An in vitro digestion of selected starches
by Ronald Lyle Davis

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Animal Science
Montana State University

Abstract:
Three vitro digestion trials were conducted comparing 5 barley starches, corn starch, milo starch, amylose and amylopectin. Within each trial, substrates were subjected to digestion on each of 3 different days. The phenol-sulfuric acid procedure used to analyze for residual starch was compared to the enthrone method of Moore et al. (1962).

In Trial I the starches of corn, milo, wheat, Oderbrucker barley and Compana barley were compared. Oderbrucker starch was digested significantly faster ($P<.01$) or equal to corn starch and the other starches. In most instances wheat and milo starch were digested slower than corn starch.

The starches of corn and the barley varieties, Compana, Newpaha, Betzes and Unitan were compared in Trial II, Corn starch digested significantly faster ($P<.01$) or equal to the other starches in all but one instance. Unitah starch digested significantly slower ($P<.01$) or equal to the other starches in all but one instance.

In Trial III corn starch, Compana starch, Newpana starch, amylopectin and amylose were compared. Amylopectin digested significantly faster ($P <.01$) or equal to the other substrates. Amylose tended to digest faster than the 3 starches at the start of the fermentation period; however at the end of the period amylose digested slower than the other substrates.

In all trials at the end of the fermentation period, digestion was either greater than or approached 90 percent. All interactions excluding hours x variety for Trials I and II were highly significant ($P <.01$) Hours x variety was significant ($P<.05$) for Trial III.

The correlation between the phenol-sulfuric acid method and the enthrone method was highly significant ($r = .99$).

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ABSTRACT

Three in vitro digestion trials were conducted comparing 5 barley starches, corn starch, milo starch, amylose and amylopectin. Within each trial, substrates were subjected to digestion on each of 3 different days. The phenol-sulfuric acid procedure used to analyze for residual starch was compared to the anthrone method of Moore et al. (1962).

In Trial I the starches of corn, milo, wheat, Oderbrucker barley and Compana barley were compared. Oderbrucker starch was digested significantly faster ($P < .01$) or equal to corn starch and the other starches. In most instances wheat and milo starch were digested slower than corn starch.

The starches of corn and the barley varieties, Compana, Newpana, Betzes and Unitan were compared in Trial II. Corn starch digested significantly faster ($P < .01$) or equal to the other starches in all but one instance. Unitan starch digested significantly slower ($P < .01$) or equal to the other starches in all but one instance.

In Trial III corn starch, Compana starch, Newpana starch, amylopectin and amylose were compared. Amylopectin digested significantly faster ($P < .01$) or equal to the other substrates. Amylose tended to digest faster than the 3 starches at the start of the fermentation period; however at the end of the period amylose digested slower than the other substrates.

In all trials at the end of the fermentation period, digestion was either greater than or approached 90 percent. All interactions excluding hours x variety for Trials I and II were highly significant ($P < .01$). Hours x variety was significant ($P < .05$) for Trial III.

The correlation between the phenol-sulfuric acid method and the anthrone method was highly significant ($r = .99$).

INTRODUCTION

Barley is Montana's most important livestock feed grain. Many varieties are grown each year and new varieties are continually being developed. There is a need to determine if there is a difference in the microbial digestion of the numerous barley varieties.

Emphasis on high-energy feeding programs for ruminants has resulted in widespread use of rations in which starch is the primary source of energy. Very little digestibility data, either *in vitro* or *in vivo*, is available on high concentrate rations.

The *in vitro* procedure has made a major contribution to the understanding of cellulose digestion in the ruminant; however relatively little attention has been given to the study of starch digestion by rumen microorganisms.

Because barley is the major source of energy in Montana's livestock feeding programs, it was deemed necessary to study starch digestion of several varieties produced by the Montana Agricultural Experiment Station at Bozeman. The major objectives of the trials reported in this manuscript were to determine if differences exist among barley varieties in regard to starch digestion by rumen microorganisms, to evaluate the *in vitro* method as a basis for comparing digestion of starch and to evaluate the phenol-sulfuric acid method of starch determination as compared to the more difficult anthrone method used by Moore *et al.* (1962).

REVIEW OF LITERATURE

The suggestion that microorganisms were responsible for cellulose disappearance as feed passed through the alimentary tracts of ruminants was made as early as 1874-1882, Johnson (1963). It was not until the early 1940's that the *in vitro* digestion technique was developed. Since that time, *in vitro* digestion has been widely used in cellulose digestibility studies. *In vitro* techniques have also been used to measure the production of volatile fatty acids, to study utilization of various nitrogenous compounds by rumen microflora and to predict forage nutritive values.

Warner (1956) established criteria for the validity of *in vitro* studies with rumen microorganisms. Warner stated that motility of the bacteria appeared to be a sensitive measure of normal rumen function. For the *in vitro* microbial population to remain normal in numbers, it was necessary to use as test substrates only substances similar to the diet fed the animal from which the innoculum was taken.

Barnes *et al.* (1964) compared several *in vitro* methods of cellulose digestion. As the length of fermentation increased, the standard deviation between methods decreased. The variability was therefore associated with the initial lag phase period of 6 and 12 hour fermentations. Donefer *et al.* (1960) stated that the prerequisite of a useful *in vitro* technique was its ability to measure the effects on cellulose digestion of the early lag differences in fermentation.

Correlations Between *In Vitro* and *In Vivo* Methods

In order to effectively evaluate the *in vitro* method of digestibility, the *in vitro* and *in vivo* methods were compared.

Barnett (1957) reported that results calculated for cellulose digestibility coefficients were within ±5.6 percent of determined *in vivo* and *in vitro* digestion. It was shown that the variability associated with cellulose digestibility *in vivo* was only slightly smaller than the variability associated with replicate determinations of cellulose digestibility *in vitro*.

Quick (1959) compared *in vitro* and *in vivo* digestibilities of several grass hays and legumes. There was no significant difference between the two methods.

Forty-eight hour fermentations conducted by Lefevre and Kamstra (1960) yielded cellulose digestion coefficients similar to values obtained *in vivo* ($r = 0.84$). Cellulose digestion in twenty-four hour fermentations was below that obtained *in vivo*. Digestion coefficients obtained *in vitro* with sheep and cattle rumen fluids as inoculum were similar.

Correlations between *in vitro* and *in vivo* measures of digestibility were conducted by Bowden and Church (1962). These correlations were as follows: (1) *in vitro* dry matter digestibility and *in vivo* dry matter digestibility were correlated ($r = 0.93$), (2) *in vitro* cellulose digestion and *in vivo* dry matter digestibility were correlated ($r = 0.87$), (3) *in vitro* dry matter digestibility with crude protein of the forage was correlated ($r = 0.94$), (4) *in vitro* cellulose digestion with crude protein content of the forage was correlated ($r = 0.93$).

Baumgardt *et al.* (1962b) supported the work by Bowden and Church by finding that the percent forage cellulose digested *in vitro* was significantly correlated with total digestible nutrients, digestible dry matter

and digestible energy determined in conventional digestion trials. The correlation between *in vitro* cellulose digestion and *in vivo* digestible energy was highly significant (r = 0.85). Hershberger *et al.* (1959) found a correlation of 0.92 between cellulose digested *in vitro* and the digestible energy of the forage.

Digestible protein was estimated *in vitro* by Baumgardt *et al.* (1962b). When the crude protein was known, the correlation between crude and digestible protein was highly significant (r = 0.999).

Pigden and Bell (1955) found that the artificial rumen procedure, when used with the proper regression equations, gave estimates of total digestible nutrients; and that digestibility of crude protein from eleven forages was in close agreement with values derived from conventional digestion trials using sheep. Baumgardt *et al.* (1962a) conducted research in which the *in vitro* fermentation of forage carbohydrate was measured. Estimates of total digestible nutrients were significantly correlated with conventional digestibility data; however these estimates were consistently underestimated.

High correlations between *in vitro* and *in vivo* digestion indicate that *in vitro* fermentation has a definite value in estimating the nutritive value of forages.

Factors Affecting Cellulose Digestion

Inoculum

A method for the study of cellulose digestion by washed cell suspensions was developed by Cheng *et al.* (1955). Washed suspensions of rumen

microorganisms were sensitive enough to detect the effect of small amounts of unidentified factors stimulatory to cellulose degradation. Washed suspensions were relatively free from any metabolic end products produced by microorganisms while still in the rumen.

Church and Peterson (1960) noted that the amount of rumen liquor had appreciable influence on the extent of *in vitro* digestion of cellulose or dry matter. Marquardt and Asplund (1964) found that cellulose digestion increased as the volume of inoculum increased. Increased bacterial numbers and additional nutrients possibly increased the rate of fermentation by decreasing the initial lag phase of growth and/or by increasing the rate of growth and metabolism during the logarithmic growth phase. Small changes in mineral medium concentrations had only negligible effect on *in vitro* digestion of dry matter and cellulose.

Based on the composition of ruminant saliva, McDougall (1949) suggested a mineral medium which adequately supported *in vitro* digestion. Several variations of this media have been used and all support *in vitro* digestion.

Marquardt and Asplund (1964) noted that unless the nutrient medium in artificial rumen studies was completely adequate, standardization of inocula or statistical control was necessary.

Cheng *et al.* (1955) reported the optimum pH for cellulose digestion to be from 6.8 to 7.6. Rumen microorganisms exhibited a greater tolerance to a high pH than to a low pH. Church and Peterson (1960) concluded the optimum pH was 6.0 to 6.7; however the pH tended to vary according to substrate and/or source of rumen liquor.

Burroughs et al. (1950a) noted that cattle and sheep fed rations of a widely varient nature indicated differences in the predominating types of rumen microorganisms. The same general type of organisms were present in each animal even though the predominating type was different.

Minerals and vitamins

Burroughs et al. (1950b) reported that rumen microorganisms have nutritional requirements which must be fulfilled if they are to digest roughage efficiently. It was suggested that these nutrients are found in ample quantities in certain good quality roughages and are inadequate in certain poor quality roughages. Cellulose digestion, using the in vitro technique, has shown needs for varying amounts of the elements sodium, potassium, calcium, magnesium, phosphorous, sulfur and chlorine. Burroughs et al. (1951) noted that iron and phosphorous found in the ashes of plants or plant products have a stimulatory effect upon cellulose digestion in vitro.

Several workers have observed the effects of water extracts and ash of good and poor quality roughages. Marquardt (1964) noted that grass hay extract supported very poor cellulose digestion even when it contained high levels of protein, ash, calcium and phosphorous. Comparable to other extracts, the high carbohydrate content may have suppressed cellulose digestion. Burroughs et al. (1950a) reported a beneficial influence of the ash of alfalfa upon roughage digestion which has been referred to in in vivo experiments. Bentley et al. (1951) provided evidence which indicated that the low phosphorous content of the poor quality forage was a

limiting factor in cellulose digestion *in vitro*. Hunt *et al*. (1954) found that more cellulose was digested and more urea utilized when inoculum was obtained from steers fed alfalfa hay than from steers fed poor quality hay. Marquardt (1964) showed that many of the nutrients required by rumen microorganisms for the fermentation of cellulose were present in aqueous extracts of forages; however phosphorous, sulfur and in some cases nitrogen were not present in adequate amounts.

Burroughs *et al*. (1951) found that urea utilization like cellulose digestion was dependent to a large extent upon the nutrient requirements of rumen microorganisms. They suggested that a roughage was utilized most efficiently when each of the nutrient requirements was adequately supplied.

Hunt *et al*. (1954) observed that microorganisms which synthesize riboflavin, niacin and vitamin B_{12} and also digest cellulose and utilize urea for protein synthesis have a sulfur requirement. Bentley *et al*. (1954) found that biotin, vitamin B_{12}, para-aminobenzoic acid, xanthine, uracil, guanine and adenine when added to the basal medium improved cellulose digestion. The amount of cellulose digested was always less than that digested in flasks which contained rumen juice supernatent, a water extract of alfalfa, yeast, or molasses. This implied that there were factors in natural products which enhanced the microbial activity in *in vitro* fermentation.

Hubbert *et al*. (1958) found sulfur, magnesium and calcium to be the inorganic nutrients most likely to be deficient in a prepared fermentation medium. Extremely low levels of copper, cobalt, zinc and boron depressed cellulose digestion.

Vitamin B_6 showed stimulatory activity in cellulose digestion according to Macleod and Murray (1956).

Carbohydrates

A very important factor affecting cellulose digestibility is the amount and type of concentrate fed with the cellulose containing material. Head (1961) reported the main effect of concentrates was due to their content of starch, which lowered the digestibility of roughage.

Head (1961) and el-Shazly (1961) reported that with low nitrogen containing cellulose substrates, the organisms digesting sugars or starch used all the available soluble nitrogen sources in the rumen liquor, thus starving the cellulolytic organisms of this nutrient.

Mills et al. (1941) reported that when adequate fermentable carbohydrate was included in the ration, urea was utilized at a maximum rate and a maximum efficiency in the rumen of the cow. He suggested the carbohydrate served as a readily available energy source for the microorganisms enabling them to build new protoplasm in which the nitrogen from the urea was incorporated.

Baker (1942) found that the addition of increasing amounts of starch to rumen contents incubated in vitro resulted in a parallel increase in the rate of multiplication of microorganisms of all types. Marston (1948) noted that in diets containing a high proportion of starch, the rate of proliferation of the microflora in the rumen was limited usually by the nitrogen supply. When additional nitrogen was provided, a large proportion was converted to protein.

Arias et al. (1951) found that increasing the energy content of the fermentation medium resulted in an increased urea utilization with all sources of energy tested. This was true whether the energy source was a soluable carbohydrate such as dextrose or sucrose, or whether the carbohydrate was more complex such as cellulose. It was also noted that rumen microorganisms have definite energy requirements and the degree to which these requirements are fulfilled has considerable influence upon their utilization of urea or other ammonia supplying compounds. Small amounts of readily available carbohydrate aided cellulose digestion which in turn increased urea utilization. Large amounts of readily available carbohydrate inhibited cellulose digestion. Hunt et al. (1954) supported Arias' work in that rumen microorganisms obtain their energy requirements from the more readily available source, instead of breaking down cellulose.

Macleod and Murray (1956) noted that glucose stimulated cellulose digestion at a concentration of .05 percent to 2 percent. Decreased digestion was observed at higher concentrations of glucose.

Cline et al., (1958) reported a greater synthesis of valeric acid from the more readily available carbohydrate. These workers offered this as an explanation for increased in vitro cellulose digestion observed by Arias et al. (1951) and Hunt et al. (1954) with a 1:2 starch-cellulose mixture versus cellulose alone.

Belasco (1956) found the optimum starch-cellulose ratio to be 1:4. Here urea utilization, cellulose digestion and volatile fatty acid production reached a peak. The inclusion of large amounts of dextrose inhibited cellulose digestion. These data also supported Arias et al. (1951)

which showed a need for readily available carbohydrate in the proper proportions. Salsbury (1961) found that if the proportion of readily available carbohydrate was too high it was doubtful that cellulose digestion could be maintained at a significant level. Johnson et al. (1960) noted that when the starch-cellulose ratio reached 1:1 or higher, cellulose digestion was completely or severely depressed. At higher ratios of starch to cellulose, both in vivo and in vitro cellulose digestion was completely inhibited and was not recovered by additional quantities of nitrogen. The effect of high levels of glucose was shown by Huhtanen and Elliot (1956). They postulated that the inhibitory effect of high levels of glucose may be explained on the basis of availability of energy sources for the rumen bacteria. If enough glucose was present, the organisms used it for a carbon source, rather than the less available cellulose.

Kane et al. (1959) in a conventional digestion trial showed that high levels of corn starch had a depressing effect upon the digestion of alfalfa hay; however when a preliminary period for adjustment to corn starch was used, no depression in the digestion of alfalfa hay was observed.

el-Shazly et al. (1961) proposed several theories to explain the depression of cellulose digestion by starch in a ration. These are as follows: (1) production of an inhibitor by starch digesting microorganisms, (2) decrease in rumen pH due to acids produced from starch fermentation, (3) competition for essential nutrients with the result that starch digesting microorganisms proliferate preferentially, (4) predominance of starch digesting microorganisms in the rumen of animals on high starch diets.

Urea appeared to be the most efficient of all nitrogen supplements tested by el-Shazly in alleviating the depression of cellulose digestion caused by starch in a ration.

Lignin

The degree of lignification of cellulose is an important factor in cellulose digestion because the digestibility of cellulose is lowered by the presence of lignin, Head (1961). Sullivan (1955) reported that the percent lignin and the digestion coefficients of cellulose are correlated from -0.92 to -0.94. Percentage of lignin is significantly correlated with digestibility of all insoluble carbohydrates. Tomlin et al. (1965) found that as grasses and legumes matured the digestibility of cellulose decreased and lignin content increased.

Gaillard (1962) noted a strong correlation between the digestibility of organic matter and the percentage crude fiber, percentage lignin and percentage cellulose plus lignin. A decrease in digestibility of the plant was observed as the plant matured.

Some evidence has been obtained that lignin itself is digestible under certain conditions. Kane et al. (1951) observed the products of metabolism of the lignin molecule in ruminant urine. The amount of lignin actually digested was very small and no microorganisms capable of attacking lignin have been found.

The grinding of feeds has been tried as a method of increasing digestibility of cellulose. The expected effect of grinding is to increase total surface area and give bacteria more surface to attack. This has not proven

too useful due to the fact that smaller particles pass through the digestive system faster and lower digestibility rather than increase it.

Dehority and Johnson (1961) found the total amount of cellulose digested increased with the time of ball-milling. Amount of forage cellulose digested by rumen bacteria decreased with increasing maturity and lignification of the plant. The increase in *in vitro* cellulose digestibility due to ball-milling became larger as the forage matured and lignin deposition was increased. There was a basic difference in grasses and legumes in regard to amount of cellulose which could be digested per given amount of lignin in the plant. This evidence presented by Dehority and Johnson substantiated the theory that lignin in forages acts as a physical barrier between the cellulose and cellulolytic rumen bacterial.

Enzymatic Starch Digestion

Balls and Schwinmer (1944) found that grains of corn starch were more rapidly broken down than grains of wheat starch. Potato starch was much more resistant to enzyme activity. It was also noted that the size of the starch grains did not appear to be a limiting factor.

Leach and Schoch (1961) explained that high linear corn starch is least affected by enzyme activity. They also observed that normal pea starch is attacked more rapidly than high linear starch from wrinkled peas; therefore the presence of a linear fraction probably retards solubilization by enzymes.

According to Sandstedt (1955) when amylase acts on raw starch, the enzyme enters at the narrow edge of the granule and following the path of

least resistance digests its way into the granule. Sandstedt (1960) also noted that starch which has been damaged will digest easier than undamaged starch granules. Enzymes do not penetrate freely into the molecular lattice of the granule, but are limited to certain accessible surfaces or regions, Leach and Schoch (1961).

In Vitro Starch Digestion

Moore et al. (1962) studied the use of the in vitro system of starch fermentation. A high speed centrifugal procedure was used to obtain the predominate rumen bacteria. Maximal starch fermentation was measured colorimetrically. The difference between concentrations of anthrone reactive material in inoculated flasks at the time of inoculation, and after a period of incubation was taken as an index of fermentation. Maximal starch digestion occurred at about 12 hours which amounted to 90 percent of the added starch. Starch was added at the rate of 1.5 grams per 100 ml. of liquid.

Loper et al. (1966) used a gravimetric technique to study starch digestion in vitro. A series of experiments based on washed cell suspensions of rumen microorganisms was conducted to develop an accurate, simplified technique. Starch remaining after fermentation was measured gravimetrically. Results obtained by this method were highly correlated ($r = 0.94$) with the results using the anthrone procedure. The fermentation was carried out at a pH of 6.8. Maximal starch digestion occurred at 12 hours, which amounted to 70 percent of the added starch. Starch was added at the rate of 0.5 percent.

There has been very limited work conducted on *in vitro* starch digestion. The foregoing literature on *in vitro* starch digestion studies is the only work reported.

PROCEDURES

General Procedures

Barley varieties were selected on the basis of known or expected structural differences observed in laboratory analysis of their respective starches, Goering et al. (1957) and Goering and Imsande (1960). Varieties selected were Compana, hulless Compana (Newpana), Betzes, Oderbrucker and Unitan. Corn starch was used as a standard for comparison since corn is a major source of carbohydrate in livestock rations. Milo and wheat starches were also included because wheat is being used in Montana livestock rations and milo is used extensively as a feed grain in other areas of the United States.

Barley starch was separated from barley flour by Dr. K. J. Goering using the method of Dimler et al. (1944). Barley flour used in the above procedure was obtained from the Montana State University Cereal Quality Laboratory.

Three trials were conducted to measure differences in the in vitro digestibility of starches from selected varieties of grain. Amylose and amylopectin were included in one comparison. Identical in vitro procedures were employed in each trial. Starches compared in this study were as follows: Trial 1 - corn, Compana, Oderbrucker, milo and wheat; Trial 2 - corn, Compana, Newpana, Betzes and Unitan and Trial 3 - corn, Compana, Newpana, amylose, and amylopectin.

Residual starch was measured by the phenol-sulfuric acid, colorimetric method for hexoses described by Whistler (1962). The percent starch digested was calculated using as the initial amount of starch the values

obtained from control tubes in which bacterial action was not allowed to take place. Absorbances were measured on a Bausch and Lomb Spectronic 20.

Fermentation vessels were analyzed for residual starch every 2 hours for a maximum of 12 hours. Blanks consisting of rumen liquor and nutrient media but without added starch were analyzed at zero time to correct for starch present in the rumen liquor.

The phenol-sulfuric acid method was compared to the anthrone colorimetric method described by Moore et al. (1962) using corn starch as the substrate.

Absorbance curves were prepared with a Beckman DK2 spectrophotometer to insure maximum absorbance for all substrates.

A 3 x 5 x 6 factorial design was used in all trials as described by Li (1965).

In Vitro Procedure

Each experiment was initiated at 5:00 A.M. Rumen contents were obtained from a three-year-old fistulated Hereford steer maintained on a ration consisting of 1/3 steam rolled barley, 1/3 dehydrated alfalfa pellets, and 1/3 beet pulp pellets with salt provided free choice. The steer was fed this ration 10 days prior to the initial collection. Rumen contents were collected and strained through four layers of cheese-cloth into a 39° C. prewarmed thermos. Rumen liquor was then centrifuged at 2,000 x G for 20 minutes using an international size 2 centrifuge. The supernatent was mixed with pre-warmed (39° C.) carbon dioxide saturated nutrient medium at a rate of 3 parts rumen liquor to 4 parts nutrient

medium. The composition of the nutrient medium used is as follows:

KH_2PO_4	0.600 gm/liter	NaCl	4.00 gm/liter
Na_2HPO_4	1.600 gm/liter	$FeSO_4.7H_2O$	0.075 gm/liter
$NaHCO_3$	3.500 gm/liter	$CaCl_2$	0.550 gm/liter
KCl	4.00 gm/liter	Urea	2.000 gm/liter

Carbon dioxide was bubbled through the mixture for 10 minutes and the pH adjusted to 6.8. Fifty ml. of rumen liquor and nutrient medium was added to 100 ml. fermentation vessels which were fitted with rubber stoppers containing inlet and outlet glass tubing for the introduction of carbon dioxide. Starch was added at the rate of 0.5 percent or 250 mg. starch per fermentation vessel. Blank vessels containing no added starch were included to correct for starch added *via* the rumen liquor. Fermentation vessels were placed in a 39° C. water bath. Carbon dioxide was bubbled through the tubes slow enough to prevent excessive splashing. Fermentations were carried out for a maximum of 12 hours.

Measurement of Starch Fermentation

Phenol-sulfuric acid procedure

One ml. of unknown containing between 20 and 50 ug. substrate was pipetted into a colorimetric tube. One ml. of 5 percent distilled phenol solution was added and contents mixed. Five ml. concentrated sulfuric acid were added to each tube. Each tube was agitated during acid addition. After standing 10 minutes, tubes were shaken and placed in a 25-30° C. water bath for 20 minutes. Absorbances were measured at 490 mu. After subtracting the absorbances of the blank, the amount of sugar was deter-

mined from a standard curve prepared from corn starch.

Anthrone colorimetric method

Ten ml. of anthrone reagent in a series of test tubes were immersed in a cold water bath (10-15° C.) and carefully overlayed with 5 ml. of sample containing from 20-40 ug./ml. of starch. After the addition of starch was made, the tubes were agitated and replaced in the cold water bath. When the series was completed the tubes were heated for 16 minutes in a boiling water bath and then cooled. Absorbances were read at 625 mu. and after subtracting the absorbances of the blank, the amount of sugar was determined from a standard curve prepared from corn starch.

RESULTS AND DISCUSSION

Trials I, II and III

The pertinent data of Trials I, II and III showing the percent digestion for the 5 substrates every 2 hours on each of 3 days are given in Tables I, III and V respectively. Analysis of variance of the data for the respective trials are given in Tables II, IV and VI.

Analysis of this data revealed considerable variance and several highly significant interactions in all trials. All interactions excluding hours x variety for Trials I and II were highly significant ($P < .01$). The hours x variety interaction was significant ($P < .05$) for trial III. Since the subsequent digestion rates of the substrates were significantly different ($P < .05$) and the hours x variety interaction was not significant, some general inferences can be made concerning the differences in digestibilities of the starches.

In Trial I, Oderbrucker starch was digested significantly faster ($P < .01$) or equal to corn starch and the other starches. In no case was Oderbrucker digested significantly slower. In most instances wheat and milo starch were digested slower than corn starch; however on day 1 wheat tended to digest faster than corn starch. Digestion of all substrates was very rapid on day 3 of Trial I; therefore it is difficult to detect any real differences. On days 1 and 3 at the end of 12 hours fermentation, the percent digestion had exceeded 95 percent. On day 2, 90 percent digestion was approached at 12 hours. Replications were highly significant ($P < .01$) for Trial I indicating that the reproducibility of measuring residual starch in the triplicate fermentation vessels was unsatisfactory.

TABLE I. PERCENT DIGESTION OF STARCHES IN TRIAL I.

		Percent Digestion 1/				
	Hours	Corn	Oder-brucker	Compana	Milo	Wheat
Day 1	2	18.0^{a}	17.7^{a}	17.9^{a}	18.5^{a}	12.6^{b}
	4	37.8^{a}	36.1^{a}	36.3^{a}	36.8^{a}	30.7^{b}
	6	48.7^{a}	60.4^{c}	55.5^{b}	50.7^{a}	52.8^{b}
	8	72.9^{d}	87.5^{c}	79.6^{a}	67.2^{b}	80.6^{a}
	10	88.6^{ab}	94.0^{c}	92.9^{bc}	86.5^{a}	93.4^{c}
	12	99.2^{a}	99.3^{a}	99.4^{a}	99.0^{a}	99.1^{a}
Day 2	2	9.6^{ab}	19.0^{c}	18.3^{c}	12.6^{b}	8.0^{a}
	4	40.6^{c}	36.5^{bc}	33.5^{ab}	31.2^{a}	22.5^{d}
	6	44.7^{c}	40.7^{bc}	41.5^{bc}	38.2^{ab}	35.9^{a}
	8	60.0^{ab}	63.5^{b}	56.4^{a}	56.3^{a}	50.0^{c}
	10	78.0^{ab}	84.2^{c}	80.0^{bc}	75.3^{a}	78.5^{ab}
	12	90.2^{ab}	92.1^{b}	89.9^{ab}	86.3^{a}	92.4^{b}
Day 3	2	37.2^{a}	43.3^{b}	43.1^{b}	33.6^{a}	37.7^{a}
	4	77.5^{b}	75.5^{ab}	77.8^{b}	71.7^{a}	76.6^{b}
	6	93.7^{a}	91.9^{a}	93.7^{a}	93.0^{a}	91.4^{a}
	8	95.4^{a}	96.0^{a}	95.9^{a}	95.6^{a}	94.0^{a}
	10	95.8^{a}	95.2^{a}	95.7^{a}	95.1^{a}	95.0^{a}
	12	97.5^{a}	96.9^{a}	97.0^{a}	97.1^{a}	96.9^{a}

1/ Values expressed are an average of 3 replications.

a, b, c, d Means on the same line bearing different superscript letters are significantly different ($P < .01$) as determined by Duncan's multiple range test.

TABLE II. ANALYSIS OF VARIANCE OF PERCENT DIGESTION FOR STARCHES IN TRIAL I (CORN, ODERBRUCKER, COMPANA, MILO, WHEAT).

Source	D.F.	S.S.	M.S.	F.
Replication	2	66.05	33.03	8.00**
Days	2	42,696.55	21,348.28	5,172.80**
Hours	5	164,648.65	32,929.73	20.72**
Variety	4	893.14	223.29	4.36*
Days x Hours	10	15,891.69	1,589.17	385.06**
Days x Variety	8	409.53	51.19	12.40**
Hours x Variety	20	620.63	31.03	1.06
Days x Hours x Variety	40	1,168.82	29.22	7.08**
Error	178	734.66	4.13	
TOTAL	269	227,129.72		

* $P < .05$
** $P < .01$

It can be seen from the data of Trial II presented in Table III and from the analysis of variance (Table IV) that there were large variations and highly significant interactions; however the hours x variety interaction was considerably smaller than in Trial I. In Trial II corn starch was digested significantly faster ($P < .01$) or equal to the other starches in all but one instance. On day 2 at 2 hours, Unitan and Newpana were digested significantly faster ($P < .01$). Unitan starch was digested significantly slower ($P < .01$) or equal to the other starches; however on day 2 at 2 hours Unitan was digested significantly faster than Betzes, Compana or corn starch. Compana, Newpana and Betzes starch were intermediate between corn starch and Unitan; however it is difficult to determine how they did compare because of day to day and hour to hour variation. At the

TABLE III. PERCENT DIGESTION OF STARCHES IN TRIAL II.

		Percent Digestion 1/				
	Hours	Corn	Compana	Newpana	Betzes	Unitan
Day 1	2	16.6^{b}	9.4^{a}	6.4^{a}	7.2^{a}	9.0^{a}
	4	22.9^{b}	18.6^{ab}	15.5^{a}	18.7^{ab}	9.5^{c}
	6	48.6^{b}	37.0^{a}	33.9^{a}	33.9^{a}	11.0^{c}
	8	65.2^{b}	59.6^{a}	57.2^{a}	58.6^{a}	34.4^{c}
	10	83.6^{a}	83.8^{a}	81.4^{a}	80.3^{a}	70.7^{b}
	12	96.0^{b}	95.3^{ab}	94.9^{ab}	94.4^{ab}	91.0^{a}
Day 2	2	8.7^{ab}	7.2^{a}	14.8^{cd}	12.7^{bc}	18.5^{d}
	4	21.8^{b}	23.1^{b}	23.7^{b}	16.4^{a}	20.6^{ab}
	6	39.7^{b}	33.8^{a}	41.3^{b}	37.4^{ab}	33.3^{a}
	8	58.1^{c}	50.6^{ab}	48.3^{a}	53.7^{bc}	48.5^{a}
	10	80.2^{b}	74.7^{a}	71.4^{a}	73.5^{a}	62.2^{c}
	12	92.6^{b}	89.5^{a}	89.0^{a}	89.8^{a}	83.8^{c}
Day 3	2	17.4^{d}	8.8^{bc}	3.1^{a}	9.4^{c}	4.2^{ab}
	4	22.4^{b}	23.0^{b}	19.7^{ab}	21.2^{ab}	17.4^{a}
	6	36.2^{bc}	37.7^{c}	31.6^{ab}	32.1^{ab}	29.8^{a}
	8	49.4^{a}	46.8^{a}	46.9^{a}	47.7^{a}	47.7^{a}
	10	66.0^{a}	67.8^{a}	65.7^{a}	67.3^{a}	66.4^{a}
	12	86.9^{bc}	90.8^{c}	83.9^{ab}	84.3^{ab}	82.2^{a}

1/ Values expressed are an average of 3 replications.

a, b, c, d Means on the same line bearing different superscript letters are significantly different ($P < .01$) as determined by Duncan's multiple range test.

end of 12 hour fermentations in almost all cases the substrates reached 90 percent digestibility. It should be noted that replications were not significantly different in Trial II.

TABLE IV. ANALYSIS OF VARIANCE OF PERCENT DIGESTION FOR STARCHES IN TRIAL II (CORN, COMPANA, NEWPANA, BETZES, UNITAN).

Source	D.F.	S.S.	M.S.	F.
Replications	2	3.63	1.81	.4267
Days	2	988.57	494.28	116.25**
Hours	5	213,830.98	42,766.20	244.67**
Variety	4	2,631.77	657.94	4.30*
Days x Hours	10	1,747.93	174.79	41.11**
Days x Variety	8	1,224.45	153.06	36.00**
Hours x Variety	20	894.66	44.73	.8147
Days x Hours x Variety	40	2,196.05	54.90	12.91**
Error	178	756.81	4.25	
TOTAL	269	224,274.85		

* $P < .05$
** $P < .01$

The analysis of variance (Table VI) and the data from Table V show that for Trial III there were again large variations and highly significant interactions. Unlike Trials I and II, the hours x variety interaction was significant ($P < .05$) indicating that the substrates did not digest in the same manner at the different hours. Due to the fact that all interactions were significant, general conclusions cannot be made; however from an inspection of the data there are obvious differences, namely amylopectin and amylose digestion. In all cases amylopectin

TABLE V. PERCENT DIGESTION OF SUBSTRATES IN TRIAL III.

		Percent Digestion 1/				
	Hours	Corn	Compana	Newpana	Amylo-pectin	Amylose
	2	11.4^{a}	11.3^{a}	9.9^{a}	18.9^{b}	20.1^{b}
	4	25.8^{a}	23.9^{a}	24.1^{a}	56.7^{c}	42.0^{b}
Day 1	6	41.8^{a}	39.8^{a}	36.7^{a}	75.1^{c}	57.4^{b}
	8	55.9^{a}	53.6^{a}	56.5^{a}	86.1^{c}	74.4^{b}
	10	70.3^{a}	68.1^{a}	71.5^{a}	90.7^{c}	78.6^{b}
	12	89.6^{b}	91.4^{b}	88.2^{ab}	92.5^{b}	83.4^{a}
	2	13.4^{a}	16.2^{a}	18.9^{a}	57.0^{c}	31.6^{b}
	4	24.0^{a}	24.3^{a}	22.7^{a}	90.1^{c}	49.9^{b}
Day 2	6	45.6^{a}	51.0^{a}	49.5^{a}	95.8^{c}	64.4^{b}
	8	81.0^{ab}	83.3^{b}	76.3^{a}	96.1^{c}	66.0^{d}
	10	93.9^{a}	95.9^{a}	94.1^{a}	98.1^{a}	79.7^{b}
	12	97.0^{a}	98.1^{a}	97.1^{a}	98.7^{a}	84.2^{b}
	2	39.3^{a}	38.7^{a}	29.3^{c}	45.7^{b}	42.5^{ab}
	4	76.9^{a}	75.8^{a}	74.6^{a}	91.5^{a}	48.9^{b}
Day 3	6	95.9^{a}	94.9^{a}	92.9^{a}	95.7^{a}	77.3^{b}
	8	97.7^{a}	98.2^{a}	97.9^{a}	97.4^{a}	82.2^{b}
	10	99.0^{a}	99.1^{a}	99.1^{a}	98.8^{a}	86.0^{b}
	12	99.0^{a}	99.1^{a}	99.2^{a}	98.9^{a}	90.6^{b}

1/ Values expressed are an average of 3 replications.
a, b, c, d Means on the same line bearing different superscript letters are significantly different ($P < .01$) as determined by Duncan's multiple range test.

digested significantly faster ($P<.01$) or equal to the other substrates. On day 1 amylose digested significantly faster ($P<.01$) than corn, Compana or Newpana; however at 12 hours amylose was significantly slower ($P<.01$) than corn, Compana and amylopectin. On day 2 amylose digested significantly faster ($P<.01$) than corn, Compana or Newpana at 2, 4 and 6 hours; however at 8, 10 and 12 hours amylose was significantly slower ($P<.01$). On day 3 amylose digested faster at 2 hours but beyond 2 hours amylose digested slower than the other substrates. As in Trials I and II, digestion was either greater than or approached 90 percent at the end of 12 hours fermentation. Replications were again non-significant as in Trial II.

TABLE VI. ANALYSIS OF VARIANCE OF PERCENT DIGESTION FOR STARCHES IN TRIAL III (CORN, COMPANA, NEWPANA, AMYLOPECTIN, AMYLOSE).

Source	D.F.	S.S.	M.S.	F.
Replications	2	18.82	9.41	1.29
Days	2	33,775.36	16,887.68	2,317.28**
Hours	5	145,119.51	29,023.90	35.57**
Variety	4	14,605.97	3,651.49	4.10*
Days x Hours	10	8,159.56	815.96	111.96**
Days x Variety	8	7,100.47	887.56	121.79**
Hours x Variety	20	7,226.75	361.34	1.94*
Days x Hours x Variety	40	7,456.57	186.41	25.58**
Error	178	1,297.21	7.29	
TOTAL	269	224,760.22		

* $P<.05$
** $P<.01$

The results of these trials indicate that if the digestibility of starches is to be compared by the *in vitro* method, improved techniques must be employed.

It became evident as the trials progressed that an improved inoculum should be tried which would contain a more representative starch digesting bacterial population. Strained, centrifuged rumen liquor as used in these studies probably contained many types and strains of bacteria plus enzymes which would digest starch. Moore *et al.* (1962) used a differential centrifugation to prepare a bacterial sediment that was microscopically representative of the mixed culture found in the whole rumen fluid of sheep. Loper *et al.* (1966) used a washed cell suspension following the method of Cheng *et al.* (1955) to obtain a predominate bacterial population. An inoculum similar to that used by Moore and Loper could possibly eliminate some of the variability due to abnormal bacterial populations. There are however many factors which affect the bacterial populations. Johnson (1963) reported that numbers of species and types of microorganisms which digest starch in the rumen are considerably greater than for cellulose. This is complicated further by the fact that inoculum taken from a given animal on a standard starch ration may differ from day to day in predominating species of microorganisms. Moore *et al.* (1962) noted the variability in the amylolytic flora of sheep. Variability in the microflora within the animal can be a result of rumen volume, Purser and Moir (1966a) and Purser and Moir (1966b), or ration characteristics, Christiansen *et al.* (1964). By providing the donor animal with a constant ration and sampling at a constant time after feeding variability in microflora can be reduced.

Structural differences between starches should be examined when comparing differences in starch digestion. A microscopic examination can be made to determine differences in granular size and shape. It has been reported that structural differences affect starch breakdown. Baker et al. (1950) noted a distinct difference between maize starch and potato starch when they were subjected to in vivo digestion by oxen. Goering et al. (1957) reported that the amylose content of 44 different barley varieties varied from 13 to 24 percent. Leach and Schoch (1951) found that high linear corn starch (i.e. corn starch with a high percentage of amylose) is least affected by bacterial enzyme activity. Harris (1962) and Greenwood (1964) stated that starch granules in general contain more amylopectin than amylose. These authors imply that starches with a high percentage of amylopectin will digest faster than those starches with a high percentage of anylose. In Trial III, amylopectin digested faster than amylose, indicating that a high linear barley starch may digest slower. If starches with high percentages of amylopectin repeatedly digest faster than high linear starches, then it may be possible that starches can be ranked in regard to digestibility by their percentages of amylopectin and amylose.

Phenol-Sulfuric Acid - Anthrone Comparison

The data for the comparison of the phenol-sulfuric acid and anthrone procedures is shown in Table VII. There was a highly significant correlation ($r = 0.99$) between the two methods. The percentage of residual starch measured was comparable except at the 2 and 4 hour determinations. These results indicate the phenol-sulfuric acid method used in this study

was equivalent to the anthrone method employed by Moore et al. (1962).

TABLE VII. COMPARISON BETWEEN THE ANTHRONE METHOD AND THE PHENOL-SULFURIC ACID METHOD USING CORN STARCH ON DAY 3 TRIAL III.

	Percent Digestion	
Hours	Anthrone	Phenol-Sulfuric Acid
	29.9	37.0
2	27.9	38.0
	29.2	36.5
	84.1	77.1
4	82.7	78.5
	82.6	77.0
	92.8	93.7
6	91.8	93.2
	93.0	94.2
	98.0	95.3
8	97.8	95.4
	97.6	95.4
	98.9	95.8
10	99.2	95.9
	99.2	95.8
	98.9	97.4
12	98.6	97.6
	98.1	97.7

r = .99

The phenol-sulfuric acid colorimetric method has worked very well in the trials reported in this manuscript. Dubois et al. (1956) observed that the phenol-sulfuric acid procedure was simple, rapid and sensitive and gave reproducible results. The reagent is inexpensive and stable and a given solution requires only one standard curve for each sugar.

There are several inherent problems in the anthrone procedure used by

Moore et al. (1962). The concentration of acid in the reagent and the manner in which the reagent and test solution are brought together determine the nature and intensity of the color developed, Johanson (1954). Morris (1948) described some of the problems associated with the anthrone procedure. These are as follows: (1) the reagent is difficult to prepare, (2) if cooling is produced before the reaction is finished, error can be introduced, (3) a known sugar standard must be run with each series of unknowns, (4) fresh reagent must be prepared daily and (5) aging of the reagent causes a color change.

Moore et al. (1962) using the anthrone procedure recovered 130 $\pm$ 5.7 percent of the added starch. Using the phenol-sulfuric acid method in this laboratory the recovery of added starch was 109 $\pm$ 6.5 percent (Table VIII).

With the evidence presented the phenol-sulfuric acid procedure because of simplicity can be used in preference to the anthrone method for estimating in vitro starch digestion.