observation in this trial was the highly significant increase in digestion of the cellulose of certain

forages due to the addition of casein hydrolysate when the low-protein alfalfa hay served as feed for the inoculum donor steer. Alternatively, this effect might be interpreted as a depression of cellulose digestion due to the absence of casein hydrolysate in the medium. In contrast, the lack of casein hydrolysate had no effect on the digestion of any of the forages when inoculum was prepared from the steer fed the high protein alfalfa hay.

Examination of the chemical composition of the forages (Appendix Table 3b) indicates that thpse substrates responding to supplementation were lowest in protein content, with the degree of the effect (increase or depression) inversely proportional to protein content. On the basis of this observation, a possible explanation of the results obtained in this trial might be that the nutrient medium prepared with inoculum obtained from the steer fed the lower protein hay was deficient in either total nitrogen or some particular nitroganous compound. This compound could have been supplied either by the native protein of the substrate, or by the addition of casein hydrolysate when the substrate itself was deficient in the substance. The inoculum prepared from the steer fed the higher protein hay might have contributed to a carry-over of this substance from the original phosphate buffer extract.

This explanation does not account for the fact that no such effect was observed in Trial 3, when purified cellulose (containing no native protein) was subjected to similar treatments. A source of non-protein nitrogen (urea) was present in all trials, but apparently did not correct any deficiency in Trial 4. The difference in results between these two trials might be related in some way to

the microbial population proliferating in the fermentation tubes. With the purified cellulose substrate, proteolytic bacteria would be eliminated from the fermentation mixture, while the presence of these organisms would be indicated in tubes containing native forage protein.

Due to the fact that cellulose digestion was the only criterion observed in these trials, no direct explanation of the results can be offered.

G. SUMMARY.

The purpose of the experiments described in this section (IV) was to study the effect of various factors on the *in vitro* cellulose digestion of both purified and native forage cellulose substrates. This information was sought in order to define various procedures as well as to determine the nutrient composition of the *in vitro* medium capable of supporting maximum and consistent cellulolytic activity. Definition of the *in vitro* procedures was considered necessary before studies of forage nutritive value could be instigated.

Because of the preliminary nature of this work, principles of proper experimental design could not be adhered to in many cases. This resulted in confounding and incomplete replication of several factors. This difficulty was aggravated by the unexpected nature of some of the results, as illustrated by the fact that many comparisons made in this section were not originally anticipated, i.e. the affect of forage fed inoculum donor steers on cellulose digestion.

Using the *in vitro* system as defined in this section, it was shown that the nutrient medium used by the Ohio workers (Quicke *et al.*, 1959a) could be improved upon by the addition of 50 mg. of casein hydrolysate per fermentation tube, and that the cellulolytic activity due to the addition of the casein hydrolysate could not be accounted for by added valeric acid or a mixture of 4 amino acids. In studies with purified cellulose substrate, it was shown that levels of valeric acid higher than 15 mg. per tube did not result in any further increase in cellulose digestion. Increasing the amino acid mixture level over a total of 12 mg. per tube resulted in a decrease in cellulose digestion.

When the cellulolytic activity of inoculum prepared from steers fed a relatively low-protein alfalfa hay was compared to that of donor steers fed higher protein alfalfa hay, the following observations were made:

1. With a purified cellulose substrate, no differences due to inoculum source were noted when valeric acid, casein,hydrolysate, or amino acid mixture were added to the basal medium.

2. With native forage cellulose substrates, however, several of the forages (generally those lower in protein content) responded significantly to casein hydrolysate supplementation - when low-protein hay served as the inoculum donor's feed.

3. Cellulose digestion for all forages appeared maximal - regardless of supplementation - when the inoculum was obtained from the donor steer fed the higher protein alfalfa hay.

The differential effect of casein hydrolysate supplementation on native forage cellulose digestion appeared to be related to a nitrogenous substance(s) present either in the forage substrate, inoculum, or casein hydrolysate. When not supplied by any one of these sources, lack of this substance in the fermentation mixture apparently can result in a depression of cellulolytic activity.

Summarizing the preliminary information presented in this section, it would appear that in order to obtain maximum digestion of all cellulose substrates, the nutrient medium of Quick et al. (1959a) should be supplemented with casein hydrolysate, and in addition, the bacterial inoculum should be prepared from steers fed a high-protein alfalfa hay.

V. PREDICTION OF THE NUTRITIVE VALUE OF A FORAGE FROM IN VITRO RUMEN FERMENTATION DATA.

A. TRIAL 5. THE ESTABLISHMENT OF A CORRELATION BETWEEN THE IN VITRO CELLULOSE DIGESTIBILITY OF A FORAGE AND ITS NUTRITIVE VALUE AS DETERMINED IN VIVO.

1. Introduction.

There have been many successful attempts in recent years to correlate *in vitro* forage cellulose or dry matter digestibility with various digestibility coefficients (cellulose, dry matter, energy) as determined *in vivo*. These procedures are generally limited not by the accuracy or precision of the *in vitro* techniques, but rather by the failure of the chosen *in vivo* criterion to completely describe forage nutritive value.

That the Nutritive Value Index (N.V.I.), as proposed by Crampton *et al.* (1960), is the most complete description of forage nutritive value which can be determined practically, is suggested by the fact that it takes into consideration both the digestible energy content and the extent to which a forage will be voluntarily consumed. The N.V.I. is thus a measure of the total digestible energy potential of a forage.

Crampton (1957) has postulated that the voluntary consumption of a forage is limited by the rate of microbial digestion of its cellulose and hemicellulose components. Trial 5 was designed to compare the rate of *in vitro* cellulose digestion of forages with their N.V.I. and its components (Relative Intake and energy digestibility) as determined *in vivo*.

2. Experimental procedure.

The *in vitro* system and procedures used in this trial were as described in Section IV, part B, with the following additions and

modifications:

a) Substrates.

Forage samples were used to supply cellulose substrate level of approximately 800 mg. per fermentation tube. The forage samples used in this trial had been collected during the in vivo sheep feeding trials in which voluntary consumption and digestibility of proximate principles and energy had been determined. These forages consisted of 5 harvested in 1956, artificially dehydrated, and fed to sheep in the chopped form (Smith, 1958) and 4 harvested in 1957, and fed under similar conditions (Beacom, 1959). The N.V.I.'s and their components (Relative Intake and energy digestibility), as determined for these forages in vivo, are presented in Table 7. Their chemical composition is given in Appendix Table 3b (samples 1-9). The digestibility of purified cellulose (Solka Floc) was determined in all fermentation runs.

b) Forage fed to inoculum donor steer.

"High-protein" alfalfa hay (No.3)[1] was fed to an inoculum donor steer during the entire course of this trial.

c) Fermentation periods and runs.

A randomly selected tube of each forage tested in a fermentation run was withdrawn after 3, 6, 12, 24, and 48 hours of fermentation. A single determination at each time period for 5 different forages and purified cellulose was made in each run, with 4 runs constituting a test. Data for the 9 forages studied in this trial were thus collected in 2 tests of 4 runs each.

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[1] Appendix Table 3a.

TABLE 7

RELATIVE INTAKE, ENERGY DIGESTIBILITY, AND N.V.I. OF FORAGES AS DETERMINED IN VIVO.

Forage	Relative Intake	Energy Digestibility (%)	N.V.I.
1956:			
(1) Alfalfa	79	63	50
(2) Red clover	106	67	71
(3) Birdsfoot trefoil	99	63	63
(4) Bromegrass	71	60	43
(5) Timothy	56	61	34
1957:			
(6) Red clover, early bloom	98	55	54
(7) Red clover, late bloom	92	53	49
(8) Timothy, early bloom	66	58	38
(9) Timothy, late bloom	69	50	34
Pooled standard deviation:	±10	±2	±7

3. Results and discussion.

Cellulose digestibility coefficients after the various fermentation periods are summarized in Table 8 for all forages and purified cellulose. These data are graphically illustrated in Figure 13 (1956 forages) and Figure 14 (1957 forages). Individual cellulose digestibility determinations are presented in Appendix Table 1e.

As illustrated in the graphic presentations, lag periods in the start of cellulose digestion appear to be related to forage species; i.e. initiation of cellulose breakdown in the grasses 9 bromegrass and timothy - lags several hours behind that of the leguminous species. This differential fermentation start is reflected in the level of cellulose digested up to the 12-hour determination. Once active fermentation started, there appears to be only minor differences in rate of digestion trends between the forages. After 12 hours, the rate of digestion decreased(except with purified cellulose), with little if any digestion observed between 24 and 48 hours for the leguminous species. The greater rise in digestion between 24 and 48 hours observed for the 1956 timothy and bromegrass samples is of questionable significance, because of the lengthy time interval involved. That the decrease in rate of digestion after 12 hours is related to lignification of the forages is suggested by the behavior of the unlignified Solka Floc (94% cellulose), which in the early stages of fermentation was closely related to the grasses, but unlike them did not increase in fermentation rate until it has been digested almost to completion. It is of interest that the general characteristics of these fermentation curves are also found upon examination of *in vitro* cellulose digestibility time curves presented by Hershberger *et al.* (1959).

TABLE 8

IN VITRO CELLULOSE DIGESTIBILITY OF FORAGE AND PURIFIED CELLULOSE SUBSTRATES AT VARIOUS FERMENTATION TIMES. TRIAL 5.

Substrate	Fermentation time (hours)				
	3	6	12	24	48
1956:					
(1) Alfalfa	8	26	47	54	56
(2) Red clover	8	28	55	62	63
(3) Birdsfoot trefoil	6	22	51	60	62
(4) Bromegrass	0	8	37	56	68
(5) Timothy	0	8	37	56	66
1957:					
(6) Red clover, early bloom	4	20	46	53	58
(7) Red clover, late bloom	4	25	47	53	59
(8) Timothy, early bloom	1	11	38	57	63
(9) Timothy, late bloom	1	5	27	46	54
Solka Floc (purified cellulose)	1	2	37	92	100
Pooled standard deviation:		±5	±3	±2	±2

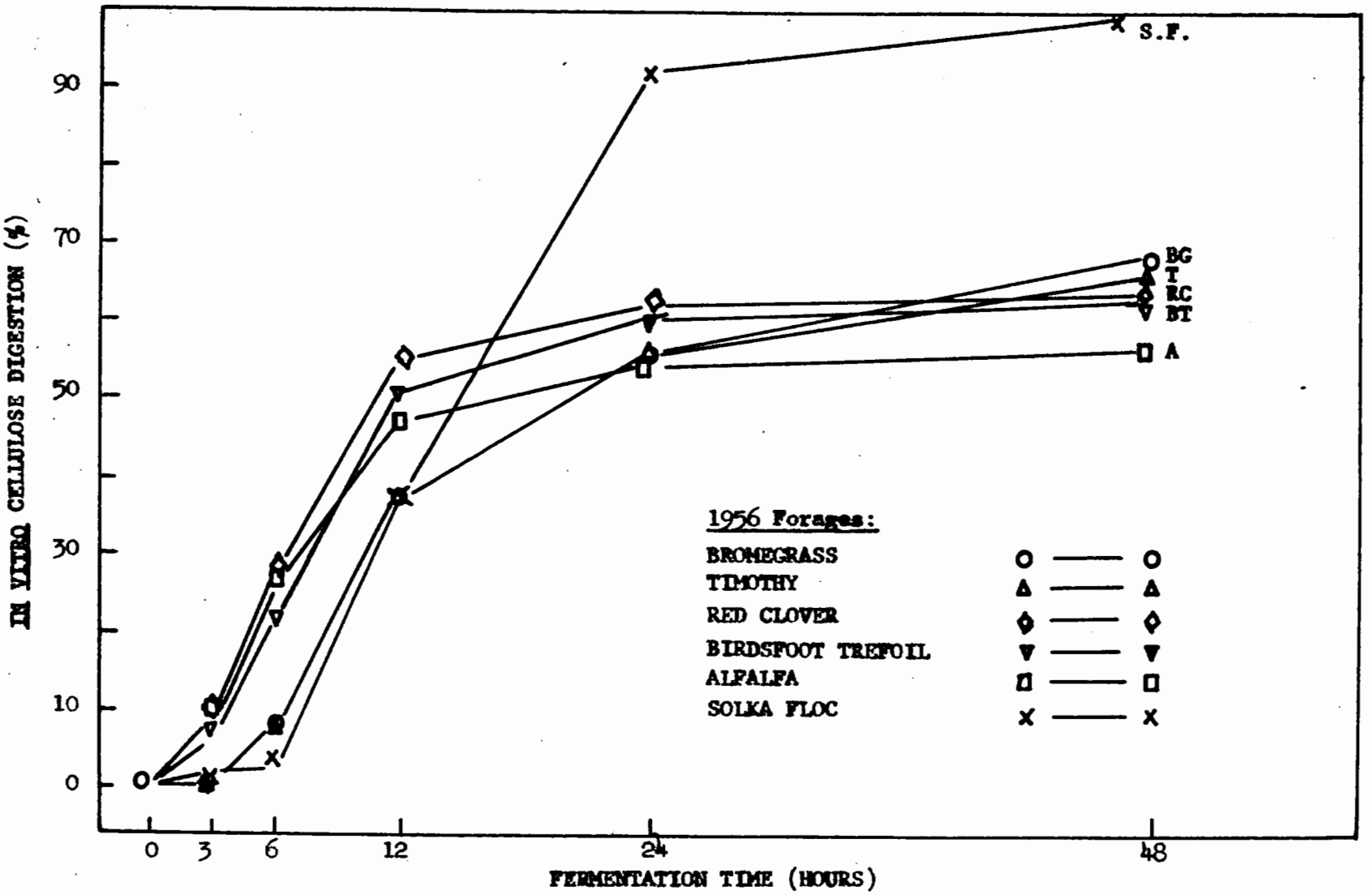

FIGURE 13. Rate of <u>in vitro</u> cellulose digestion, 1956 forages.

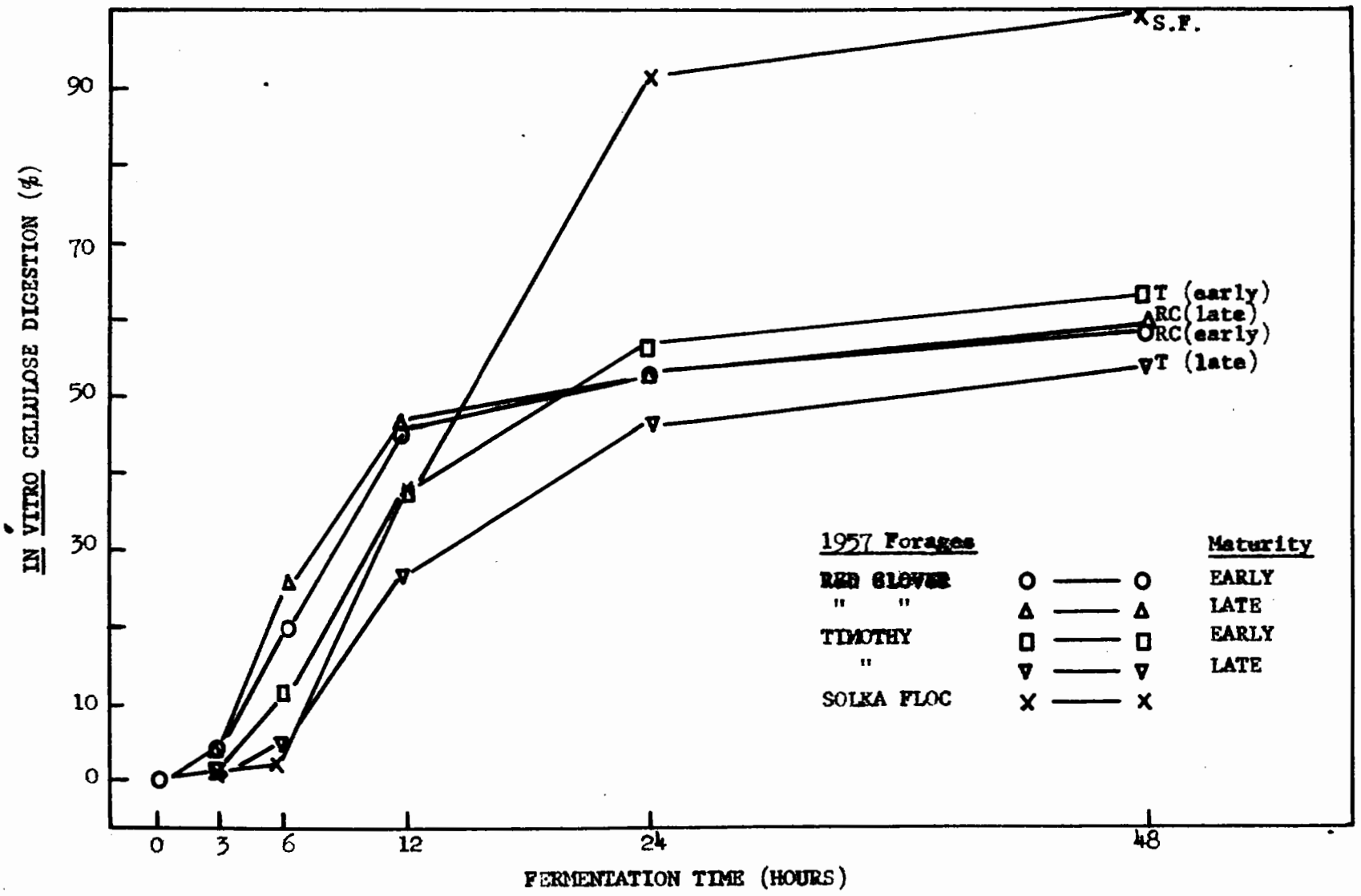

FIGURE 14. Rate of <u>in vitro</u> cellulose digestion, 1957 forages.

In order to determine what relationships existed between *in vitro* cellulose digestion and *in vivo* measures of nutritive value obtained for the same forages, simple correlation coefficients were determined between all possible pairs of the *in vitro* and *in vivo* data. Only those correlations shown to be statistically significant are presented in Table 9.

TABLE 9

SIGNIFICANT CORRELATIONS BETWEEN THE *IN VITRO* AND *IN VIVO* DATA. TRIAL 5.

Variables correlated	Coefficients r	r^2
Nutritive Value Index with:		
6-hour *in vitro* cellulose digestion	0.84**	0.71
12-hour *in vitro* cellulose digestion	0.91**	0.82
Relative Intake with:		
12-hour *in vitro* cellulose digestion	0.83**	0.69
Energy digestibility (%) with:		
24-hour *in vitro* cellulose digestion	0.87**	0.76

** Highly significant (P=0.01).

That 12-hour *in vitro* cellulose digestion was highly correlated with voluntary consumption (Relative Intake) is of great interest. If the lag periods which characterize the 12-hour *in vitro* determination also occur when a forage is introduced into the intact rumen, they may serve to explain factors which affect level of intake. This hypothesés was presented by Crampton *et al.* (1960) who suggested that reduction of rumen load, as a consequence of rate of microbial

degradation of cellulose and hemicellulose, determines the length of period between recurring hunger, and thus the voluntary consumption characteristic of a particular forage.

The highly significant correlation between 24-hour *in vitro* cellulose digestion and *in vivo* energy digestibility is one that has been demonstrated by other workers if the following assumptions are made: (i) That the 24-hour *in vitro* determination is essentially a measure of the characteristic "leveling off" of rate of digestion (Kamstra *et al.*, 1958). (ii) That *in vivo* indices such as TDN, digestible dry matter, and digestible cellulose, in fact measure or are related to the available energy content of a forage. Therefore, *in vitro* cellulose digestion at 24 hours and beyond essentially measures the digestible energy available per unit of forage consumed.

Of special interest is the highest correlation observed - i.e. that between the N.V.I. and 12-hour cellulose digestibility (Figure 15). Based on the limited data presented, this *in vitro* criterion can thus predict with a high degree of accuracy the total digestible energy potential of a forage, using the equation $Y = -7.8 + 1.31X$, where Y equals the predicted N.V.I. and X the 12-hour *in vitro* cellulose digestibility.

It is of interest that the pooled standard deviation[1] for the 12-hour *in vitro* determination is ±3 (Table 8). This within-forage variability is low, considering that each value is an average of 4 single determinations, each made in a separate fermentation run.

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[1] Average of the standard deviation obtained for each of the forages.

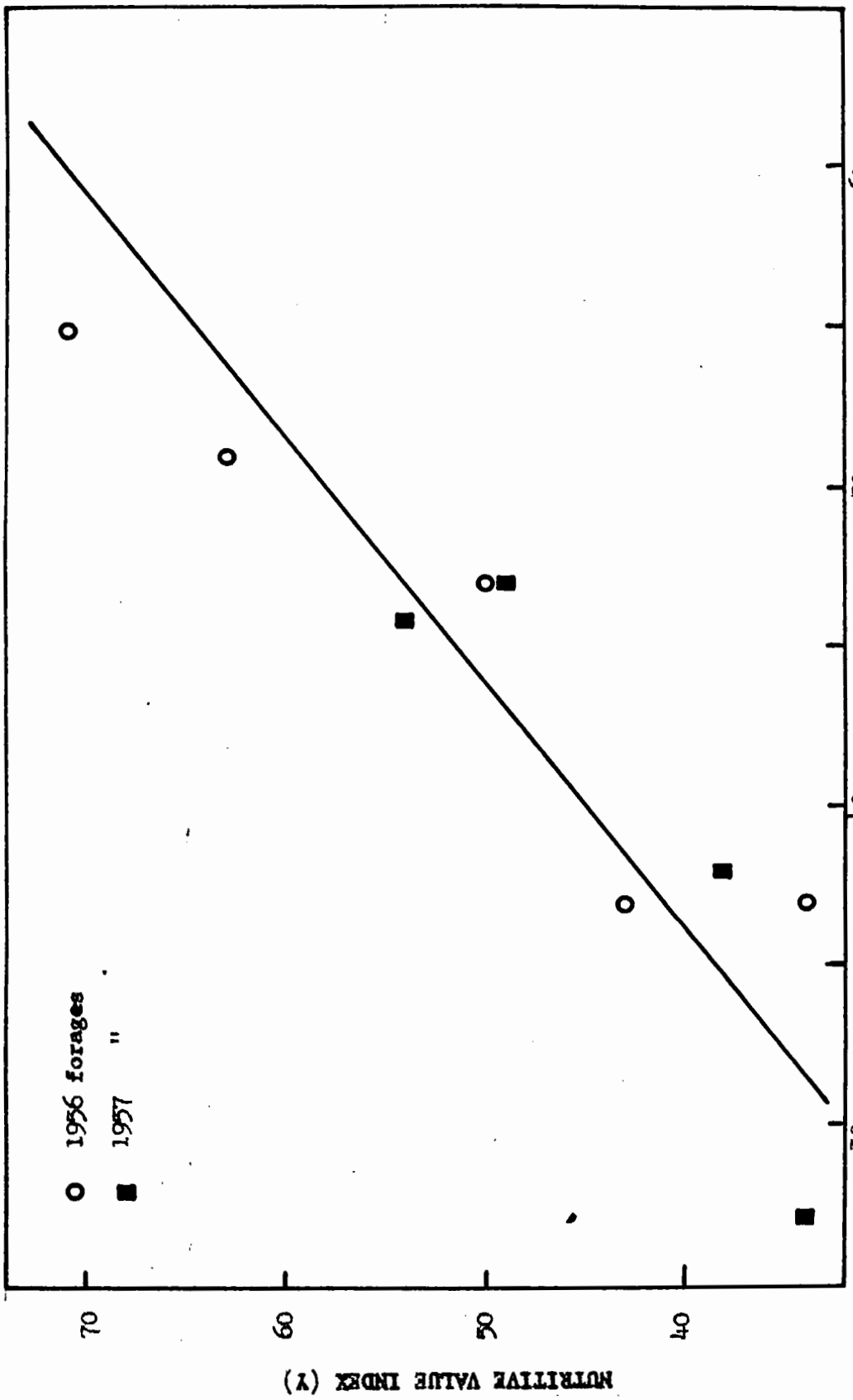

FIGURE 15. Regression of Nutritive Value Index on 12-hour *in vitro* cellulose digestion. Trial 5.

Perhaps even greater error control could be achieved if the *in vitro* cellulose digestion of a forage was determined in duplicate or triplicate within a run, with 2 or 3 fermentation runs constituting a test. In contrast, the high variability of ±10 (Table 7) is associated with the measurement of voluntary consumption (Relative Intake), and is reflected in the pooled standard deviation for the N.V.I. (±7). This shortcoming in the measurement of voluntary consumption, as described by McCullough (1959), necessitates the observation of a larger experimental animal "population" in order to obtain reliable values, and to show significant differences between forages. This fact increases the usefulness of the *in vitro* technique, since once the basic relationships between the *in vivo* and *in vitro* values are well defined, the prediction of the N.V.I. by *in vitro* procedures can be highly accurate.

Although the *in vitro* results described in this trial, and their relationships to the N.V.I. were obtained using a well-defined *in vitro* rumen fermentation procedure, other *in vitro* techniques also might be applicable. The prerequisite of a useful *in vitro* technique must be its ability to measure the effect on cellulose digestion of early lag differences between forages. Hershberger *et al.* (1959), using an *in vitro* system characterized by rumen liquid inoculum obtained from sheep, have presented fermentation time curves for cellulose digestion which indicate marked differences between forages in the early stages of digestion (less than 10 hours). As in Trial 5, these early fermentation differences of Hershberger *et al.* do not appear to be related to the ultimate *in vitro* cellulose digestibility of the forages studied. Unfortunately, it is not possible to examine

the relationship between early *in vitro* fermentation and voluntary consumption of the forages studied by these workers, since the latter criterion was not determined in their *in vivo* sheep trials.

The use of a standard substrate in all fermentation runs might enable comparisons as well as permit corrections to be made for the observed data according to some previously defined standard response. Thus, by taking into account and correcting for various levels of cellulolytic activity in different fermentation runs, it may be possible to reduce within-forage variability of the *in vitro* determinations. The only standard cellulose substrate which was readily available and chemically defined was Solka Floc. Although it is not a forage but a purified cellulose, it nevertheless was incorporated in all fermentation runs. There are several disadvantages in the use of a purified cellulose substrate, related to the observation that it does not necessarily behave similarly to native forage cellulose in the fermentation mixture. The average Solka Floc cellulose digestion at 12 hours in this trial was 37% with a standard deviation of ±6, the latter being twice that observed for the forages and suggesting that it might be over-sensitive as a standard. This would indicate that the purified cellulose substrate might respond to variations in the *in vitro* procedures which would have a smaller effect on the cellulose digestion of forage substrates.

It might be of interest to speculate as to causes of the differential lag periods observed in initiation of *in vitro* cellulose digestion of forages of varying nutritive value. In developing this *in vitro* rumen fermentation procedure for testing forages an attempt was made to eliminate variables that were not related to the nutritive

value of the substrates. To this end, the forage samples were all uniformly prepared for in vitro digestion by grinding to pass the same mill screen. Likewise, the composition of the nutrient medium was designed to support maximum digestion of a purified cellulose substrate. This latter point would indicate that no gross nutritional deficiencies due to the nutrient composition of the forages would affect the length of the lag periods, although the intricacies of the interactions between the multitude of rumen microorganisms and their specific nutrient requirements perhaps cannot be ignored.

Oglinsky and Umbreit (1954) note that in the lag phase of the bacterial growth cycle, the lag is in cell division not in the synthesis of new protoplasm. These workers suggest that factors contributing to the magnitude of the lag phase include carry-over of metabolites necessary for cell growth and division (shortening lag phase); and adaptation to new medium requiring elaboration of new enzymes for using different sources of nitrogen and carbon (lengthening lag phase). Since the in vitro rumen fermentation procedure described in these trials involves a common inoculum and nutrient medium source, it would appear that the differential lag period is related to some chemical and/or physical property characteristic of the forage substrate.

Microscopic examination of digested feed particles obtained from rumen contents (Baker and Harriss, 1947), indicated that the cellulolytic bacteria are in very close proximity or "attach" themselves to the substrate being degraded. On this basis, differential lag periods may be the result of factors affecting "attachment" of the bacteria to the forage substrate. Such factors could

include the presence and distribution of lignin and/or other complex and relatively indigestible carbohydrates. Using a purified, delignified cellulose (Solka Floc) Salsbury *et al*. (1960) shortened the lag phase in *in vitro* cellulose digestion by pre-incubation of the substrate with rumen microorganisms. Since washing or autoclaving of the pre-incubated substrate restored the original lag phase period, they suggested that the effect of pre-incubation was due to attachment of the organisms rather than to a change in the substrate.

If it can be demonstrated that the factors influencing early initiation of *in vitro* cellulose digestion are related to physical properties of the forage substrates, it might be possible to measure forage resistance to degradation by the use of cell-free cellulolytic enzyme preparations. Such an *in vitro* procedure would greatly simplify forage evaluation since fermentation with the fastidious rumen microorganisms could thus be eliminated. The present unavailability of such enzyme preparations which can actively degrade native forage cellulose substrates makes this an academic question at this time.

B. TRIAL 6. THE EFFECT OF FORAGE FED TO INOCULUM DONOR STEER ON IN VITRO CELLULOSE DIGESTIBILITY OF 1957 FORAGES AT VARIOUS FERMENTATION TIMES.

1. Introduction.

It was demonstrated in Trial 4 that the extent of *in vitro* cellulose digestibility of a forage could be influenced by the nature of the forage fed to the inoculum donor steer. In particular, the 30-hour cellulose digestibility of certain low-protein forage samples was depressed when inoculum was obtained from a steer fed a low-protein alfalfa hay. When the fermentation tubes containing these forages were supplemented with casein hydrolysate, cellulose digestion was normal.

In Trial 6, the rate of cellulose digestion, as influenced by the nature of the forage fed the inoculum donor steer, was measured for several forages for which the N.V.I. had been determined *in vivo*.

2. Experimental procedures.

This trial was run in conjunction with Trial 5, using the identical *in vitro* procedures.

a) Substrates.

The 4 forages harvested in 1957 were used in this trial (Nos. 6-9; Appendix Table 3b).

b) Forage fed to inoculum donor steer.

Inoculum used in this trial was prepared from rumen contents of a steer fed a low-protein alfalfa hay (No.2; Appendix Table 3a). Comparative data for the 1957 forage substrates obtained using inoculum prepared from the steer fed the higher protein alfalfa hay (No.3; Appendix Table 3a), were those determined in Trial 5.

3. Results and discussion.

The results of this trial are summarized in Table 10 and illustrated graphically in Figure 16.

Statistical analysis of the data (Appendix Table 2e) discloses a highly significant difference (P=0.01) between inoculum sources. This difference in cellulose digestion, in favor of the inpculum prepared from the donor steer fed the higher protein alfalfa hay, was essentially confined to the 6- and 12-hour fermentation periods. In contrast, cellulose digestion using inocula prepared from the different sources was not significantly different (P=0.01) in the 24- and 48-hour fermentation periods when the forages are compared on an individual basis. The fact that increasing fermentation time decreases the differences due to inoculum sources suggests that the differences obtained in Trial 4, after a 30-hour fermentation period, would have been of a greater magnitude if observed at an earlier fermentation time.

Since the 12-hour cellulose digestibility determination of a forage has been shown to be highly correlated (P=0.01) with its N.V.I. (Trial 5), a more detailed examination of the effect of inoculum source at this fermentation time is justified. It appears that the main effect due to inoculum source at 12 hours is the lower overall cellulolytic activity observed for the "low-protein" source inoculum, with the forages in the same relative positions regardless of inoculum source.

When the relationship between the N.V.I.'s and the 12-hour in vitro cellulose digestion coefficients (using either source of inoculum) was determined, the following correlation coefficients

TABLE 10

THE EFFECT OF FORAGE FED TO INOCULUM DONOR STEER ON IN VITRO CELLULOSE DIGESTIBILITY OF 1957 FORAGES AT VARIOUS FERMENTATION TIMES. TRIAL 6.

	Fermentation time (hours)[1]							
	6		12		24		48	
Alfalfa hay fed donor steer, protein:	L[2]	H[3]	L[2]	H[3]	L[2]	H[3]	L[2]	H[3]
Forages (1957):								
(6) Red clover, early	12	20**	38	46**	51	53	55	58
(7) Red clover, late	12	25**	42	47	50	53	53	59*
(8) Timothy, early	4	11*	32	38*	51	57*	62	63
(9) Timothy, late	0	5	17	27**	42	46	49	54

[1] Data from 3-hour fermentation period not included because of difficulty in accurately measuring low levels of cellulose digestion.

[2] Low-protein alfalfa hay (No.2).

[3] High-protein alfalfa hay (No.3). Fermentation data from Trial 5.

** Highly significant increase (P=0.01) due to high-protein inoculum source.

* Significant increase (P=0.05) due to high-protein inoculum source.

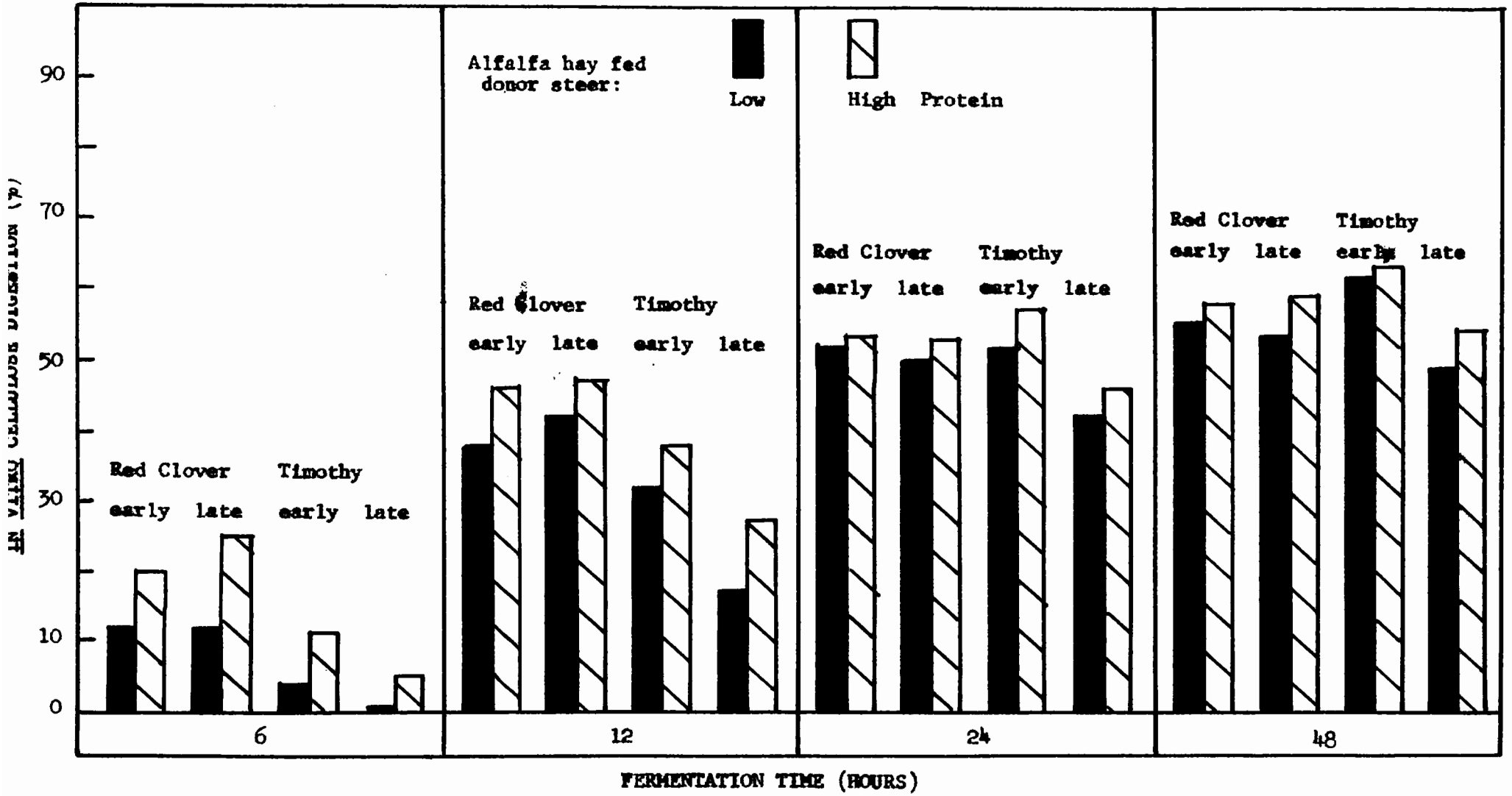

FIGURE 16. The effect of forage fed to inoculum donor steer on in vitro cellulose digestibility of 1957 forages at various fermentation times (Trial 6).

were obtained:

Inoculum from steer fed high-protein alfalfa hay, r = 0.91
" " " " low- " " " , r = 0.85.

Because of the limited number of comparisons, neither of the correlation coefficients are statistically significant (P=0.05), although they do indicate that the cellulose digestion obtained using inoculum obtained from the steer fed the higher protein hay was possibly more closely related to the in vivo N.V.I. of the forages.

In all cases, the nutrient medium used in this trial contained casein hydrolysate. This would further indicate that in vitro cellulose digestibility differences at the earlier fermentation times were more likely due to a generally low level of cellulolytic activity for all forages, rather than to a differential effect on low-protein forage substrates, as observed in Trial 4 when casein hydrolysate was omitted from the medium. It would thus appear that the initially low cellulolytic activity observed in this trial from inoculum prepared from the donor steer fed the lower protein alfalfa hay, was related to quantity and/or type of bacteria appearing in the inoculum. The fact that the level of cellulolytic activity increased with fermentation time with the "low-protein source" inoculum would indicate a "catching-up" by the bacteria as they proliferated with time.

It might be postulated that the use of inoculum possessing lower cellulolytic activity would be just as accurate in predicting the N.V.I. of a forage, if some fermentation period greater than 12 hours were chosen. Since reports in the literature indicate that some of the in vitro systems used by other workers are characterized by a lower level of cellulolytic activity than that reported in this study,

the question of determining the optimum fermentation time for these systems is of some importance, if this forage testing procedure is to find wide applicability. It would appear, although data is not available to substantiate this postulation, that the optimum fermentation period to predict the N.V.I. of a forage using any reasonably accurate in vitro system, could be determined with test forage samples of known nutritive value (N.V.I.). The procedure, as used in Trial 5, would consist of determining the fermentation curves (cellulose digestion × time) for the standard forage samples, and determining at which time period the maximum correlation with N.V.I. is obtained. Such standard forage samples would be obtained from a laboratory where the N.V.I. had been determined in animal feeding trials. This would form a basis for standardization of in vitro results between various laboratories.

C. TRIAL 7. THE RELATIONSHIP BETWEEN 12-HOUR CELLULOSE DIGESTIBILITY AND THE IN VIVO N.V.I. OF FORAGES FED IN THE CHOPPED AND GROUND FORM.

1. Introduction.

The results of Trial 5 indicated a highly significant correlation between 12-hour *in vitro* cellulose digestibility and the *in vivo* N.V.I. of forages fed to sheep in the chopped form. Since only a limited number of samples fed in one physical form were tested in Trial 5_4 the applicability of the *in vitro* procedure to testing forages of widely differing qualities fed in various physical forms was not established.

Trial 7 consisted of an extension in the number of *in vivo* - *in vitro* comparisons (including forages which were fed in each of two physical forms - chopped and ground), in order to determine the effect of the extended data on the *in vitro* prediction equations for the N.V.I. of a forage.

2. Experimental procedure.

The *in vitro* system and procedures used in this trial were as described in Section IV, part B, with the following additions and modifications:

a) Substrates.

The number and form of the forages studied in this trial were as follows (Table 11):

TABLE 11

FORAGES STUDIED IN *IN VIVO* - *IN VITRO* COMPARISONS. TRIAL 7.

Source	Year harvested	Physical form fed	
		Chopped	Ground
Macdonald College	1956	5[1]	-
" "	1957	4[1]	4
" "	1958	-	6
" "	1959	6	6
Saskatchewan (Swift Current)	1955	11	-

[1] Data from Trial 5.

The 1956 and 1957 forages fed in the chopped form were as described previously. The 1957 forages were also fed in the ground form with the results reported in this trial. The 1958 forages included 4 stages of maturity of artificially dehydrated hay fed only in the ground form (Jeffers, 1960). The 1959 forages represented 3 stages of maturity of alfalfa hay and of bromegrass hay, each maturity stage fed in both the chopped and ground form. The Saskatchewan samples, from the Swift Current Research Station of the Canada Department of Agriculture, were obtained from forages fed in sheep trials as described by Troelsen and Campbell (1959). The Nutritive Value Indices of these forages were calculated on the basis of the *in vivo* data presented in their report.

b) Fermentation runs and periods.

Since only 12-hour in vitro cellulose digestibility was determined for most of the forages reported in this trial, it was possible to make duplicate determinations for 15 forages in a fermentation run, with 3 runs constituting a test.

c) Forage fed to inoculum donor steer.

"High-protein" alfalfa hay (No.3 or 4)[1] was fed to the inoculum donor steer during the course of this trial.

3. Results and discussion.

The results of the 12-hour cellulose digestibility determinations for the forages fed in chopped or in ground form are presented in Tables 12 and 13, respectively. Since the physical form in which a forage is fed may have considerable effect on its voluntary intake, and subsequently on the calculation of its N.V.I., the same forage fed in various forms may have a different N.V.I. for each of these forms. The in vitro fermentation test, however, is always based on a finely ground sample and the results are thus independent of the actual form in which the forage was fed. It is for this reason that separate regression analysis are required for each of the physical forms studied, as presented in Table 14. Graphical illustration of the regression lines are presented in Figure 17.

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[1] Appendix Table 3a.

TABLE 12

IN VIVO (N.V.I.) AND IN VITRO (12-HOUR CELLULOSE DIGESTION) COMPARISONS FOR FORAGES FED IN CHOPPED FORM.

Forage	Description	N.V.I. (in vivo)	12-hr. in vitro cellulose digestion (%)
Macdonald College, 1956[1]:			
Alfalfa	early bloom	50	47
Red clover	" "	71	55
Birdsfoot trefoil	" "	63	51
Bromegrass	" "	43	37
Timothy	" "	34	37
1957:[1]			
Red clover	early bloom	54	46
" "	late "	49	47
Timothy	early "	38	38
"	late "	34	27
1958:			
Alfalfa	early bloom	71	52
"	full "	62	50
"	post "	64	47
Bromegrass	early "	51	51
"	full "	42	44
"	post "	42	35
Saskatchewan, 1955:			
Crested wheatgrass	unfertilized	36	30
" "	fertilized	35	29
Intermediate "	unfertilized	36	34
" "	fertilized	33	32
Streambank "	unfertilized	36	31
" "	fertilized	37	29
Russian wild ryegrass	unfertilized	46	36
" " "	fertilized	42	37
Reed canary grass	unfertilized	26	29
Tall wheatgrass	"	27	27
Alfalfa	"	45	43

[1] Data from Trial 5.

TABLE 13

IN VIVO (N.V.I.) AND IN VITRO (12-HOUR CELLULOSE DIGESTION) COMPARISONS FOR FORAGES FED IN GROUND FORM.

Forage	Description	N.V.I. (in vivo)	12-hour in vitro cellulose digestion (%)
Macdonald College			
1957:			
Red clover	early bloom	71	46
" "	late "	60	47
Timothy	early "	47	38
"	late "	43	27
1958:			
Timothy	early bloom	75	47
"	half "	59	34
"	full "	46	27
"	post "	36	23
Red clover	early "	79	55
Alfalfa	early "	74	53
1959:			
Alfalfa	early bloom	70	52
"	full "	69	50
"	post "	66	47
Bromegrass	early "	58	51
"	full "	51	44
"	post "	47	35

TABLE 14

REGRESSION OF N.V.I. (DETERMINED IN VIVO WITH CHOPPED AND GROUND FORAGES) AND 12-HOUR IN VITRO CELLULOSE DIGESTION. TRIAL 7.

Y	X	Regression equation	r	Standard[1] deviation
N.V.I. (chopped forage)	12-hr. in vitro cellulose digestion	Y = -6.3 + 1.30X	0.91**	±5.5
N.V.I. (ground forage)	12-hr. in vitro cellulose digestion	Y = 12.0 + 1.12X	0.87**	±6.7

** Highly significant (P=0.01)

[1] Standard error of estimate or standard deviation of Y for fixed X.

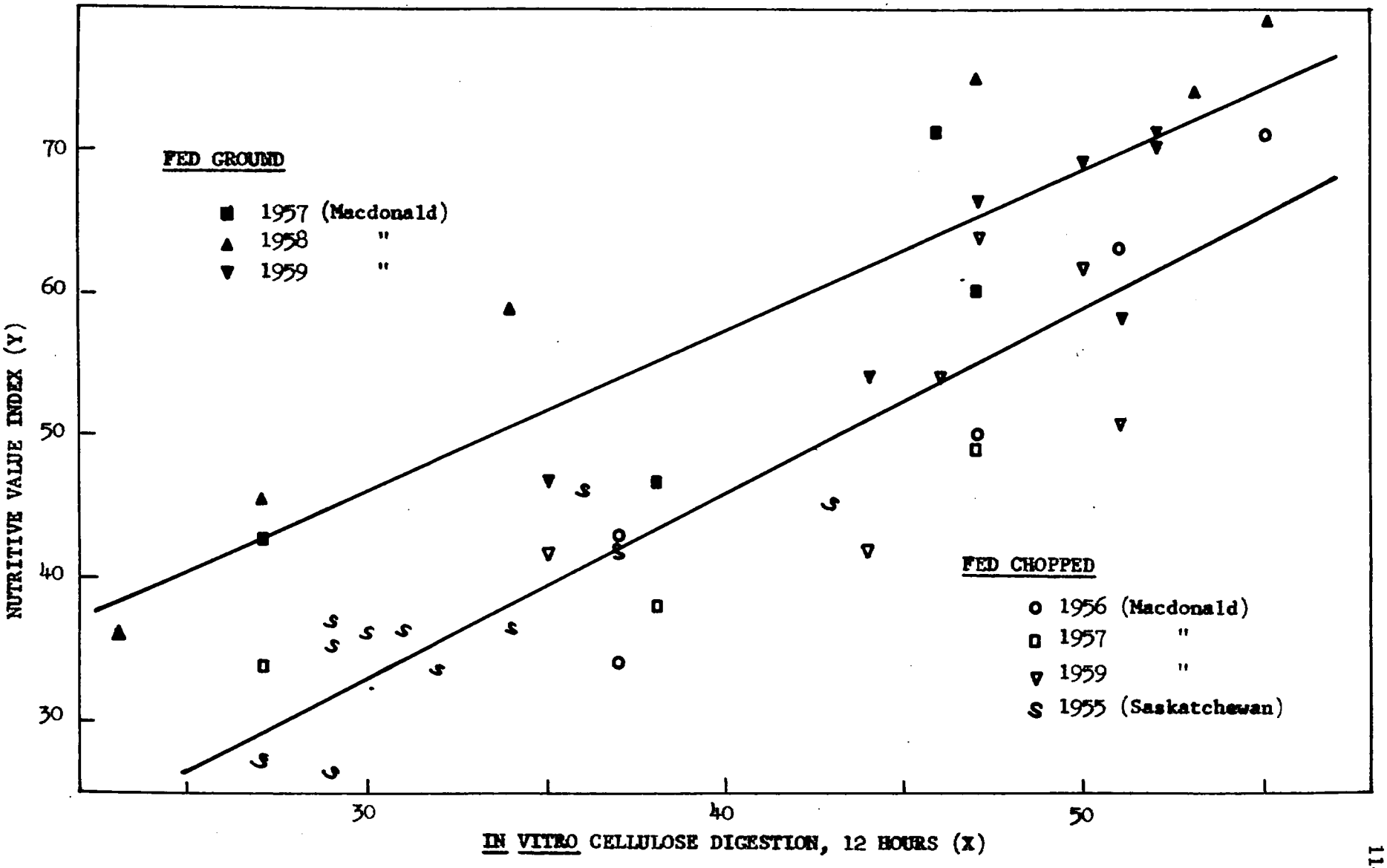

Figure 17. Regression of N.V.I. (determined in vivo with forages fed in the chopped and ground form) and 12-hour in vitro cellulose digestion (Trial 7).

It is of interest to note that the extension in the number of *in vivo* - *in vitro* comparisons, for forages fed in the chopped form, from the original 9 observations (Trial 5) to the 26 reported in this trial fully substantiated the highly significant correlation previously established. The regression coefficient (b = 1.3) remained unchanged, with the newly determined regression line essentially coinciding with the former one.

The Saskatchewan forages, representing various grasses grown and harvested under prairie conditions, were found to be lower in nutritive value than the artificially dehydrated legumes grown under more humid conditions in Eastern Canada. This contrast is borne out in both the *in vivo* and *in vitro* measurements, suggesting that the *in vitro* test as described can be used to compare forages of widely differing nutritive qualities.

Although not parallel, the slope of the regression line for the forages fed in the ground form deviated only slightly from that representing the chopped forages. However, appropriate statistical analysis showed these two regression coefficients to be homogeneous at a probability level of 1%. They were therefore pooled to give a single weighted regression coefficient of 1.23, with the resultant regression equations presented in Table 15, and illustrated in Figure 18.

TABLE 15

REGRESSION OF N.V.I. (DETERMINED IN VIVO WITH CHOPPED AND GROUND FORAGES) AND 12-HOUR IN VITRO CELLULOSE DIGESTION ASSUMING HOMOGENEITY OF REGRESSION COEFFICIENTS. TRIAL 7.

Physical form fed	Regression equation
Chopped:	$Y = -3.5 + 1.23X$
Ground:	$Y = 7.4 + 1.23X$
	or
	$Y = -3.5 + 1.23X + 10.9$

To illustrate the possible use of the relationship between chopped and ground forages, it would only be necessary to feed a forage in one physical form in an in vivo trial, and to estimate the N.V.I. of the other form by adding or subtracting the factor 10.9. In the case where the N.V.I. is being predicted solely by in vitro means, the predicted value can be expressed depending on the physical form in which the forage is to be fed.

The results of this trial are in agreement with reports from other workers that grinding causes a greater increase in nutritive value of poorer quality forages (basically by increasing voluntary consumption). At a N.V.I. of 30 (chopped), an increase of 10.9 units on grinding is equal to 36%, while at a N.V.I. of 60 (chopped) the percent increase on grinding is only 18.

Although the relationship between 12-hour in vitro cellulose digestion and the in vivo N.V.I. of a forage fed in different physical forms has been emphasized in this discussion, it should

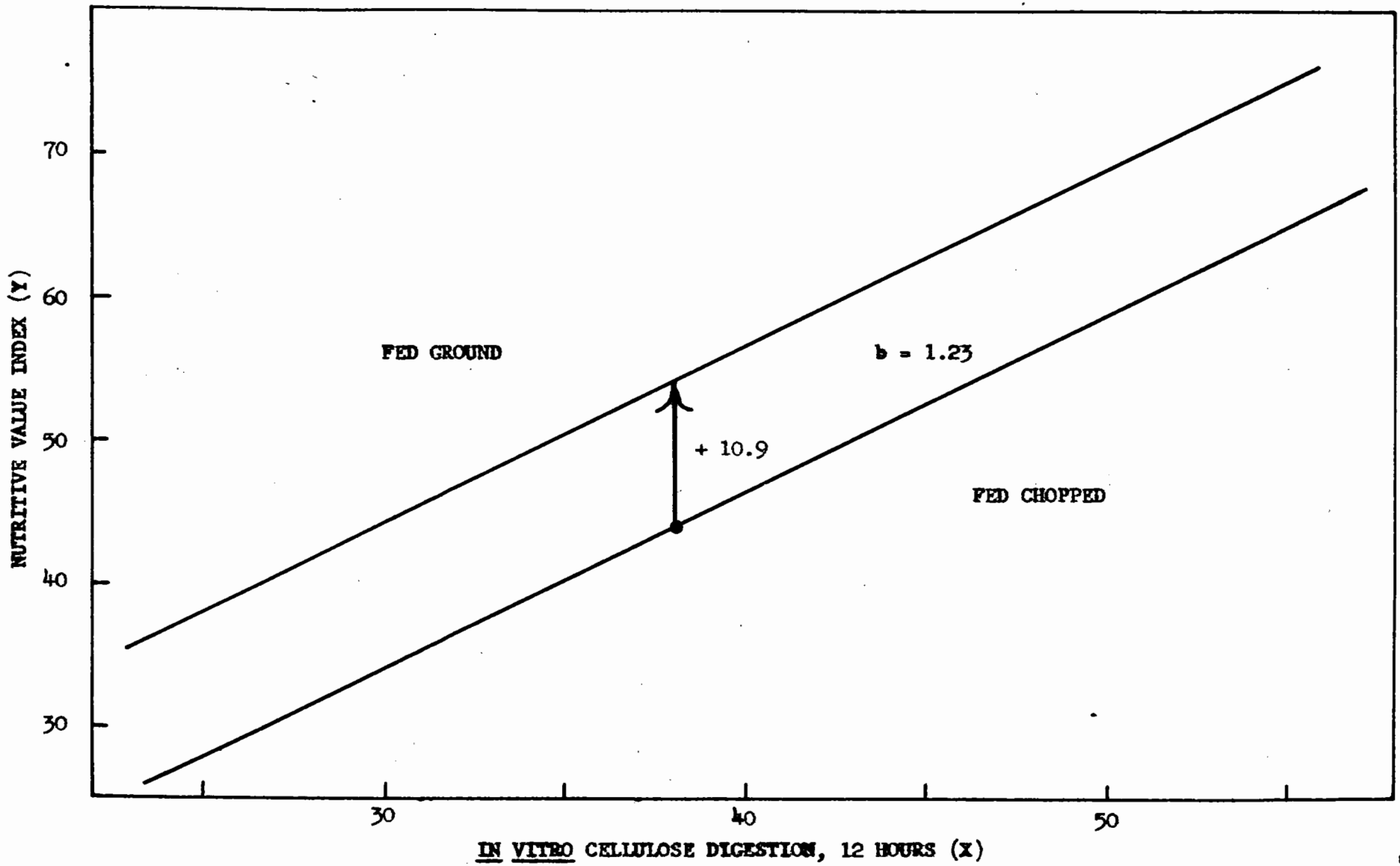

FIGURE 18. Regression of N.V.I. (determined in vivo with chopped and ground forages) and 12-hour in vitro cellulose digestion, assuming homogeneity of regression coefficients. (Trial 7).

be pointed out that the most important consideration is that early *in vitro* cellulose digestion (12 hours in the case of this study) has been shown to rank forages according to a meaningful criterion of nutritive value. In this respect the *in vitro* rumen fermentation procedure could be used by agronomists interested in selecting nutritionally superior forage species or strains, as well as in evaluating various forage species mixtures. The advantages of the *in vitro* procedure, namely the requirement for only a small sample (5-10 grams), and the rapidity of results (1-2 days), indicate its potential usefulness both to nutritionists and agronomists. The above advantages notwithstanding, the most important attribute of this *in vitro* technique is its close relationship to the total digestible energy potential of a forage as determined *in vivo*.

D. SUMMARY.

Section V. dealt with the possibility of using the previously described in vitro rumen fermentation system to predict the nutritive value of forages. In vitro cellulose digestibility coefficients of forages of known nutritive value (as determined in vivo) were determined after various fermentation periods. As a result of such a study with 9 samples of forages which had been fed to sheep in the chopped form, a highly significant (P=0.01) correlation was established between early in vitro digestion (12 hours) and the in vivo N.V.I.

When in vitro cellulose digestibility was determined for a group of 4 of these forage samples using inoculum obtained from a steer fed a "low-protein" alfalfa hay and compared to that obtained in the original study where "high-protein" alfalfa hay was fed, a significant depression was observed in digestion after 6 and 12 hours. This depression appeared to be caused by an overall lower level of cellulolytic activity, which uniformly affected all of the different forage substrates. It was postulated that such "low activity" inoculum might be as useful in predicting N.V.I.'s if a fermentation period of longer than 12 hours was used.

In a concluding test, in vivo - in vitro comparisons were made on a total of 42 forage samples to confirm if possible the accuracy of the N.V.I. prediction equation obtained from the original 9 comparisons. The final collection of forage samples included 26 which had been fed in the chopped form and 16 in the ground form. The statistical results of the 26 comparisons essentially duplicates the original fer hypothesis (bound.) and correlation coefficients (r = 0.91) obtained for the original chopped forages. The regression coefficients for both

the ground and chopped hay comparisons were found to be homogeneous (P=0.01) and justified calculation of a single weighted average b value. The resultant prediction equations with Y = N.V.I. and X = 12-hour *in vitro* cellulose digestion, were as follows:

$$\text{Chopped forages: } Y = -3.5 + 1.23X$$

$$\text{Ground forages: } Y = 7.4 + 1.23X$$

or

$$-3.5 + 1.23X + 10.9$$

The last prediction equation illustrates the difference in N.V.I. due to physical form fed, as it affects the position of the regression lines.

The results described in this section indicate that early *in vitro* cellulose digestibility can be used to accurately predict the N.V.I. of a forage fed in either the chopped or ground form.

VI. INTEGRATING DISCUSSION.

The ultimate object of this research was the development of an *in vitro* rumen fermentation system which could be used to predict the nutritive value of a forage. To successfully meet this requirement, an *in vitro* procedure would necessarily be accurate, reproducible, rapid, and most important of all be highly correlated to some concise measure of forage nutritive value as determined *in vivo* with some ruminant species.

There were two main aspects of the experimental work. The first was the establishment of an *in vitro* system characterized by a high level of cellulolytic activity, using well-defined and easily duplicated procedures. These studies, as described in Section IV, were mainly concerned with the composition of the nutrient medium, and the effect of different sources of rumen inoculum. Since the type of *in vitro* system used in this study was based on the one already developed by Bentley and co-workers at the Ohio Agricultural Experiment Station, this aspect of the research was essentially confirmatory, and covered only a small area of the potential questions of technique that could arise. The use of an *in vitro* system whose merit had already been demonstrated in ruminology studies was deliberate in that major emphasis could then be placed on the other aspect of this research - the development of an *in vitro* test for predicting forage nutritive value.

The primary question in the first part of this research was an examination of the adequacy of the *in vitro* nutrient medium to support maximum cellulose digestion with either purified or forage

cellulose sources. In a distinguished series of *in vitro* studies, Bentley and co-workers had demonstrated that maximum cellulolytic activity could be achieved if either valeric acid or a combination of several amino acids replaced the heterogeneous rumen liquid in the nutrient medium. In the present study it was shown that the resuspended bacterial cell inoculum contributed a minimum amount of nutrients to the medium as demonstrated by the low level of purified cellulose digestion achieved on a basal medium lacking valeric acid, amino acids, or a source of rumen liquid factors. In contrast, the addition of varying levels of these substances to the medium resulted in increases of 100 to 200% in cellulose digestion. Of all the supplements added to the nutrient medium, none could achieve the total cellulolytic activity demonstrated by an enzymatically prepared casein hydrolysate. Since the addition of this material to the Ohio medium (containing valeric acid) slightly increased the level of purified cellulose digested in 30-hours, it was included in the complete nutrient medium in subsequent forage studies.

Although the use of inoculum prepared from fistulated steers fed different supplies of alfalfa hay had no apparent effect on the *in vitro* digestion of purified cellulose, this was not the case when digestion of native forage cellulose was examined. Several of the forages were shown to respond favorably to casein hydrolysate supplementation when inoculum was prepared from an inoculum donor steer fed a low protein alfalfa hay (13%,dry matter basis). However, cellulose digestion of all forages appeared maximal - regardless of supplementation - when a higher protein alfalfa hay (16%, dry matter basis) served as the inoculum donor"s feed. Since those forages

which responded to casein hydrolysate supplementation were low in protein content, it was postulated that this effect was due to lack of some nitrogenous substance(s) that was present either in the forage substrate, inoculum prepared from the high protein alfalfa source, or casein hydrolysate.

With the completion of the preliminary studies on in vitro techniques, the evaluation of forage quality was undertaken. As a result of these preliminary studies the in vitro system was modified for the forage studies so that the nutrient medium contained casein hydrolysate, with resuspended cell inoculum prepared from the rumen contents of a steer fed a high-protein alfalfa hay. The in vitro forage studies were dependent upon the availability of samples of forages of known nutritive value, as determined in vivo. In these in vivo trials digestibility by sheep of the proximate principles and energy, as well as voluntary consumption of the forages had been determined. These observations formed the basis for the calculation of N.V.I.'s for each of the forages - this criterion of forage quality being an expression of the total digestible energy potential of a forage.

The initial study concerned the in vitro rate of forage cellulose digestion, since it had been postulated that the Relative Intake and thus N.V.I. of different forages was largely a consequence of their in vivo rate of cellulose degradation by ruminal micro-organisms. This study, involving samples of 9 forages which had been fed in the chopped form, revealed that there were characteristic lag periods preceding the initiation of in vitro cellulose digestion. These lag periods, which appeared to be of greater duration for

timothy and bromegrass hay than for the leguminous species, were reflected in the level of cellulose digested after 12 hours of fermentation. Confirmatory statistical analyses showed that of all the *in vivo* - *in vitro* comparisons, the highest correlation was observed between the *in vivo* N.V.I. and the 12-hour *in vitro* cellulose digestion, with a highly significant ($P = 0.01$) coefficient of 0.91. This relationship suggests that the same principle affecting *in vivo* voluntary consumption might be operative in the *in vitro* system. The differential lag periods observed between forages were suggested as being related to some chemical and/or physical characteristic of the forage affecting the "attachment" of the rumen bacteria to the substrate structure.

Another highly significant correlation ($P = 0.01$) of importance was that observed between *in vivo* energy digestibility and 24-hour *in vitro* cellulose digestion. This relationship confirms that noted by other workers between the digested energy per unit of forage consumed and *in vitro* cellulose or dry matter digestion (generally) after 18 hours. This is of importance since it demonstrates that by altering the length of fermentation periods, different *in vivo* - *in vitro* relationships can be determined using the same *in vitro* system.

Rate of cellulose digestion was also determined for 4 of the forages studied in the previous trial, but in this instance inoculum prepared from the rumen contents of a steer fed the "low-protein" alfalfa hay was used. When these results were compared to those previously obtained for these forages, a highly significant ($P = 0.01$) depression in cellulose digestion was noted after 6- and 12-hour

fermentation periods, apparently due to lower protein inoculum source. This depression was interpreted as being caused by a reduced level of cellulolytic activity due to deficiencies in number and/or type of certain rumen bacteria. However the ranking of the 4 forages after the 12-hour digestion period was similar regardless of inoculum source. Since there are indications that *in vitro* systems used by other workers are characterized by varying levels of cellulolytic activity, it was suggested that standard forage samples, of predetermined N.V.I. might be exchanged between different laboratories so that the optimum period for predicting the N.V.I. of test forages could be determined for each system.

In order to confirm the original quantitative relationship observed between the N.V.I. and 12-hour cellulose digestion of a forage, a series of *in vivo* - *in vitro* comparisons were made between 42 forage samples. These comparisons included a wide variety of forage species, harvested at different stages of maturity, and fed to sheep in the chopped and/or ground form. A 3-fold increase in the number of forage samples fed in the chopped form did not appreciably alter the original prediction equation - both the regression and correlation coefficients remained the same. The increase in N.V.I. due to grinding as calculated by the *in vivo* - *in vitro* comparisons was equal to 10.9 units. When the N.V.I.'s for the chopped or ground forages were compared to the *in vitro* data the regression coefficients were found to be homogeneous ($P=0.01$). For this reason they were pooled to give a single value ($b = 1.23$).

The results of this research lead to the conclusion that the total digestible energy potential (N.V.I.) of a forage fed in either the chopped or ground form can be predicted from *in vitro* cellulose digestibility data. If the *in vitro* system used for forage evaluation is characterized by easily duplicated procedures with a controlled source of rumen microorganism inoculum, a high degree of precision and accuracy is obtainable.

VII. CONCLUSIONS

From the results obtained using the *in vitro* system and procedures described in this thesis, the following conclusions have been drawn:

1. The supplementation of the nutrient medium with casein hydrolysate resulted in a higher level of *in vitro* digestion ($P = 0.01$) of a purified cellulose substrate than was achieved by the addition of either valeric acid or an amino acid mixture (leucine, isoleucine, valine, and proline).

2. The addition of more than 15 mg. of valeric acid per fermentation tube did not result in any further increase in the *in vitro* digestion of a purified cellulose substrate. Increasing the amount of supplementary amino acid mixture from 12 to 14 mg. per fermentation tube resulted in a highly significant depression ($P = 0.01$) in cellulose digestion.

3. With a purified cellulose substrate, no differences in cellulose digestion due to inoculum source (inoculum prepared from donor steer fed either "high" or "low" protein alfalfa hay) were noted when either valeric acid, casein hydrolysate, or an amino acid mixture was added to the basal medium.

4. With native forage cellulose substrates, several of the forages tested responded significantly ($P = 0.01$) to casein hydrolysate supplementation. This observed increase in cellulose digestion occurred when "low" protein alfalfa hay was fed to the inoculum donor steer. When inoculum was obtained

from a donor steer fed "high" protein alfalfa hay, cellulose digestion for all forages appeared maximal, regardless of supplementation.

5. When samples of 9 forages (5-1956; 4-1957 harvest) which had been fed in the chopped form were studied, their digestible energy potential (as measured in vivo by the N.V.I.) was found to be highly correlated ($r = 0.91$) with their 12-hour in vitro cellulose digestibility.

6. The energy digestibility (as determined in vivo) of the same 9 forages was found to be highly correlated ($r = 0.87$) with their 24-hour in vitro cellulose digestibility.

7. When four 1957 forages were tested using inoculum obtained from a steer fed a "low" protein alfalfa hay, a significant depression ($P = 0.01$) was observed in in vitro cellulose digestion when the results were compared to those obtained when a "high protein source" inoculum was used. This depression, noted particularly after the 6- and 12-hour fermentation periods, appeared to be caused by an overall lower level of cellulolytic activity, which uniformly affected all of the different forage substrates. At the 12-hour fermentation period, forages were ranked in the same relative order regardless of inoculum source.

8. An increase in the number of comparisons between the in vivo N.V.I. and 12-hour in vitro cellulose digestibility, to a total of 26 forages fed in the chopped form, resulted in identical regression ($b = 1.3$) and correlation ($r = 0.91$) coefficients to those originally observed with 9 comparisons.

9. When samples of 16 forages which had been fed in the ground form were studied, their *in vivo* N.V.I. and 12-hour *in vitro* cellulose digestibility were found to be highly correlated ($r = 0.87$). The regression coefficients obtained with forages fed either chopped or ground were found to be homogeneous ($P = 0.01$), thus justifying the calculation of a single weighted average coefficient.

10. Based on the homogeneity of these regression coefficients, the following prediction equations, with Y = N.V.I. and X = 12-hour *in vitro* cellulose digestibility, were calculated:

Chopped forages: $Y = -3.5 + 1.23X$

Ground forages: $Y = 7.4 + 1.23X$

or

$Y = -3.5 + 1.23X + 10.9.$

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APPENDIX TABLE 1a. Individual cellulose digestibility determinations, Trial 1.

Supplements:	Amount added per fermentation tube								
valeric acid (mg.)	0	0	2.5	5	2.5	0	2.5	0	
casein hydrolysate (mg.)	0	0	0	0	0	0	50	50	
amino acid mixture (mg.)	0	0	0	0	0	12	12	0	
supernatant (ml.)	0	5	0	0	5	0	0	0	
Run 19 (24 June, 1958)	36	57	68	77	76	78	74	84	
Inoculum donor steer A, fed mixed	37	47	69	71	78	79	70	83	
hay (1)	35	51	66	71	75	77	74	83	
	31	54	70	73	76	76	74	80	
Average	35	52	68	73	76	78	73	83	67
Run 20(30 June, 1958)	12	52	64	74	74	76	82	83	
Inoculum donor steer A, fed mixed	12	54	65	74	74	78	83	83	
hay (1)	12	54	68	71	75	76	83	84	
	10	56	66	72	77	79	86	81	
Average	12	54	66	73	75	77	84	83	65
Run 21 (3 July, 1958)	36	60	79	83	84	79	78	81	
Inoculum donor steer B, fed mixed	34	59	75	84	85	77	80	81	
hay (1)	35	62	74	81	84	78	80	81	
	29	61	77	85	84	80	79	82	
Average	34	61	76	83	84	79	79	81	72
Trial average	27	56	70	76	78	78	79	82	

APPENDIX TABLE 1b. Individual cellulose digestibility determinations, Trial 2.

Forage fed inoculum donor steer	Supplements:	Amount added per fermentation tube							
	valeric acid (mg.)	0	0	0	20	15	0	0	
	casein hydrolysate (mg.)	0	0	0	0	0	50	100	
	amino acid mixture (mg.)	0	24	12	0	0	0	0	
"low-protein" alfalfa hay (No.2)	Run 25 (24 July, 1958) Steer B	11	64	69	78	80	85	89	
		13	63	66	76	77	82	86	
		12	61	60	76	75	84	87	
		18	65	69	77	77	86	87	
	Average	14	63	66	77	77	84	87	67
	Run 26 (29 July, 1958) Steer A	18	70	73	90	87	94	97	
		17	70	72	91	90	94	97	
		18	72	78	92	91	96	97	
		18	71	78	91	91	95	97	
	Average	18	71	75	91	90	95	97	77
	25 + 26 Average	16	67	71	84	84	90	92	72
"high-protein" alfalfa hay (No.3)	Run 71 (7 July, 1959) Steer A	17	76	78	84	84	93	94	
		16	75	80	84	82	92	94	
		17	77	79	83	85	93	94	
		16	74	77	82	84	93	94	
	Average	17	76	79	83	84	93	94	75
	Run 73 (25 Aug,,1959) Steer A	16	59	70	80	84	90	90	
		14	57	67	77	81	88	90	
		18	58	63	85	84	90	91	
		15	57	66	83	87	90	90	
	Average	16	58	67	81	84	90	91	69
	71 + 73 Average	16	67	73	82	84	91	92	72
	Trial Average	16	67	72	83	84	90	92	

APPENDIX TABLE 1c. Individual cellulose digestibility determinations, Trial 3.

Supplements:	Amount added per fermentation tube				
valeric acid (mg.)	15	30	15	15	
casein hydrolysate (mg.)	0	0	100	50	
Run 32 (26 Aug., 1958)	93	96	96	97	
Steer A, fed alfalfa hay (2)	94	95	96	96	
Average	94	96	96	97	95
Run 34 (30 Sept., 1958)	90	89	90	92	
Steer A, fed alfalfa hay (2)	86	85	90	92	
Average	88	87	90	92	89
Run 36 (15 Oct., 1958)	96	98	99	99	
Steer B, fed alfalfa hay (2)	97	98	100	99	
Average	97	98	100	99	98
Trial average	93	94	95	96	

APPENDIX TABLE 1d. Individual cellulose digestibility determinations, Trial 4.

Forage fed inoculum donor steer	Nutrient medium	Fermentation run	Forage cellulose digestion (%)					
			Alfalfa (1)	Red Clover (2)	B.Trefoil (3)	Brome g. (4)	Timothy (5)	
"low-protein" alfalfa hay (2)	Control	31 (20 Aug.,1958)	55	57	55	56	54	
		Steer A	55	60	60	54	54	
		Average	55	59	57	55	54	56
		32 (26 Aug.,1958)	58	56	54	54	49	
		Steer B	57	55	55	52	53	
		Average	58	56	55	53	51	54
		Average	56	57	56	54	52	55
	+ Casein hydrol.	31	55	57	60	61	61	
			57	60	62	62	62	
		Average	56	59	61	62	62	60
		32	56	50	61	61	60	
			57	61	62	61	61	
		Average	57	56	62	61	61	59
		Average	56	57	61	61	61	59
"high-protein" alfalfa hay (3)	Control	51 (18 Feb., 1959)	56	60	62	62	64	
		Steer A	54	63	63	62	51	
		Average	55	62	62	62	58	60
		70 (2 July, 1959)	58	63	64	61	63	
		Steer A	58	66	61	61	62	
		Average	58	65	63	61	63	62
		Average	57	63	63	62	60	61
	+ Casein hydrol.	51	58	65	60	62	56	
			56	57	60	61	62	
		Average	57	61	60	62	59	60
		70	57	63	62	62	64	
			58	63	63	62	64	
		Average	58	64	63	62	64	62
		Average	57	62	61	62	62	61

APPENDIX TABLE 1e. Individual cellulose digestibility determinations at various fermentation times, Trial 5.

Substrate	Fermentation time (hours 3	6	12	24	48
1956:					
(1) Alfalfa	8	28	47	54	54
	11	25	45	54	56
	4	22	47	52	57
	8	29	47	54	55
Average	8	26	47	54	56
(2) Red clover	7	24	55	63	61
	13	26	51	65	63
	5	31	57	60	63
	5	31	56	61	64
Average	8	28	55	62	63
(3) Birdsfoot trefoil	11	26	55	60	61
	4	18	50	62	60
	4	18	49	60	64
	5	26	50	58	62
Average	6	22	51	60	62
(4) Bromegrass	0	15	36	56	67
	0	5	33	58	67
	0	4	39	54	67
	0	9	40	54	69
Average	0	8	37	56	68
(5) Timothy	0	11	37	60	63
	1	0	38	55	66
	0	8	36	51	67
	0	13	38	57	69
Average	0	8	37	56	66

APPENDIX TABLE 1e (continued).

Substrate	Fermentation time (hours) 3	6	12	24	48
1957:					
(6) Red clover early bloom	7	18	49	51	59
	4	30	46	50	60
	0	14	50	56	58
	3	18	40	54	55
Average	4	20	46	53	58
(7) Red clover late bloom	9	26	46	57	59
	5	31	48	49	61
	0	18	49	52	59
	3	24	44	54	57
Average	4	25	47	53	59
(8) Timothy early bloom	5	5	39	57	64
	0	8	32	59	65
	0	13	41	57	62
	0	17	38	56	59
Average	1	11	38	57	63
(9) Timothy late bloom	0	5	33	48	54
	5	1	30	48	52
	0	2	19	43	55
	0	13	25	44	53
Average	1	5	27	46	54
Solka Floc (purified cellulose)	0	3	42	92	100
	0	5	32	89	100
	1	1	36	93	100
	0	4	40	90	100
	4	2	26	91	99
	0	3	35	93	99
	0	0	41	95	99
	0	1	44	96	100
Average	1	2	37	92	100

APPENDIX TABLE 2a. Analysis of variance of purified cellulose digestion, Trial 1.

Source of variation	d.f.	variance	F
All causes	95		
Between subgroups:	23		
Treatments	7	4219	1172**
Fermentation runs	2	393	109**
T × F	14	121	34**
Remainder	72	3.6	
		(S.D=1.9)	

** Highly significant (P=.01). Coefficient of variability = 5.3%

L.S.R. (0.01) = 2.1 to 2.3 (for p = 2 to 8, n = 12).

APPENDIX TABLE 2b. Analysis of variance of purified cellulose digestion, Trial 2.

Source of variation	d.f.	variance	F
All causes	111		
Between subgroups:	27		
Treatments	6	11,151	3,261**
Fermentation runs	3	585	171**
T × F	18	39	11**
Remainder	84	3.4 (S.D.=1.9)	

** Highly significant (P=0.01) Coefficient of variability = 2.6%

L.S.R. (0.01) = 1.7 to 1.9 (for p = 2 to 7, n = 16).

APPENDIX TABLE 2c. Analysis of variance of purfied cellulose digestion, Trial 3.

Source of variation	d.f.	variance	F
All causes	23		
Between subgroups:	11		
Treatments	3	12.7	7.9**
Fermentation runs	2	169	106 **
T × F	6	2	1.3
Remainder	12	1.6 (S.D. = 1.3)	

**Highly significant (P=0.01) Coefficient of variability = 1.4%

L.S R. (0.01) = 2.2 to 2.4 (for p = 2 to 4, n = 6).

APPENDIX TABLE 2d. Analysis of variance of native forage cellulose digestion, Trial 4.

Source of variation	d.f.	variance	F
All causes	79		
Between subgroups:	39		
Forages	4	33	4.9**
Runs	3	103	15.4**
Supplement (casein hydrol.)	1	97	14.5**
Interactions:			
F × R	12	8.9	1.3
F × S	4	20	3.0*
R × S	3	30	4.5**
F × R × S	12	6.1	>1
Remainder	40	6.7 (S.D. = 2.6)	

* Significant (P=0.05) Coefficient of variability = 4.4%

** Highly significant (P=0.01)

L.S.R. (0.01) = 5.0 (for p = 2, n=4).

APPENDIX TABLE 2e. Analysis of variance of native forage cellulose digestion, Trial 6.

Source of variation	d.f.	variance	F
All causes	126		
Between subgroups	31		
Forages	3	1057	56**
Time	3	13,036	689**
Inoculum	1	1047	55**
Interactions:			
F × T	9	164	8.6**
F × I	3	3.7	>1
T × I	3	46	2.4
F × T × I	9	14	>1
Remainder	95	19.0 (S.D = 4.4)	

** Highly significant (P=0.01) Coefficient of variability = 11.4%

L.S.R. (0.05) = 6.2 (for p = 2, n = 4)

(0.01) = 8.1 (for p = 2, n = 4).

APPENDIX TABLE 3a. Some chemical constituents of forages fed to inoculum donor steer.

No.	Forage	Year harvested	(expressed as percent of dry matter) Protein (N × 6.25)	Cellulose	N.F.E.	Ether Extract	Ash	Gross Energy Cals/gm.
(1)	Mixed hay	1957	15.9	35.3	37.3	2.0	9.5	4.47
(2)	Alfalfa hay	1958	13.4	36.1	41.5	1.6	7.4	4.42
(3)	Alfalfa hay	1958	16.1	36.0	38.9	1.3	7.7	4.41
(4)	Alfalfa hay	1959	20.8	32.0	36.5	1.3	9.4	4.53

APPENDIX TABLE 3b. Some chemical constituents of forage substrates.

No.	Substrate	(expressed as percent of dry matter) Protein (N × 6.25)	Cellulose	Gross Energy (Cals/gm.)
	1956:			
(1)	Alfalfa	17.6	36.0	4.52
(2)	Red Clover	15.9	29.7	4.41
(3)	Birdsfoot trefoil	14.7	35.4	4.62
(4)	Bromegrass	9.0	34.0	4.51
(5)	Timothy	7.2	35.9	4.45
	1957:			
(6)	Red clover, early	15.6	33.0	4.33
(7)	" " , late	17.2	33.9	4.41
(8)	Timothy, early	8.0	34.7	4.37
(9)	" , late	6.8	32.6	4.29