EFFECT OF SUPPLEMENTING CORN SILAGE WITH ISOACIDS AND UREA ON PERFORMANCE OF HIGH PRODUCING COWS

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ABSTRACT

EFFECT OF SUPPLEMENTING CORN SILAGE WITH ISOACIDS AND UREA ON PERFORMANCE OF HIGH PRODUCING COWS

By

Arthur Felix

The effect of supplementing high urea-corn silage-based rations for Holstein cows with isoacids on nitrogen utilization, growth, and milk production was studied over a period of five years. A total of 112 high producing lactating cows and 20 growing heifers were used in four separate milk production trials and one growth study. One *in vitro* experiment was also conducted. The addition of isoacids to urea rations increased rumen microbial activity *in vitro*, improved rate of growth of young animals, increased persistency of lactation, and improved nitrogen retention in lactating cows.

In the *in vitro* study, five experiments were carried out using gas production as a parameter to estimate the effect of different concentrations of isoacids on rumen microbial activity. Concentrates or corn starch and filter paper were used as the source of energy. The addition of

isoacids to urea depressed the rate of gas production irrespective of the source and the amount of substrate used in the medium. This study showed that the isoacids affected the fermentation process, and therefore, enhanced rumen microbial activity.

In the growth study, isoacids and phenylacetate were added with urea to low quality grass hay fed to 20 dairy heifers averaging from 171 to 327 kg body weight. Isoacids and phenylacetate increased the growth rate of the younger animals but not of the older animals.

In the first milk production trial the isoacids and phenylacetate were fed with urea to 24 lactating cows in a randomized block design using soy protein as a positive control, urea control, and urea plus isoacids and phenylacetate treatment. Corn silage was used as the only roughage and concentrate was not fed. Soy protein was most effective in increasing milk production, persistency of lactation and body weight. Isoacids improved milk production, persistency of lactation and body weight over the urea alone.

In the second trial, corn silage was supplemented with urea plus isoacids fed to 28 lactating cows in a randomized block design consisting of a positive control, a negative control, urea, and urea plus isoacids. Concentrate was fed according to the level of milk production. Isoacids improved corn silage dry matter intake and persistency of lactation over the urea control.

The third milk production trial used 30 lactating cows fed corn silage in a cross-over design consisting of a urea control and urea plus two different levels of isoacids. Concentrate was fed according to milk production. A nitrogen balance trial was also conducted at the end of each period. The addition of isoacids to urea improved fat corrected milk, persistency of lactation, decreased plasma urea nitrogen and rumen ammonia nitrogen, increased rumen acetate and nitrogen retention over urea fed alone.

In the fourth trial, 30 lactating cows fed corn silage and concentrate and urea as supplemental crude protein, were assigned to two treatment groups in a continuous feeding experiment. Isoacids increased the persistency of lactation and total feed intake.

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Arthur Felix

A DISSERTATION

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DEDICATED TO

The memory of my mother, Elucia; my father, Darius, whose many sacrifices and courage have enriched my life,

and

my wife, Jeanine, whose patience and encouragement have made this work possible.

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INTRODUCTION

The world population is still growing at a rate sufficient to double its current number every thirty years. The most important concern is how to properly feed the people in the world today. Of almost equal importance is how to provide improved diets for people in the future. The rate of population growth is faster than the rate of food production, at least in many countries of the world (Hodgson, 1971). Requirements to meet projected food needs are indeed alarming. In 1968, estimates indicated that to provide about 2,400 kcal of energy and 35.1 g of protein per capita per day to the world population, the supply will have to be increased by about 38 percent over the 1970 needs by 1985 (Agr. Statistics, 1968). Further estimates suggest that supplies of food crops, sugar, starchy food, vegetable and oil seed crops, as well as milk, meat, eggs, and fish all would need to be increased by 37 to 39 percent to meet human physiological caloric needs (Hodgson, 1971). The shortage of protein-rich foods to meet future world demands is constantly being stressed. Current estimates indicate that more than 50 percent of the world population is suffering

from diet protein shortages. United Nations agencies recommend that priority be given to increasing world protein supplies.

In more affluent countries such as the United States, animal products become more expensive to produce as world demands for cereals and protein supplements increase. Scientists all over the world are striving to increase the production of conventional protein of high quality and to develop procedures for preparing new proteins suitable for feeding both man and animals. Two foods, rich in protein of high biological value, are milk and meat, in the production of which the bovine holds the key position. Because of the microbial population inhabiting the rumen, the ruminant animal is unique in its ability to convert to milk or meat for human consumption those feeds which are metabolically less available to other species. For example, if ruminant animals are fed forages and other feeds inedible by man along with limited amounts of cereal grains the efficiency of producing proteins for humans in terms of total resource utilization can be enhanced (Moore et al., 1967). A report by Hardin and Rogers (1970) indicated that about 29 percent of total land area is forested, 38 percent is apparently unused, and 22 percent is in permanent meadow and pasture. The latter area is a large supplier of feed for the world's ruminant population. Only 11 percent of the total land is in permanent crop production and an undetermined amount of this is in rotation forage crops of various kinds. A

considerable portion of the forested land does, or can, provide grazing. Also, the forage and crop refuse from land under permanent crops can be added to that forage supply. The grassland crops have a limited role in human food production except as feed for runinants, especially dairy cattle, which have the highest efficiency in converting feed protein to food protein.

The case for the ruminant animal as a producer of protein is even stronger when their capacity to use non-protein nitrogen (NPN) is considered. Many feeding experiments with various non-protein nitrogen compounds have been performed. One of these compounds most widely used and most thoroughly investigated is urea. The use of the feed-grade urea in the United States in 1973 was approximately 800,000 tons (Allen, 1974). The use of this quantity of urea spares approximately 4.5 million tons of 50 percent protein supplement that can be used for feeding man and other monogastric animals. The real ability of ruminants to utilize urea as the sole source of nitrogen for protein synthesis is most clearly seen when urea is fed to milk-producing cows, which require protein in especially high amounts (Virtanen, 1966). Moore et al. (1967), calculating the protein input and output of a cow producing 5,295 kg of milk annually with usual feeding practices of the U.S., found that the recovery of protein in the milk was about equal to the protein consumed as grain and oilseed concentrates. However, when urea was used to partially replace protein concentrates, the protein

in the milk averaged about 21 kg more per cow per year than was consumed in cereals and legumes. Because of the increasing and severe competition for natural protein by human beings and non-ruminant animals, it becomes necessary that a portion of the natural protein in ruminant diets be replaced with non-protein nitrogen. But a most outstanding feature of urea feeding is the extent of its utilization with the basic diet especially by high-producing cows.

Protein is the nutrient which often limits the performance of high-producing cows. Because it is quantitatively the major component of animal tissue dry matter, because of various physiological and biochemical functions of proteins in the animal body, and because of high rates of milk production and the relatively high content of milk, high-producing lactating cows are more likely to encounter a shortage of protein than any other ruminants. An often forgotten feature of protein nutrition in ruminants is its dualistic nature: the microbial metabolism in the forestomachs on one hand, and the non-microbial metabolism in the remainder of the alimentary tract and the tissues of the animal on the other hand.

Thus, the fate of the dietary protein fed will depend upon digestive processes as well as the physiological status of the animal. Furthermore, cows producing more than 35 kg of milk daily may fail to produce to their genetic capacity because of the lack of some key nutrients such as protein. In some cases, a cow's physical capacity limits

her feed intake so that nutrient supply to the mammary gland is not enough for maximum production of milk and milk protein. Thus, in many cases, the high producing lactating cow, at the peak of lactation is deficient in energy, protein and calcium. One of the most important goals for the animal nutritionist to pursue is to increase the efficiency of protein utilization to fulfill the requirement of the high-producing cows. In cases where the digestive processes improve the quality of protein reaching the absorptive sites of the small intestine, they would be classified as beneficial such as the case of urea utilization (Chalupa, 1972).

However, a major problem in the efficient use of a high level of dietary urea is its rapid hydrolysis into ammonia and the subsequent inability of the rumen microorganisms to utilize the ammonia at a rate comparable to its production. A considerable portion of the excess ammonia nitrogen is lost through metabolic excretions. Furthermore, the ability of the rumen bacteria to utilize ammonia depends on the availability of a suitable source of energy and carbon skeletons. If the nutritional status of high-producing cows is to be improved, it is imperative that the key nutrients be identify, nutrients which may be in short supply to the microbial population, and be provided in the time of greatest need.

Several investigators have reported that supplementing urea-based rations with the isoacids* (n-valerate, isobutyrate, isovalerate and 2-methylbutyrate) improved the nitrogen economy of the animals. There is considerable evidence that rumen microorganisms utilize the carbon skeletons of the isoacids in the synthesis of the corresponding branched-chain amino acids and for microbial protein formation. But, to date, there exists no evidence of the beneficial effect of feeding a combination of urea and these isoacids on the performance of high-producing lactating cows.

The objective of the studies reported herein was to investigate the influence of the direct supplementation of the isoacids to high urea-corn silage-based rations on nitrogen utilization and on the subsequent performance of high-producing lactating cows.

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*The term isoacids for the purposes of this dissertation refers to a mixture of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate.

LITERATURE REVIEW

Feeding Corn Silage and Urea to Dairy Cattle

The use of corn silage as a basic roughage constituent for dairy cattle has increased rapidly because of its high energy yields ease of mechanization and storage, and uniform feeding value. The number of hectares of corn harvested as corn silage has nearly doubled in the United States during the past two decades. In Michigan, corn silage is the most important silage crop with over four million metric tons produced in 1973 (Mich. Agr. Stats., 1974). Cattle feeders have become increasingly aware of the excellent forage characteristics of corn silage and its value in the ruminant ration.

The corn plant is similar to other forage grasses in that it contains most of the same components, although the concentrations are somewhat different from those in other forages. The high digestibility of its crude fiber by ruminants, and high concentrations of starch and other soluble carbohydrates are peculiar to corn silage and enhance its energy value compared to most forage crops. Research on corn silage diets for dairy cattle has been reviewed (Riley, 1967; Coppock and Stone, 1968; Huber, Polan and Hillman,

1968a). Several trials comparing corn silage to other forages have been reported (Holter et al., 1973; Belyea et al., 1975a, 1975b; Thomas et al., 1970; Vandersall et al., 1970). These trials showed that corn silage or corn silage plus hay gave similar intake and production responses and that there appeared to be no advantage for including hay in corn silage rations. Because corn silage is widely recognized as a high quality roughage, it has been used as a standard of comparison for many other silages. As a source of energy for milk production, corn silage has been shown to be equal or superior to dried-corn silage, alfalfa-bromegrass silage, corn-treated meadow-crop silage, broomcorn silage, beets and many other types of silages (Coppock and Stone, 1968).

The voluntary consumption of corn silage dry matter appears to increase as the dry matter content increases, at least within certain limits (Huber et al., 1965). Corn silage has a relatively low crude protein content of 8 to 9 percent on a dry matter basis. Various approaches have been used to increase the protein content of corn silage or to supplement it economically with non-protein nitrogen (NPN). One supplemental method, which is widely practiced, is to feed corn silage in combination with high-protein legumes or grass and legume mixture (Coppock and Stone, 1968). Another procedure which is increasing rapidly in popularity is to add urea or other non-protein nitrogen compounds to the whole corn plant at ensiling. Experiments conducted by Huber et al. (1965, 1967, 1968b, 1971) and by Schingoethe

and Beardsley (1975) have shown that urea added to corn silage at ensiling is efficiently utilized as a nitrogen source for milk production. However, Coppock and Stone (1968) reported that the higher protein content of the urea-corn silage is not due to the urea *per se*, but rather to its sparing action since it appears to reduce the degradation of protein during the silage fermentation.

Corn silage is also a convenient carrier for urea. The feeding urea with corn silage masks the undesirable taste of urea, and also allows more intake of urea over the whole day and minimizes possible excesses in the blood compared to including the urea in concentrate which is consumed in relatively short periods (Huber *et al.*, 1968a). Polan and co-workers (1968) reported that adding urea to silage at ensiling to supplement as much as 38 percent of the total nitrogen in the ration did not significantly reduce milk production. A more uniform intake of NPN during the day may partially explain those results (Huber *et al.*, 1968). Some losses of urea-nitrogen usually occur during storage and feeding. These losses may be even greater if corn is deficient in energy. Conrad and Hibbs (1968) point out that about 1 kg of rapidly fermentable carbohydrate is required per 100 g of urea for maximum utilization of urea in the adapted dairy cow. It was later stipulated that at least two-thirds of that fermentable carbohydrate should be in the form of starch.

However, the problem of using urea to supplement corn silage rations lies not in the question of whether to use it, but rather under what condition and by what techniques and routes of administration of appropriate levels urea can be most effective.

Huber and co-workers (1967) compared the utilization of nitrogen by lactating cows fed corn silage (ad libitum as the only forage) which had been ensiled with 0.0, 0.5, or 0.7 percent urea. The level of urea in the silage did not significantly influence the level of milk production, silage or total intake. But in a second trial it was discovered that cows fed a 0.85 percent urea-corn silage ration were in severe negative nitrogen balance because of lowered protein digestibility and large urinary nitrogen losses. This was substantiated by the work of Polan et al. (1968, 1970) showed that adding urea to corn silage at ensiling at levels of 0.5 to 0.85 percent resulted in increases in urinary nitrogen and decreases in nitrogen retention, although milk production was not affected. It was then postulated that the cows receiving the high urea (0.85 percent) silage were drawing heavily on endogenous protein supplies.

The benefits of using both urea and natural protein to supplement corn silage for balancing rations for high-producing cows have been studied (Huber and Thomas, 1971; Conrad and Mugerwa, 1970; Van Horn and Jacobson, 1969). However, divergent results have been observed concerning the optimum level of urea which can be successfully used for

milk production. Huber and associates (1967a) observed a depression in milk production when high corn silage rations were supplemented with urea in the concentrate to supply 21 percent or more of the total nitrogen in the diet. But when urea supplied only 11 percent of the nitrogen, no depression occurred. Later Van Horn and Jacobson (1971) found that the addition of urea beyond 11.4 percent dietary protein was of little benefit. In a earlier study the same investigators (Van Horn and Jacobson, 1967) observed a reduction in feed intake by cows consuming a dairy concentrate containing 2.2-2.7 percent urea. However, addition of 0.5 percent urea to corn silage and 1.0 percent to concentrate did not affect feed intake or milk production. Holter et al. (1968) found no depressing effect on feed intake and milk production, but decreases in nitrogen retention were found when high quality multi-ingredient concentrate mixtures containing up to 2.5 percent urea were fed with corn silage forage. Their highest level of urea provided about 300 g of urea per day. Urea increased rumen ammonia nitrogen from 15 mg to 30 mg/100 ml fluid during the first hour after feeding. The concentrate mixture contributed to the high feed intake and excellent performance.

Boman et al. (1969) studied restricted concentrate supplementation with corn silage ad libitum. Diets compared were:

1. Corn silage plus a 20 percent protein grain supplement;
2. Corn silage plus 45 percent cottonseed meal; and
3. Urea-corn silage (0.5 percent) urea plus cottonseed meal added to shelled corn.

Differences among treatment groups were small in total dry matter intake, but there was a trend in favor of groups fed restricted concentrate and more urea with respect to milk production and weight gain. Consumption of the urea-corn silage was depressed during the first month of the study. This subject deserves more attention in high-producing cows early in lactation.

Conrad and Hibbs (1967b) pointed out that ingestion of approximately 0.4 to 0.5 kg of NPN per 100 kg of body weight would result in depression of feed intake and milk production in cows fed urea treated corn silage as the only forage. High-producing cows are apparently affected more adversely by high urea than are low producers (Huber et al., 1967a).

Van Horn and Jacobson (1971) suggested that for near optimum performance of lactating dairy cows fed a corn silage and urea ration, some high quality dietary protein such as soybean meal is required.

Some of the Dietary Factors that Affect Microbial Protein Synthesis

The celluloytic bacteria produce the enzyme cellulase which hydrolyzes the insoluble cellulose to soluble

cellulodextrins and/or sugars (Bryant, 1973). These products are fermented by the organisms to obtain energy for growth. The final products of the rumen fermentation of cellulose are mainly acetate, propionate, butyrate, carbon dioxide, methane and microbial cells. These compounds are not produced by celluloytic species alone, but by a complex interacting microbial population. This population includes:

1. The cellulolytic species;
2. Other carbohydrate-fermenting species;
3. Species that degrade compounds such as succinate and formate, and, in some cases, lactate formed by the carbon dioxide fermenting species; and
4. Methanogenic bacteria that obtain energy for growth by reducing carbon dioxide to methane (Hungate, 1966; Hungate et al., 1970).

The efficiency with which rumen bacteria digest the cellulose of plants depends upon many factors. These factors are mainly: ammonia, readily available energy, carbon skeletons, vitamins, minerals, growth stimulators or inhibitors (antibiotics, hormones, anabolics), and factors that influence the chemical and physical environment such as the amount of associated lignin and silica (Van Soest, 1969), pH, temperature, diet particle size and density, salivation and rumination, presence or absence of oxygen, etc. (Bryant, 1973; Moore, 1964; Leatherwood, 1973).

The most important bacterial species found in the rumen of domestic animals are Ruminococcus flavefaciens

(Sijpesteijn, 1951), Ruminococcus albus (Hungate, 1957), Bacteroides succinogenes (Hungate, 1950), and Butyrivibrio fibrisolvens (Bryant and Small, 1956). A few other species including a number of the genus Clostridium (Shane et al., 1969) and Cillobacterium cellulosolvens (Bryant et al., 1958, Van Gylswyk and Hoffman, 1970), have been found, but they are less important. The four major species seem to obtain their energy for growth only via carbohydrate fermentation (Bryant, 1973). Among these species, Bryant (1973) found Bacteroides succinogenes to be in general, the most actively celluloytic organism and to digest the more resistant cellulose, such as cotton fibers and mature hay, to a far greater extent than the other species.

Growth and yields of rumen microbes are an important determinant of ruminant animal performance. Furthermore, the rumen microbes are an important source of protein for the animal, and also influence the rates of fermentation. Low microbial growth rates might reduce fermentation and as a result reduce intake of low energy rations. The effects of low microbial growth rates upon amino acid availability to the animal and upon intake are particularly relevant when low protein roughages, by-product feed and non-protein nitrogen are fed. Thus, it is important to identify factors which influence rumen microbial protein synthesis.

One of the important nutrients for rumen microbial growth is ammonia. The increasing substitution of urea and other non-protein nitrogen sources for plant protein in the

rations of dairy and beef cattle is economically important because worldwide demand for natural protein exceeds available supplies. However, although cows have demonstrated an ability to lactate and reproduce on purified diets containing only NPN (99 percent) as a source of nitrogen (Oltjen and Bond, 1967; Virtanen, 1966, 1969), only partial replacement of dietary plant protein nitrogen with NPN has been recommended for lactating dairy cows. The variable factors involved in urea feeding have been well-reviewed (Reid, 1953; Freitag et al., 1966; Oltjen, 1969, Chalupa, 1968, 1970, 1972, 1973; Helmer and Bartley, 1971). Irrespective of the source of dietary nitrogen or non-protein nitrogen, ammonia is a central intermediate in the degradation and assimilation of nitrogen and it is the main nitrogenous nutrient for ruminal microbial growth (Bryant and Robinson, 1961, 1962; Bentley et al., 1955; Bryant, Robinson and Chu, 1959; Dehority, 1963; Hungate, 1966).

The relative importance of ammonia as compared to amino acids in the nutrition of rumen bacteria was discussed by Bryant and Robinson (1962). They examined the nitrogen requirement of 44 strains of rumen bacteria and found that 82 percent could be grown with ammonia as the sole nitrogen source, 30 percent would not grow unless ammonia was present, and 56 percent could use either ammonia or amino acid nitrogen (Bryant and Robinson, 1962). Information summarized by Bryant (1963) and Hungate (1966) indicates that ammonia is an essential nutrient for growth of Bacteroides succinogenes,

Ruminococcus flavefaciens, Ruminococcus albus, Bacteroides amylophylus, Methobacterium ruminantium and Eubacterium ruminantium, even when preformed organic nitrogen is present in the media (Warner, 1955; Abou Akkada and Blackburn, 1963). Under such conditions, ammonia is not essential, but may stimulate growth of some strains of Succinovibrio dextrinosolvens, Butyrivibrio fibrisolvens, Bacteroides ruminicola, and Streptococcus bovis (Bryant and Robinson, 1962; Gill and King, 1958; Shane et al., 1969; Chalupa, 1972).

The positive relationships between conversion of nitrogen sources to ammonia and the in vitro magnitude of both cellulose and starch degradation indicate the importance of ammonia as an essential nutrient for fiber--and starch--digesting bacteria (Acord et al., 1966; Chalupa et al., 1963). Therefore, it is reasonable to assume that dietary NPN will be of little benefit to the ruminant animal unless it is first converted into ammonia and then utilized for microbial protein synthesis in the rumen. Slyter and co-workers (1968) found that 7.4 percent of 403 strains of rumen bacteria possessed urease activity. Some strains which possessed ureolytic activity were identified as Propionibacterium sp., Bacteroides sp., Ruminococcus sp., Streptococcus bovis, and an anaerobic Lactobacillus. Allison (1969, 1970) reported that ammonia nitrogen is primarily incorporated into bacterial cells and appears in protozoal cells as a consequence of ingestion of bacteria by protozoa. Al Rabbat et al. (1971) observed that 61 percent of microbial nitrogen

was derived from ammonia and 39 percent was from ammino acid and peptide nitrogen. A similar observation has been reported by Pilgrim and associates (1970) indicating that 70 percent and 64 percent of bacterial nitrogen was derived from ammonia when animals were fed wheaten hay and lucerne hay, respectively.

The percentage of microbial protein derived from urea was greatest with a diet containing low protein (Nikolic Anna, 1972). A more recent in vitro study by Maeng and co-workers (1976) indicated that the optimum ratio of non-protein nitrogen for microbial growth was 75 percent urea nitrogen and 25 percent amino acid nitrogen. Allison (1969) stipulated that when microbial growth is limited by the availability of nitrogen, it is probable that ammonia concentration is critical and supplementation or replacement of dietary protein with urea may, in certain instances, increase bacterial or protozoal concentrations. But when dietary protein is adequate, addition of urea to the ration may have little effect on microbial growth (Satter and Roffler, 1975). The point at which the ammonia concentration becomes limiting for growth of ruminal bacteria has not been clearly defined. Neither has the optimum concentration of ruminal ammonia required for maximum cell yield been established. This is probably because the concentration depends upon such factors as level of feeding, solubility of dietary protein, availability of carbohydrates and minerals to the microbes, frequency of feeding, etc. Recent in vitro research by Satter

and Slyter (1974) indicated that increasing the ammonia concentrations above 5 mg NH_3-N per 100 ml fluid was of little benefit to the ruminant animals. However, *in vivo* results by Slyter *et al.* (1973) showed that nitrogen retention of steers was improved by maintaining ruminal ammonia concentrations above 8 mg/100 ml. Miller (1973) also studied this problem *in vivo* and reported that the greatest microbial flow from the rumen was achieved with rumen ammonia concentrations of approximately 28 mg NH_3-N/100 ml fluid. Probably the increased performance with the higher ammonia concentrations *in vivo* over that *in vitro* is due partially to the possible beneficial effects of ammonia outside the rumen, such as synthesis of non-essential amino acids in the liver (National Research Council, 1976).

As indicated earlier, ammonia is the common denominator in the utilization of NPN by ruminants (Hungate, 1966). If the rumen microorganisms cannot degrade the compound in question to yield free ammonia, it is useless as a nitrogen source to the microorganisms. The ability of rumen microorganisms to utilize ammonia is dependent upon the presence of a suitable source of energy (Lewis, 1961, 1962; Briggs, 1967; McDonald, 1948; Otagaki *et al.*, 1955). Briggs (1967) and McDonald (1948) demonstrated that available energy enhanced microbial protein synthesis. It is well established that readily fermentable carbohydrates, starches or grains, rather than roughages are required for optimum utilization of urea (Conrad and Hibbs, 1968; Belasco, 1956). The

availability of energy from different sources is the key to evaluating their dietary effects in the ruminant. Adequate available energy is essential for evaluating the utilization potential of any nutrient. Lewis (1961) demonstrated the effect of different carbohydrate sources and concluded that the type of carbohydrate present regulates ammonia concentration in the rumen and the extent of microbial protein synthesis. This conclusion is substantiated by the widely successful use of urea as the supplementary source of nitrogen in high-grain diets. Chalupa (1968) reported that rumen microorganisms have a definite energy requirement and that the degree to which this requirement is met definitely influences the utilization of urea. In experiments in which animal performance appears to be superior on preformed protein-supplemented diets over performance on NPN-supplemented diets and when protein needs were low, the differences were likely due to the energy contributions of the preformed portein in low-protein diets. Burroughs et al. (1971a, 1971b, 1974) proposed a system for the evaluation of feeds based on estimated urea fermentation potential (UFP). This system recognizes that microbial protein synthesis is primarily dependent upon energy availability and that the conversion of rumen degraded protein into microbial protein represents an energy cost.

In addition to furnishing energy, dietary preformed proteins are sources of the branched-chain carbon skeletons (isobutyrate, isovalerate and 2-methylbutyrate). Rumen

microorganisms can use NPN for protein synthesis if the necessary carbon skeletons are present or if these can be synthesized fast enough from dietary carbohydrate or alternate carbon sources. Bryant (1972) reported that strains of cellulolytic species usually required some carbon sources other than those used as the energy source. Primary sources of carbon fragments that arise from carbohydrate fermentation are carbon dioxide and volatile fatty acids (VFA's).

The use of carbon dioxide by the rumen microbes as the source of carboxyl carbon has been reported by Otagaki et al. (1955) and Wright (1960), showing that ^{14}C from $^{14}CO_2$ was utilized in protein synthesis by mixed rumen bacteria. Other investigators have noted this requirement and most of the work was summarized by Dehority (1971). The species fall roughly into two groups. The first group includes Bacteroides succinogenes and R. flavefaciens which have an absolute requirement and require large amounts of these compounds for optimal growth since they fix CO_2 into pyruvate in the path of succinate (White, Bryant and Caldwell, 1962). Without carbon dioxide, these species are unable to obtain energy for growth. They also use CO_2 for biosynthetic purposes (Allison, 1969, 1970). The second group includes Butyrivibrio fibrisolvens and Ruminococcus albus which may or may not require a small amount of CO_2 for initiation of growth but require a small amount for optimal growth (Dehority, 1971). These species probably require CO_2 mainly for biosynthetic purposes. In their review, Kay and

Hobson (1963) noted that when rumen microorganisms were grown in a medium containing casein hydrolysate, isobutyrate, isovalerate and $^{14}CO_2$ (in carbonate form), 2 percent fo the cellular lipid carbon was from ^{14}C of the $^{14}CO_2$, whereas 17 percent and 77 percent were in nucleic acid and protein respectively. The label in the protein was found in 15 amino acids including the three branched-chain amino acids, leucine, isoleucine and valine. All the radioactivity was observed in the carboxyl carbon of the amino acids. The investigators concluded that carbon dioxide is an important precursor of the carboxyl group of these amino acids. From this observation the same workers listed the following sources of carbon: carbohydrates, carbon dioxide and volatile fatty acids (VFA). However, there are specific requirements for isobutyrate, isovalerate, 2-methylbutyrate, phenylacetate, and indole-3-acetate to provide for the synthesis of the specific amino acids (Allison, 1969). There are potential sources of keto acids in rumen fluid, but the branched-chain VFA's arise mainly from the deamination of branched-chain amino acids provided by dietary protein (El-Shazly, 1952a; Annison, 1954). It is significant that the feeding of protein-free diets causes a depression in the concentration of these acids (Orskov and Oltjen, 1967; Oltjen, 1969; Chalupa et al., 1970), with isovalerate and isobutyrate being greatly influenced (National Research Council, 1976). The high levels of the enzyme activity present in the rumen would indicate that insufficient carbon skeletons could limit ammonia assimilation

(Hoshino et al., 1966; Chalupa, 1972). This observation is in agreement with the calculations of Balch (1967) showing that the level and type of dietary carbohydrate has a large influence on the efficiency of NPN utilization.

Several investigations have been conducted, which showed that a number of rumen microorganisms require one or more of the volatile fatty acids, n-valeric, isovaleric, isobutyric and 2-methylbutyric acids for growth (Allison, Bryant and Doetsch, 1959, 1962a; Allison, 1969, 1970, 1965; Robinson and Allison, 1967; Bryant and Doetsch, 1955; Kunsman, 1970; Slyter and Weaver, 1971). Bryant and Doetsch (1962a) observed that two fatty acid components are necessary for growth of Bacteroides succinogenes; one of these components is a branched-chain volatile fatty acid which may be either isobutyric, isovaleric or DL-α-methylbutyric acids; the other component is a straight-chain acid. Both straight- and branched-chain acids or any combination of them have been found to be growth stimulatory (Bryant and Robinson, 1962). Bentley et al. (1954), Dehority et al. (1957, 1958, 1967) and Allison and Bryant (1963) reported that four and five carbon branched- and straight-chain VFA are essential for the in vitro growth of at least some of the rumen cellulolytic microorganisms. Allison et al. (1958) reported that three cellulolytic strains of R. flavefaciens and two cellulolytic strains of R. albus, which are among the most numerous and most important of the cellulolytic organisms isolated from the rumen, have nutritional requirements for

isoacids. Recently Maluszynska and co-workers (1974) isolated a cellulolytic micromonospora which was found to be stimulated by valeric acid. Bryant and Robinson (1961) extended this finding to four additional strains of R. albus, one of which was non-cellulolytic.

Non-cellulolytic rumen bacteria with isoacid requirements have been observed. Wegner and Foster (1960) isolated a bacterium and an unnamed gram-positive rod which require both branched- and straight-chain acids. Bryant (1959) noted that all of several strains of Eubacterium ruminantium required isoacids and that a number of other non-cellulolytic bacteria are greatly stimulated by these acids. It has also been reported that rumen bacteria that require 2-methylbutyric acid for growth include the Methanobacterium ruminantium strain M-1 and B. ruminicola strain H_2b (Bryant, 1965; Dehority, 1966).

The requirement of these acids by the cellulolytic bacteria emphasizes an interesting interaction among the rumen bacterial species to obtain materials often essential for their growth. When purified "poor quality" diets high in cellulose and lacking some of these essential materials are fed to ruminants, the cellulolytic bacteria grow and maintain their functions, at least to a limited extent, because of production of these factors by other bacteria.

Bentley et al. (1954, 1955) reported that n-valeric, isovaleric, isobutyric and n-caproic acids or their amino acid precursors stimulate cellulose digestion and the

conversion of urea nitrogen into protein by rumen microorganisms as measured by artificial rumen techniques. Burroughs and co-workers (1951) working with mixed cultures of bacteria from the rumen have shown that cellulose digestion occurs with ammonia or urea as the sole source of nitrogen. Cellulose digestion was stimulated if protein was included, but ammonia or urea stimulated that digestion above that observed with protein as nitrogen source. McLeod and Murray (1956) also showed that certain amino acids can replace protein in stimulating cellulose digestion. These included valine, leucine, isoleucine and proline (Dehority, 1958).

It is generally accepted that nitrogen from catabolized amino acids enters an ammonia pool. Probably there are extracellular and intracellular pools. However, the pool size of free, extracellular amino acids in the rumen is usually quite low (Allison, 1970). Extracellular concentrations of α-amino nitrogen, measured, after dialysis or ultra-filtration, are usually less than half the values for total free amino acids measured from acidified samples. It is suggested that the differences may be due to release of amino acids from microbial cells in acidified samples (Wright and Hungate, 1967a). Qualitative and quantitative estimates of the free amino acids in rumen protozoa have been made. A small proportion of the free, extracellular glycine, glutamate, or aspartate was incorporated intact into microbial cell substances, but a much larger proportion of labelled carbon from these amino acids was found in volatile fatty

acids and CO_2 (Wright and Hungate, 1967b). While some species of rumen bacteria use exogenous amino acids (Bryant and Robinson, 1963), amino acids in peptides are more efficiently utilized than are free amino acids by certain species (Pittman and Bryant, 1964; Wright, 1967). Other species are less able to use preformed amino acids and assimilate amonia nitrogen in quantities approaching or equivalent to the amount of nitrogen incorporated into the cells. This occurs even when these organisms are grown in a medium containing a complete mixture of amino acids (Allison, 1969; Bryant and Robinson, 1961, 1963). The most probable explanation for the apparent failure of amino acids to complete with amonia is the low activity of, or absence of systems for transport of amino acids into the cells (Allison, 1969; Bryant, 1973).

The ability of many rumen bacteria to assimilate ammonia nitrogen implies also that they have the ability to construct the carbon skeletons of the amino acids that constitute the protoplasm of the cells. In effect, the microflora of the rumen has been shown to be capable of both producing and utilizing isoacids. El-Shazly (1952a) was the first investigator who reported the presence of isoacids in rumen fluid. He indicated a positive correlation between the level of protein in the diet and the level of the isoacids in the rumen fluid. Later, Annison (1954) and El-Shazly (1952b) observed that the branched-chain VFA, isobutyrate, isovalerate and 2-methylbutyrate arose from the degradation of dietary protein and the subsequent

deamination of the corresponding branched-chain amino acids. They then concluded that the probable origins of isobutyrate, isovalerate and 2-methylbutyrate are valine, leucine and isoleucine, respectively. Slyter and Weaver (1969) reported that branched-chain VFA may be synthesized by ruminal bacteria when a substrate devoid of protein is supplied to a mixed rumen microbial population of cellulolytic bacteria which require them for growth. The same workers noted that even when a diet was fed which contained no branched-chain amino acids, the carbon skeleton precursors of branched-chain fatty acids, the cattle were still able to maintain a large population of cellulolytic bacteria that require fatty acids for growth. Other investigators have reported pronounced depressions in the ruminal concentrations of isobutyric and isovaleric acids (Orskov and Oltjen, 1967; Cline et al., 1966; Freitag et al., 1966), somewhat depressed levels of these acids (Clifford and Tillman, 1968) or lowered quantities of isobutyric acid (Matrone et al., 1966) when ruminants were fed a protein-free diet. Replacing isolated soy protein by urea has also resulted in lower acetic acid proportions and increased proportions of butyric acid (Orskov and Oltjen, 1967).

In other studies Oltjen and associates (1969) observed less ruminal branched-chain fatty acids from cattle fed a purified diet containing urea than in cattle fed purified diets which contained soy-protein. Of interest is the observation by Annison (1954) that the branched-chain

VFA concentration in the rumen almost certainly depends not only on the rate and extent of degradation of dietary and microbial protein but also on the rate of absorption of these acids. Conversion of leucine into isovaleric acid by Bacteroides ruminicola has been reported by Bladen, Bryant and Doetsch (1961).

Dehority et al. (1967) noted that in vitro digestion of cellulose by a mixed culture of microorganisms is stimulated by leucine, valine and isoleucine as well as by corresponding VFA's produced by anaerobic catabolism of these amino acids. The branched-chain amino acids, phenylalanine and tryptophan are deaminated and decarboxylated in the rumen and the acids produced accumulate in appreciable concentration (El-Shazly, 1952b; Menahan and Schultz, 1964; Allison, 1970). Allison (1970) stipulated that the volatile products from alanine are acetate and formate, and that phenylacetate is the major catabolic product of phenylalanine metabolism. This was confirmed by Martin (1973) showing the conversion of phenylalanine to phenylacetate excreted in the urine of sheep. He then suggested that the amount of phenylacetate excreted in the urine is a measure of the equilibrium occurring in the rumen between catabolism of phenylalanine and reutilization of products of catabolism for phenylalanine synthesis.

Yokoyama and Carlson (1974) reported that incubation of tryptophan with ruminal microorganisms resulted in formation of indole acetic acid (Lacoste, 1961). N-valerate was

found to be produced from either carbohydrate or from proline (Dehority et al., 1958; Elsden et al., 1956; Pottele et al., 1966).

Although catabolism of dietary branched-chain amino acids is a major source of branched-chain VFA, small amounts of these acids were present in the rumen when purified diets containing no protein were fed (Clifford and Tillman, 1968). This suggests that the acids were produced from amino acids synthesized by microbial cells and may indicate turn over of these cells (Bryant, 1973; Allison, 1969).

Amino Acid Synthesis by Rumen Microorganisms

Information on free amino acid pool size, turnover, and metabolic fate and on nitrogen sources for pure cultures of rumen bacteria (Wright and Hungate, 1967a, 1967b; Bryant and Robinson, 1963) suggests that a relatively small portion of the amino acids that pass through the extracellular pool are incorporated intact into microbial protein. Furthermore, evidence has already been given that amino acids in peptides are incorporated by certain rumen microbes more efficiently than are free amino acids.

Nevertheless, even in animals fed on ration containing appreciable quantities of protein, it is likely that a large portion of the amino acids in microbial proteins are synthesized de novo from intermediates or end-products of carbohydrate fermentation, or end-products of amino acid metabolism (Allison, 1970). Microbial biosynthetic

capacities are shown when ruminants grow and produce milk while fed on purified diets with NPN as the only source of nitrogen (Virtanen, 1966). Even on such diets, there are detectable quantities of branched-chain VFA in the rumen and also organisms that require them (Slyter and Weaver, 1969). Therefore, the metabolism of the branched-chain amino acids indicates a turnover of microbial protein or a metabolism of endogenous animal protein (Phillipson, 1964). Bacteriophages (Adams et al., 1966; Paynter et al., 1969) or unidentified factors (Jarvis, 1968) are potential sources, but branched-chain VFA are present in the rumen mainly as the result of degradation of dietary protein and deamination of branched-chain amino acids (Chalupa, 1972). Rumen bacteria are able to synthesize the carbon skeletons of leucine, isoleucine, valine, phenylalanine, tryptophan, alanine and glutamate by reductive carboxylation reactions not likely to function in aerobic organisms (Allison, 1970). The use of carbon skeletons of the branched-chain VFA for rumen microbial protein synthesis has been largely discussed (Allison et al., 1962a; Allison and Bryant, 1962, 1963; Allison and Peel, 1971; Allison, 1969; Cline et al., 1966; Oltjen et al., 1971; Hume, 1970; Allison, Bryant and Doetsch, 1962). The branched-chain VFA (Allison, Bryant and Doetsch, 1962; Allison and Bryant, 1963), phenylacetic acid (Allison, 1965b), and indole acetic acid (Allison and Robinson, 1967) are carboxylated and aminated by mixed cultures of ruminal microorganisms incubated in vitro and by a number of pure cultures of important

ruminal bacteria to resynthesize the original amino acid which is then incorporated into microbial protein. The pathways are different from any that have been described for biosynthesis of these amino acids (Allison, 1969). Carbon skeletons of branched-chain fatty acids were incorporated mainly into the lipid and protein portion of the cells. Higher branched-chain fatty acids and fatty aldehydes were synthesized from isovalerate and isobutyrate (Allison, Bryant, Katz and Keeny, 1962; Wagner and Foster, 1963).

In the protein fraction leucine was synthesized from isovalerate (Allison, Bryant and Doetsch, 1959, 1962; Hoover et al., 1963; Singer and Doolittle, 1975), valine from isobutyrate (Allison and Bryant, 1963; Allison and Peel, 1971; Quay et al., 1975) and isoleucine from 2-methylbutyrate (Robinson and Allison, 1969; Hungate, 1966). The tracer evidence also indicated that carboxyl groups of these amino acids were formed by carboxylation. Hoover and co-workers (1963) have observed that carbon from each of these sources C_2, C_3, C_4, C_5 was incorporated into the amino acids and that the rate of their utilization was proportional to their concentration in the incubation flasks. A recent study by Allison and associates (1974) demonstrated that four strains of R. albus utilized indole acetic acid for tryptophan synthesis. This is in agreement with reports by Lacoste (1961) and Scott et al. (1964) that indole-3-acetic acid is a major product of tryptophan metabolism by the rumen microbial population.

The possible conversion of valeric acid to proline has been suggested (Amos et al., 1971; Potter et al., 1966; Bentley et al., 1955; Dehority et al., 1957). But no real evidence has been given so far that valeric acid carbon is incorporated into proline.

It is also tempting to suggest that histidine may be synthesized from imidazole acetic acid since Land and Virtanen (1959) found that when ^{15}N ammonia was fed to cattle, little N from ammonia was incorporated into the imidazole ring of histidine.

Noteworthy is the fact that the straight-chain acids such as n-valeric are also used for biosynthesis of higher fatty acids such as n-pentadecanoic acid and for similar fatty aldehydes; these compounds are biosynthesized by the addition of two-carbon units to the carboxyl end of the valerate molecule and major components of the phospholipids of Bacteroides succinogenes (Wegner and Foster, 1963).

Results from many experiments with mixed rumen populations conducted both in vivo and in vitro indicate that synthesis of ruminal microbial protein is dependent upon the availability of carbohydrate (Hungate, 1963). The energy for amino acid biosynthetic reactions is probably mainly derived from glycolytic reactions. Intermediates produced during carbohydrate fermentation and fermentation end-products (CO_2, acetate) are primary sources of carbon for bacterial amino acid biosynthesis (Allison, 1969; Chalupa, 1968, 1970; Tillman and Sidhu, 1969; Otagaki, 1955).

Acetate has been found to stimulate growth of ruminal bacteria, especially when ammonia is the main source of nitrogen (Hungate, Bryant and Mah, 1964). This suggests that acetate is an important carbon source for biosynthesis of certain amino acids.

Apart from their importance in the metabolism of the host animal, B-vitamins may play an important part in regulating microbial interrelationships in the rumen. It has been reported that dietary urea appears to stimulate B-vitamin synthesis in the rumen (Teeri and Colovos, 1963; Briggs et al. , 1964). Because of the complexity of the rumen microflora, it is difficult to generalize about requirements for certain nutrients, especially the B-vitamins. However, it appears that specific B-vitamins are needed by certain strains of rumen bacteria; and, if they are not present at adequate concentrations in the rumen fluid, growth of some bacterial strains may cease (NRC, 1976). Also rumen bacteria are known to produce as well as to use certain B-vitamins (National Research Council, 1976). Bryant and Robinson (1961) reported that all Butyrivibrio fibrisolvens strains so far studied required one or more B-vitamins. The one strain studied in detail by Gill and King (1958) required biotin, pyridoxine, and folic acid.

Certain minerals are required by rumen bacteria and the host animal but substitution of NPN for dietary protein does not increase requirements for these beyond those stated in feeding standards (National Research Council, 1976). But,

since NPN and grain are combined in a mixture to replace protein supplements in isonitrogenous diets, it must be remembered that this combination of NPN plus carbohydrate source may contain a lower level of essential minerals than does the replaced protein supplement. Therefore, the use of NPN compounds in ruminant diets makes it quite important for nutritionists to consider the necessity for adding other mineral source in the diet. B. succinogenes was shown to have a high requirement for sodium and a relatively high need for calcium (Bryant et al., 1959). Other studies suggested that most species of the rumen anaerobes required sodium, potassium, calcium and phosphate, iron, zinc and molybdate for optimum cellulose digestion (Bryant, 1973).

Substitution of urea for natural protein removes from the diet a major source of sulfur which is essential for microbial synthesis of sulfur amino acids (Chalupa, 1968). B. succinogenes utilizes either cysteine or sulfide but not sulfate (Bryant and Robinson, 1962). Sulfide seems to be essential for some strains of R. albus (Allison et al., 1958). The study of Emery et al. (1957) suggested that Butyrivibrio fibrisolvens, but neither B. succinogenes nor ruminococci utilize ^{35}S of sulfate. The need for sulfur in the diet of ruminants has been reviewed by Garrigus (1970). Brown et al. (1960) produced a significant response by adding sulfur, sulfate or methionine to urea rations, using a purified diet deficient in sulfur. Other factors such as growth stimulators

or inhibitors have been reviewed in some detail in the recent report of the National Research Council (1976).

Supplemental Feeding of Isoacids and Urea to Ruminants

The main finding on the nutrition of the cellulolytic bacteria which has been shown to have some practical value in animal nutrition has been the beneficial effect often obtained by adding isoacids to diets. Some studies have shown that the addition of a mixture of four-and-five-carbon branched-chain and straight-chain fatty acids to low protein diets may lead to an increase in feed intake by ruminants. Hensley and Moir (1963) found that the intake of a milled oaten hay diet by sheep was increased by the addition of 0.56 percent of a mixture of isobutyrate, isovalerate and n-valerate. Later, Van Gylswyk (1970) supplemented low protein teff hay with urea plus isobutyrate, isovalerate, 2-methylbutyrate and n-valerate to sheep conditioned to these diets. He observed an increase in voluntary hay intake accompanied by increased numbers of cellulolytic bacteria per gram of rumen ingesta, and increased proportions of Ruminococci as compared to urea alone.

Other studies showed increased microbial protein (Hensley and Moir, 1963) or increased nitrogen flow rate. Hume (1970) examined the effect of supplementing a low protein diet for sheep with a mixture of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate, in terms of the amount of protein produced in the rumen. The addition of

the isoacids to urea increased protein production. A similar observation has been made by Kay and Phillipson (1964) when they infused isoacids in addition to urea into sheep fed poor quality hay. Replacing these acids with the same amounts of acetate, propionate and butyrate resulted in little effect on duodenal nitrogen, suggesting that the increase in duodenal nitrogen flow rate was due to the addition of the isoacids.

Increased nitrogen retention is one of the most current beneficial effects observed from the feeding of isoacids with urea. Cline and co-workers (1966) fed 4.18 g isobutyrate, 1.18 g n-valerate and 5.9 g isovalerate per lamb per day and observed a significant increase in nitrogen retention.

Öltjen and co-workers (1971) investigated whether a dietary addition of the three branched-chain fatty acids and phenylacetate would improve the nitrogen utilization by steers fed a protein-free diet. Increased nitrogen retention and decreased urinary nitrogen were observed with the addition of the acids. However most of the change was noted in steers fed isolated soy protein.

Oltjen et al. (1970) obtained similar results and a tendency of the isoacids to increase the corresponding plasma amino acids over the urea treatment. Umunna and associates (1975) reported higher nitrogen retention and lower urinary nitrogen loss by lambs fed high roughage rations supplemented

with urea plus isobutyrate and isovalerate as compared with urea alone.

An increase in cellulose digestion by lambs has been reported by Cline et al. (1966) when urea and isoacids were fed. Hungate and Dyer (1966) observed that for steers the addition of valeric acid and isovaleric acid to wheat straw and urea rations stimulated appetite.

The possibility that the isoacids, isobutyrate, isovalerate and valerate might be limiting growth in calves fed urea-containing diets was examined by Miron et al. (1968). They observed greater body weight gains in calves fed soybean meal alone or soybean meal plus isoacids than in those fed urea plus the isoacid treatment. As both the starter diet and the hay contained 20 percent protein, it is doubtful whether the urea nitrogen was needed.

Experimental evidence has been presented in this literature review to substantiate the beneficial effects of isoacids plus urea on animal performance. However, evidence regarding a possible need to supplement high-urea diets with isoacids is not clear-cut. Some investigators have received little response (Hungate and Dyer, 1956; Menahan and Schultz, 1964; Oltjen et al., 1971; Miron et al., 1968). Including isoacids in the diet did not change the rumen protozoa numbers, cellulolytic bacteria numbers, nor the microbial amino acid composition (Oltjen et al., 1971). Bryant suggested that further studies on the unknown nutrients essential or highly stimulatory to some cellulolytic species may lead to

the discovery of other feed additives that increase the rate or extent of ruminal cellulose digestion and protein synthesis, especially when high-forage or high-NPN-containing diets are fed.

There have been only a few studies of the effects of isoacids and urea on performance when included in dairy cattle rations. The study of Virtanen (1966) showed that urea feeding alone has a moderate effect on performance of dairy cows. Furthermore, synthesis of rumen microbial protein requires that all amino acids be present at optimum levels since a deficiency of one will limit protein synthesis to the level of the deficient amino acid. This is even more critical in the case of high-producing dairy cows which require a high level of amino acids for milk protein synthesis. Isobutyrate, isovalerate, and 2-methylbutyrate are required for synthesis of valine, leucine and isoleucine, respectively. This indicates that specific carbon precursors may be needed for other amino acids.

Cattle fed urea-containing purified diets had lowered plasma concentrations of valine, leucine, isoleucine, and phenylalanine, but increased levels of serine and glycine (Oltjen and Putnam, 1966; Oltjen, 1969) compared to cattle fed isolated soybean protein. Also, less dietary nitrogen was retained when the cattle were fed urea-containing diets. When combinations of valine, leucine, isoleucine, and phenylalanine were infused into the abomasum of steers, the utilization of the urea-containing diet was improved, almost

equaling the performance of steers fed the soy protein-containing diet (Oltjen et al., 1970). The same workers also found that the infusion of glycine and serine depressed the utilization of the soy protein diet, demonstrating the importance of amino acid balance in ruminants.

It is proposed that isoacids are not present in adequate concentrations in the rumen fluid for optimum growth of rumen microorganisms when animals are fed high levels of urea as a source of supplemental nitrogen. The purpose of the studies reported in this investigation was to determine the nutritional response of high-producing lactating cows to high-urea corn silage-based rations supplemented with isoacids.

MATERIALS AND METHODS

A series of studies was conducted over a period of five years at the Michigan State University Dairy Research Center. A total of 112 high-producing lactating Holstein cows and 20 growing dairy heifers were utilized in four separate milk production experiments and one growth study. One in vitro experiment was also conducted. A high-producing cow for purposes of these studies was considered to be one which produced an average of at least 23 kg of milk per day (or 7015 kg) during a 305--day lactation.

The objective of these studies was to investigate the effects of isoacids and phenylacetate on growth of dairy heifers and milk production of high-producing cows.

In Vitro Experiment

The main objective of this study was to determine the effects of isoacids on rumen gas production. Most of the methods used for measuring rumen microbial activity have been a measure of end-products other than gas production. Such a procedure is usually time-consuming. Furthermore, in a closed system, the measurement of metabolic activity by fiber disappearance, for example, in 48 hours may not show

differences between treatments even though there were significant differences at the times prior to 48 hours. The in vitro technique of using gas production rates as a parameter for obtaining an estimate of microbial net growth was proposed by El-Shazly and Hungate (1965) and evaluated by El-Din and El-Shazly (1969). These investigators measured the maximum fermentation rates of subsamples taken at the beginning and after 1 hour of incubation of a sample, on the assumption that with proper dilution and the substrate in excess, the fermentation rate is maximal and proportional to total microbial cells. They obtained an average net growth which was in approximate agreement with rumen turnover rate.

Five in vitro experiments were conducted during this study, with some variations in the composition of the culture media, amount and kind of substrates, acid levels and buffering system. Table 1 shows the composition of the different culture media. In the first experiment 10 g of finely ground concentrate were used as the source of energy. This amount was diluted with 200 ml of Hungate's buffer (1966). In the second experiment 5 g of concentrate diluted in 10 ml of deionized water were used. In the third experiment concentrate was replaced with 1 g of corn starch and 1 g of finely ground Whatman* filter paper (No 1), all diluted with 10 ml of water. In the fourth experiment, corn starch was increased to 1.5 g and the amount of cellulose

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Table 1.--Composition of the *in vitro* media.

Ingredient	Experiment No.				
	I	II	III	IV	V
Rumen fluid, ml	100.0	100.0	100.0	100.0	100.0
Concentrates, g	10.0	5.0	.....	.....	20.0
Buffer, ml*	200.0	.....	.....	.....	200.0
Sodium bicarbonate, g	0.5	0.5	0.5	0.5	0.5
Corn starch, g	.....	.....	1.0	1.5	.....
Cellulose, mg	.....	.....	1000.0	150.0	.....
Methionine, mg	.....	.....	.....	1.0	5.0
Urea, mg	200.0	200.0	100.0	50.0	50.0

*Hungate buffer: 1 part A + 1 part B + 4 parts deionized water.

A = 0.3% KH_2PO_4, 0.6% NaCl, 0.3% $(NH4)_2SO_4$, 0.06% $MgSO_4$, 0.06% $CaCl_2$

B = 0.3% K_2HPO_4

reduced to 150 mg, and 1 mg of methionine was added. In the fifth experiment 20 g of concentrate were diluted with 200 ml of buffer, and 5 mg of methionine were added. Different variables were used in an attempt to investigate which combination had better response in terms of gas production.

The donor of the rumen fluid was a mature, non-lactating Holstein cow fitted with a permanent fistula. The cow was fed a control corn silage ration for about six months, which was later supplemented daily with 4 kg of a high urea-corn grain-based concentrate for a three-week adaption period. The concentrate ration contained also 20 g each of four volatile fatty acids (isobutyrate, isovalerate, 2-methylbutyrate and n-valerate). The urea and acid mixture were removed from the diet 72 hours prior to sampling. The ingredient composition of the concentrate used during the adaption period and throughout the experimental treatment and in the culture media is shown in columns A and B, respectively of Table 2. Corn silage rations were fed three hours prior to taking the rumen sample, and the concentrates were fed just after sample collection. Detailed information concerning the levels of isoacids used and number of treatments per experiment is shown in Table 3. Samples were collected from 5 different parts of the rumen. A plastic tube attached to a 2000 ml Erlenmeyer flask fitted with a water pump was used for withdrawal of rumen fluid. The rumen samples were collected in a 1-liter plastic bottle filled up to the rim, tightly closed and kept in water in a thermos jar

Table 2.--Composition of the concentrates.

	A	B
Ground shelled corn, %	49.1	58.0
Oats, %	23.6	28.0
Urea, %	5.9	.....
Beet pulp, %	8.8	11.0
Molasses, %	7.8	.....
Isoacids, %	1.7	.....
Defluorinated phosphate, %	1.0	1.0
Ground limestone, %	1.0	1.0
TM salt, %	1.0	1.0
Vitamin A, IU/kg	4400.0	4400.0
Vitamin D, IU/kg	1100.0	1100.0

Table 3.--Quantities of isoacids used in the *in vitro* experiment.

Treatment	Experiment No.				
	1	2	3	4	5
Control (urea)	X	X	X	X	X
Urea + 2µl acids*	-	-	-	X	-
Urea + 4µl acids	X	X	X	X	X
Urea + 8µl acids	X	X	X	-	X
Urea + 12µl acids	X	X	-	X	X
Urea + 16µl acids	X	X	X	X	-
Urea + 20µl acids	X	-	X	-	-
Urea + 24µl acids	X	-	-	X	-
Urea + 28µl acids	X	-	-	-	X
Urea + 32µl acids	X	-	-	X	-
Blank (no urea or acids)	X	X	X	X	X

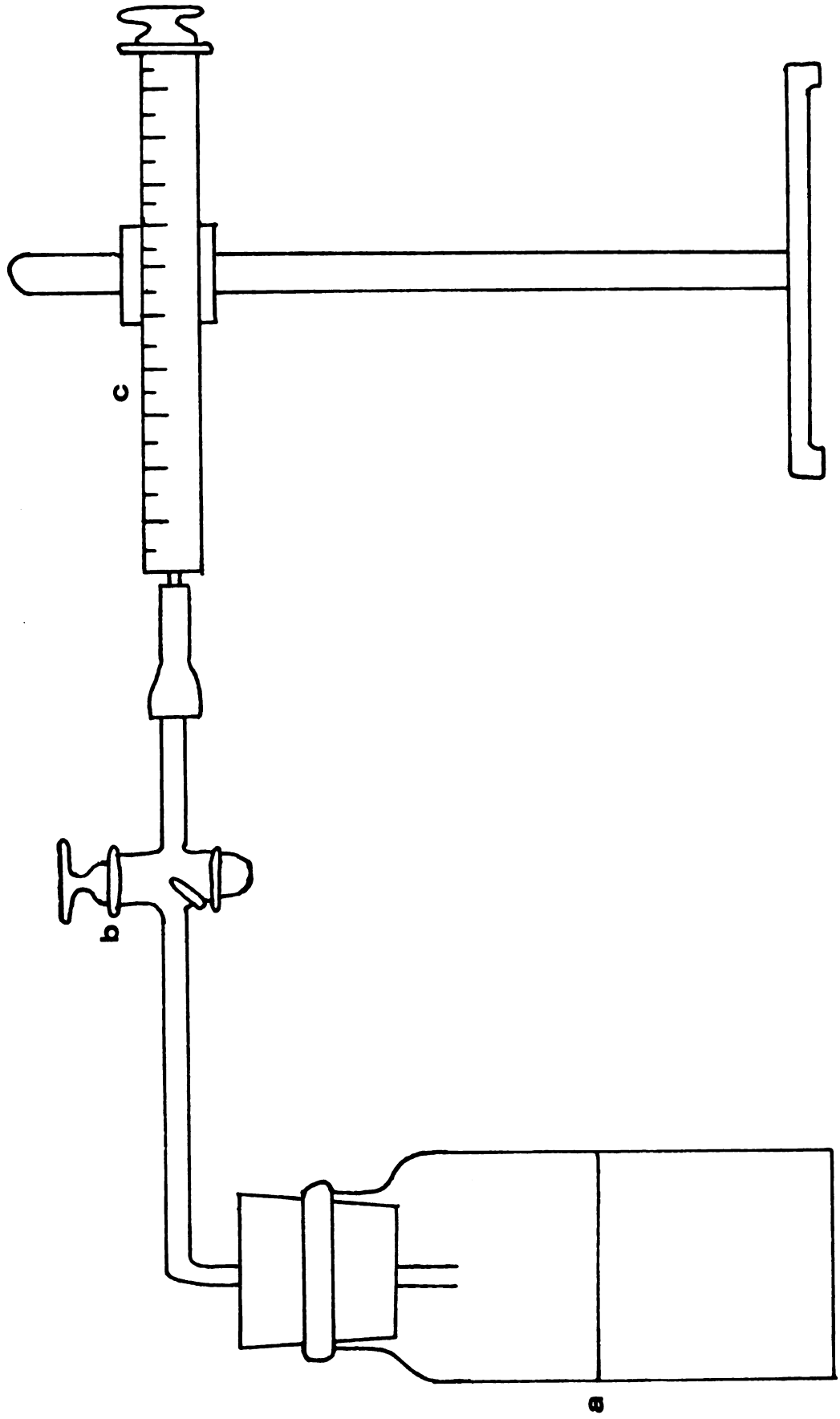
*µl of acid mixture/100 ml rumen fluid.

at 40°C and then transferred to the laboratory. The samples were then filtered through four layers of cheesecloth. One hundred milliliters of rumen fluid were transferred into each of the one-pint glass jars containing the substrates. The jars were preincubated at 40°C. They were then immediately flushed with CO_2 and tightly closed with a rubber stopper provided with a T-tube having a three-way stopcock (Figure 1). The Stopcock connected the jar to a 10 ml syringe from one side, or closed the jar and opened it to the air, allowing the syringe to empty or to collect gas for analysis. The interval between sampling and incubation was from 10 to 15 minutes. The gas measurements started thirty minutes from the beginning of the incubation. The rate of gas production during the fermentation process was measured using a 10 ml water-lubricated syringe by reading the volume of gas forced into the syringe every five minutes for a period of one hour. The pH of each incubation jar was measured before and after incubation.

Just after incubation 10 ml aliquots from each jar were transferred to plastic vials containing 1 ml of 9N sulfuric acid and saturated mercuric chloride to stop the fermentation process and to prevent nitrogen loss. The samples were centrifuged for 15 minutes at 34,800 x g. The supernatants were frozen and later utilized for ammonia and VFA determinations. One sample was taken prior to incubation and treated as the others to serve as the zero time control.

FIGURE 1.--Apparatus for measuring fermentation rates of rumen microorganisms.

a--jar of 473 ml capacity containing 100 ml of rumen contents
b--three-way stopcock
c--10 ml graduated syringe (2/10)


c
b
a

Ammonia nitrogen was determined according to the method of Fawcett and Scott (1960) as modified by Kulasek (1972).

Volatile fatty acids (VFA) were determined by the method of Ottenstein and Bartley (1971a, 1971b) with minor modifications. Three microliter aliquots were injected into a Hewlett-Packard gas chromatograph*, model 5730A, equipped with a model 7671A automatic sampler and an Integrator-Recorder, model 3880A. The all-glass column (6ft X 2mm i.d.) was packed with graphited carbon, Carbopak A**. Nitrogen was used as the carrier gas. The acid standards were Eastman compounds***, containing 0.01N each of acetic, propionic, butyric, valeric, isobutyric, isovaleric and 2-methylbutric acids in deionized water. The column was conditioned at 200°C (overnight) and the running temperature was maintained at 175°C. The column was cleaned three times after conditioning, with a 3 µl injection of deionized water. Carrier gas flow rate was 60 ml/min.

Growth Study

In many instances dairy herd replacements are wintered on high fiber-low protein roughages. Improved digestibility and, consequently, increased growth of heifers

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*Hewlett-Packard. Route 41, Avondale, Pennsylvania.

**Supel Co., Inc., Supel Co. Park, Bellafonte, Pa.

***Eastman Organic Chemicals, Eastman Kodak Company, Rochester, New York.

may be achieved if rumen microorganisms are supplemented with certain key volatile fatty acids.

The objective of this study was to investigate the effects of feeding urea plus isoacids and phenylacetate on growth rate of dairy heifers fed on low quality roughages. Twenty yearling dairy heifers weighing from 121 to 327 kg were paired on the basis of body weight and used in a 90-day feeding trial. Heifers from each pair were randomly assigned to one of two treatments. All heifers were fed timothy hay ad libitum as the sole roughage. In addition, the control group received per head day 450 g of a pelleted supplement which consisted of 80 g of urea, 50 g of molasses and 270 g of ground hay. The other group was supplemented with 454 g of the pelleted diet containing the same ingredients and in equal amount as in the control plus 50 g of the acid mixture. The acids were added to the urea-treated ground hay prior to pelleting. Both groups of animals were fed free choice a salt-mineral mixture consisting ot 50 percent trace mineralized salt and 50 percent dicalcium phosphate.

All animals were weighed for two consecutive days at the beginning, twice during the trial and at the end of the experiment. The average body weights were 245 and 246 kg for the control and treatment groups, respectively. Differences in body weight changes between the two groups were tested by the student "t"-test technique.

Effects of Isoacids on Milk Production

Urea has long been used as a source of supplemental nitrogen for cattle and sheep. But it has been recognized that carbon skeletons from branched-chain and some straight-chain volatile fatty acids (isoacids) were needed with urea for microbial protein synthesis. In a series of four feeding trials high-producing lactating Holstein cows were fed urea-based diets plus isoacids.

Trial I.--This trial was designed to evaluate the effects of feeding a mixture of isoacids and phenylacetate with urea on milk production when corn silage was fed as the sole roughage, and without concentrates, on the assumption that high-producing cows in the peak of lactation are in high energy demand. A randomized block design of 3 treatment groups and 8 animals per group was used in this trial. Blocks were based on previous milk yields. Treatment groups were balanced for age, stage of lactation and producing ability. The treatment period was for 60 days

Twenty-four lactating Holstein cows within 30 days postpartum and producing over 25 kg of milk per day were placed on a two-week preliminary period. All cows were fed control corn silage free choice and the regular herd corn grain-based concentrates at 1 kg per 3 kg of milk. The preliminary period was followed by a two-week transition period for adjustment to the experimental rations, to which the cows were randomly assigned. Cows were then gradually

converted to the experimental treatments in 5-day intervals. During the treatment period cows were fed control corn silage ad libitum and the experimental rations. The three experimental diets consisted of the following ingredients:

a. 300 g soy protein + 1200 g ground grass hay (premix);
b. 300 g urea + 454 g dry molasses + 300 g grass hay; and
c. 300 g urea + 454 g dry molasses + 300 g grass hay + 100 g isoacid mixture. The acid mixture consisted of 20 g each of isobutyrate, isovalerate, 2-methylbutyrate, n-valerate and phenylacetate.

The treatments were administered once a day and were well mixed with the top third portion of the silage. A salt mineral mixture containing NaCl, K, MgO, Ca and PO_4 was also fed free choice. Daily milk yields were recorded for each milking. Body weights were taken for two consecutive days at the beginning and at the end of the experimental period.

Trial II.--Trial I indicated that isoacids and phenylacetate fed with urea to high producing cows improve milk production as compared to urea alone. These sources of supplemental nitrogen are more economical than soybean meal. Furthermore, according to the National Research Council report (1976), an experimental diet should contain both a "negative" and a "positive control" in order to test both the animal protein needs and the efficiency of the source of nitrogen in fulfilling those needs. The "negative control" is used as a test diet known to be protein deficient.

The "positive control" is a test diet with preformed protein furnishing the supplemental protein and eliciting a positive response relative to the negative control. Trial II was then conducted to compare the effects of soybean meal (positive control), urea, urea plus isoacids and no nitrogen supplementation (negative control) on milk production by cows fed grain along with corn silage. Twenty-eight Holstein cows in early lactation and milking over 20 kg per day were alloted to 4 treatments in a randomized block design. Blocks were based on milk yields during a two-week preliminary period. Treatment groups were balanced for age, stage of lactation and breeding groups.

Treatment comparisons were established as follows:

a. Negative control
b. Positive control
c. Urea
d. Urea + isoacids

The negative control consisted of corn silage, 1.4 kg of hay and corn grain. The positive control included the same ingredients as for (a) plus soybean oil meal. The urea treatment was similar to (a) plus urea as supplemental nitrogen. The urea-isoacid treatment included the same components as (c) plus 20 g of each acid. Total crude protein concentration of each diet was 14%.

During the preliminary period cows were fed control corn silage ad libitum and the regular herd concentrate at 1 kg per 2.5 kg of milk. The concentrate ration contained

2.5 percent crude protein equivalent as urea and was well-mixed with the silage prior to feeding. Cows were gradually converted to the experimental concentrates over a 4-day period. During treatment, the control corn silage was fed and experimental concentrates at 1 kg per 2.5 kg of milk. Silage and concentrate were well-mixed before feeding.

During the first 4 weeks of treatment, the amount of concentrate offered was based on milk production during the preliminary period; this concentrate was reduced 5 percent at 28 days and 5 percent more at 56 days. Sufficient corn silage was fed and regularly adjusted to provide a 10 percent weighback of total feed. The ingredient composition of the concentrates is indicated in Table 4.

Daily milk yield was recorded five days a week from both the morning and evening milkings. Milk samples were collected from two consecutive milkings during the preliminary period, and at 2-week intervals during treatment, composite (AM and PM) samples were taken.

Corn silage was sampled three times a week for a weekly composite and frozen for dry matter determination. Concentrate samples were taken at each feed preparation and kept in the cooler for further analysis. Feed was weighed in and weighed back from each cow on a daily basis. Cows were weighed as previously described. Milk fat was

Table 4.--Composition of the concentrates for trial II.

Ingredient	Treatment			
	a	b	c	d
Ground shelled corn, %	55.5	44.5	56.2	54.0
Oats, %	27.5	22.0	23.5	25.5
Soybean meal, %	....	16.5	....	....
Urea, %	....	....	2.6	2.5
Beet pulp, %	10.0	10.0	10.4	10.0
Molasses, %	4.0	4.0	4.2	4.0
Dical phosphate, %	1.0	1.0	1.0	1.0
Ground limestone, %	1.0	1.0	1.0	1.0
TM salt, %	1.0	1.0	1.0	1.0
Isoacids, %	....	....	....	1.0
Vitamin A, IU/kg	4400.0	4400.0	4400.0	4400.0
Vitamin D, IU/kg	1100.0	1100.0	1100.0	1100.0

determined according to the method of Babcock*. Milk protein determination was performed by the Kjeldahl N procedures (N x 6.38 = CP in milk). Total solids in milk were determined in duplicates by oven drying at 100°C for three hours. Two milliliters of milk were pipetted for weighing in aluminum pans of 3 cm diameter which were used for drying. The content of solids non fat of milk was estimated as total solids % minus fat%. Feed dry matter was determined by drying in a forced air oven for 24 hours at 100°C.

Trial III.--In trial II, the use of a randomized block design showed that an equal weight of isoacids improved persist way of lactation. But trial II failed to show significant difference in milk yield between treatments. It has been reported that, using as few as eight Holstein cows per treatment in a 30-day feeding period, the probability of detecting a true mean difference in milk yield of 2 kg per day is much higher with a cross-over design as compared to a randomized block design (Gill, 1969). Therefore, in trial III a special Latin square cross-over design was used to evaluate the effects of two different blends of isoacids for milk production by Holstein cows. Thirty lactating Holstein cows producing more than 23 kg of milk per day were randomly assigned to a 3 x 3 cross-over design following a three-week

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*The fat test was performed at the center for the Dairy Herd Improvement Association, Forest Road, East Lansing, Michigan.

prelimanary period. Treatment allocation was based on milk production during the preliminary period, age, and breeding groups. The experimental design is shown in Table 5.

Table 5.--Experimental design for trial III.

Groups	Prelim. Period (days)	Treatment periods (days)		
		I	II	III
	0-21	22-49	50-78	79-107
I	....	a	c	b
II	....	b	a	c
III	....	c	b	a

Each treatment period was of 28 days duration. During the preliminary period the cows were fed control corn silage ad libitum as the sole roughage. In addition, the regular herd corn grain-based concentrate was fed at the rate of 1 kg per 3 kg of milk. Cows were gradually converted to experimental concentrates throughout the preliminary period.

During treatment cows were fed control corn silage free choice. In addition, cows on control (a) received per head per day 1 kg of concentrate per 3 kg of milk. The experimental concentrates contained 3.0 percent crude protein equivalent as urea. Cows in treatment b were fed similar concentrate to (a) plus 80 g of the acid mixture 1, containing on a molar basis 28, 24, 24, and 24 percent of

isobutyrate, isovalerate, 2-methylbutyrate and n-valerate, respectively. Cows in treatment c were offered the same treatment as (b), but the corresponding values for the acid mixture 2 were 36, 17, 17 and 30 percent. Table 6 shows the ingredient composition of the concentrates. Corn silage was fed in the morning. Urea and acid mixtures were premixed with part of the concentrates and fed well-mixed with the top third portion of the silage during the morning feeding. The remaining amount of concentrate was fed in the afternoon. Feed amounts were adjusted to average a 10 percent refusal. The procedures used for sampling silages and concentrates and for weighback recording were similar to those previously described. Daily milk yield recording and milk sampling were similar to that described in trial II.

Blood samples were collected bi-weekly from tail veins of each cow three hours after the morning feeding. The blood was drawn in a 15 ml vacuum tube containing 30 mg potassium oxalate and 33 mg sodium fluoride. Plasma was prepared by centrifuging at 2000 x g for 15 minutes. The plasma was then stored for further urea analysis. Plasma urea--N determination was performed by the colorimetric method of Fawcett and Scott (1960) as modified by Kulasek (1972). At the end of each treatment period (28 days) rumen fluid was collected from all cows by stomach tube at 3 hours post-feeding. The rumen fluid was then strained through four layers of cheesecloth, and the pH was measured with a

Table 6.--Composition of the concentrates for trial III.

	A	B	C
Ground shelled corn, %	54.0	53.5	53.5
Oats, %	26.0	25.8	25.8
Beet pulp, %	10.0	9.9	9.9
Dry molasses, %	4.0	3.9	3.9
Urea, %	3.0	3.0	3.0
Isoacids, %	.....	0.9	0.9
Defluorinated PO_4, %	1.0	1.0	1.0
Ground limestone, %	1.0	1.0	1.0
TM salt, %	1.0	1.0	1.0
Vitamin A, IU/kg	4400.0	4400.0	4400.0
Vitamin D, IU/kg	1100.0	1100.0	1100.0

Beckman pH meter*, model G. A sample was prepared for ammonia and VFA determinations, using the same procedures as described in the in vitro study. Body weights of each cow were taken at the beginning and the end of each treatment period as previously described. Proximate analysis including dry matter, crude fiber, crude protein, and ash was conducted for silages and concentrate samples according to the standard method of the A.O.A.C.** Table 7 shows the chemical composition of the diets.

Table 7.--Composition of the diets for trial III.

	Corn Silage	Concentrates
Dry matter, %	38.47	88.45
Crude protein, %	8.42	18.47
Organic matter, %	35.31	83.16
Ash, %	3.16	5.29
Crude fiber, %	23.84	7.41

A digestion trial was conducted at the end of each experimental period, using three groups of three cows each and in the same experimental sequence. Since the cows were on the same diet for three weeks, no adaptation period was

*Beckman Industries, Inc., South Pasadena, California.

**Proximate analysis was performed at the analytical lab of the Biochemistry Department, Michigan State University, East Lansing.

needed. However, the cows were equipped with urinary collection devices five days prior to the collection period. The collection period was of five days length.

The urinary collection devices were made from light transparent plastic material at the Michigan State University Veterinary Research Center. Cows were fitted with these apparati at the MSU Dairy Research Center. The devices were sutured along with a 1-foot rubber tubing at six points surrounding the cow's vagina, following local anesthesia (Rapicaine*); the devices were then well-glued to the skin with branding cement to prevent leaks during collection time. One day prior to collection, cows were transferred to the digestion stall where they were fed and milked as usual. Samples from feed and orts were collected and analyzed as previously described. Milk yields were recorded as usual. The urine was collected in a 5 gallon plastic carboy containing 30 ml of 50 percent hydrochloric acid to acidify the urine and to prevent loss of nitrogen. The plastic carboy was connected with the collection device through a 5-foot plastic tube.

Daily urine weight was recorded, and the carboy emptied following removal of a 10 percent aliquot, which was stored in the cooler. Upon termination of each collection period, samples from each cow were pooled. A 10 percent

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*Haver-Lockhart Laboratories, Shawnee, Kansas.

subsample was then taken for nitrogen determination using the Kjeldahl procedure as previously indicated. Total feces output was collected from each cow in a galvanized metal container placed immediately behind the cow. The weight of the feces was recorded once a day and a representative sample (15 percent) was taken after thourough mixing. At the end of each period a composite sample was prepared for each cow, and a 10 percent subsample was used for dry matter, crude protein, crude fiber and ash determinations. Proximate analyses for feed, orts, and feces were performed as previously described.

Trial IV.--The Latin Square cross-over design experiment was selected because it had been reported that in dairy animal feeding this type of design is more appropriate to detect differences between treatments over a relatively short period of time and with much smaller numbers of animals per treatment than the randomized block design. However, results from trial III showed that, in the particular case of the isoacids, the cross-over design cannot show differences between treatments in milk production on a 28-day period because milk production declines when the cows are changed from one ration to another. Twelve cows showed significant reduction in feed intake and, consequently, reduction in milk production when their diets were changed from control to the acid mixtures. It took them more than 2 weeks to recover. Because of the initial effect of changing

treatment on appetite, and because of the relatively short treatment period, the cows did not exhibit any positive response to the acid treatment in trial III.

Thus, trial IV utilized two treatment groups in a continuous feeding randomized block design. This trial consisted of two treatments, acid mixture and control. The treatment period was 90 days. Thirty lactating Holstein cows averaging 40 days post partum and producing over 23 kg of milk per day were paired on age, stage of lactation, milk production, body weights and breeding groups. One cow from each pair was randomly assigned to each of the two treatments for a three-week preliminary period. Control corn silage was fed free choice as the sole roughage from the start of the preliminary period throughout the entire trial. Silage rations were supplemented with a corn grain-based concentrate at 1 kg per 3 kg of milk. The grain ration was initially the same as the regular herd rations. This ration was then gradually converted to the experimental concentrate over a series of 7-day intervals throughout the preliminary period. The composition of the concentrates is shown in Table 8. Total crude protein of the rations was 14 percent. During the treatment period the experimental concentrates were fed at 1 kg per 3 kg of milk for cows producing over 25 kg of milk per day. The ratio was reduced by 10 percent for every 5 kg below that initial level. Urea and acid treatments were premixed with the concentrates and fed with silage as previously indicated. Each cow on the control diet received

Table 8.--Composition of the concentrates for trial IV.

	A*	B*
Ground shelled corn, %	57.0	56.3
Oats, %	20.0	19.6
Urea, %	3.0	3.0
Beet pulp, %	9.0	8.9
Molasses, %	8.0	7.9
Isoacids, %	....	1.1
Dical phosphate, %	1.0	1.0
TM salt, %	1.0	1.0
Ground limestone, %	1.0	1.0
Vitamin A, IU/kg	4400.0	4400.0
Vitamin D, IU/kg	1100.0	1100.0

*A = Control; B = Acid treatment.

275 g of urea per day. Cows on the acid treatment were fed per head per day an amount of urea similar to that fed the control group plus 80 g of the acid mixture. Feeding and sampling procedures were similar to those described in the previous trial except that corn silage and concentrates were fed in the afternoon (4 to 6 pm). Daily milk yields, body weights and orts were recorded; feed, milk, blood and rumen fluid samples were collected and analyzed similarly to the previous trial. The chemical composition of the rations is shown in Table 9. All data were statistically analyzed by analyses of various techniques, using a CDC 6500 computer at the Michigan State University computer laboratory. When necessary, differences between means were tested by orthogonal, Tukey's or "t"-test comparisons. Details of statistical analysis are reported in the appendix.

Table 9.--Composition of the diets for trial IV

	Corn Silage	Concentrates
Dry matter, %	30.70	88.11
Crude protein, %	7.95	18.72
Organic matter, %	26.66	82.15
Ash, %	4.04	5.96
Crude fiber, %	24.04	7.35
NFE*, %	61.92	64.12
Ether extract, %	2.25	3.92

*NFE = Nitrogen-free extract.

RESULTS AND DISCUSSION

In vitro Experiment

The effects of different concentrations of the isoacids on gas production for the five experiments are summarized in Table 10. The values represent an average of seven observations per treatment. In general, isoacids depressed ($P<.01$) the rate of gas production irrespective of the source and the amount of substrate used in the culture medium. However, the rate of gas production was different between experiments ($P<.01$; Table 13). Also the rate of gas production was higher for the experiments which contained higher levels of concentrates in the media. Thus, an excess of substrate is necessary for maximum gas production (El-Shazly and Hungate, 1965). When food is in excess and other conditions are favorable, fermentation is maximal and is a linear function of population size. The rate of gas production in this present study is in agreement with that observed by El-Shazly and Hungate (1965) and more recently by Naga and Harmeyer (1975). However, whatever level of gas production obtained is consistently depressed by the increasing concentrations of the isoacids in the media. Gas production

Table 10.--The effects of different concentrations of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate on gas production in vitro*

	Experiment**				
	1	2	3	4	5
No urea	22.90[a]	13.87[a]	12.25[a]	16.65[a]	26.90[a]
Urea	21.92[a]	11.18[b]	13.60[a]	18.17[b]	33.23[b]
Urea + 2µl***	-	-	-	17.82[b]	-
Urea + 4µl	21.28[ab]	11.05[b]	13.27[a]	18.33[b]	25.70[c]
Urea + 8µl	20.30[b]	11.75[b]	12.15[ab]	-	22.77[d]
Urea + 12µl	21.02[ab]	10.17[bc]	-	15.33[c]	19.10[e]
Urea + 16µl	16.65[c]	12.02[ad]	10.35[c]	10.13[d]	-
Urea + 20µl	19.42[d]	-	9.65[c]	-	-
Urea + 24µl	16.42[c]	-	-	6.26[e]	-
Urea + 28µl	16.96[c]	-	-	-	15.33[f]
Urea + 32µl	14.15[e]	-	-	5.26[f]	-
SD****	.99	.25	.42	.41	.52

*Acid mixture was an equal weight of each one of the four acids used.

**Gas production measured in µl/ml rumen fluid/minute.

***Amounts of acid mixtures are expressed in microliters/100ml fermentor.

[abcdef]Values in same column with different superscripts are significantly different ($P<.05$), using Tukey's Test comparison.

****Standard deviation.

was lower for corn starch or cellulose than for concentrates (experiments 1 and 5).

One would have expected higher rates on gas production from those two experiments which contained corn starch as a more readily fermentable carbohydrate. Yet such was not the case. The probable explanation for this is the presence of cellulose in combination with corn starch. The 90 minute incubation time may have been insufficient to allow a full degradation of cellulose by the microbes. Umunna (1975) reported that the source of energy affected the extent of microbial utilization of isoacids. For example, the rumen microbes were more active with corn starch than with solka floc as a substrate. The difference in rate of gas production between concentrates and corn starch plus cellulose as source of energy could be explained in the light of a recent report by Maeng et al. (1976). These workers observed that the optimum ratio of non-protein nitrogen to amino acid nitrogen for microbial growth was 75 percent urea nitrogen and 25 percent amino acid nitrogen. The concentrate contained protein and probably many other chemicals that would stimulate microbial activity.

The effects of isoacid concentrations on ammonia nitrogen output are presented in Table 11. The statistical analysis is reported in Table 13. Contrary to the gas production pattern, the ammonia nitrogen levels tended to increase with increasing concentrations of isoacids. This tendency was more marked in experiments 3 and 4 where corn

Table 11.--The effects of different concentrations of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate on rumen ammonia production *in vitro**

	Experiment**				
	1	2	3	4	5
No additions	21.6	12.2	11.5	10.6	11.2
No urea	46.1	24.1	5.5	6.2	29.1
Urea	48.7	38.3	21.7	22.3	34.7
Urea + 2µl***	-	-	-	22.8	-
Urea + 4µl	47.6	36.8	22.7	21.1	32.9
Urea + 8µl	51.1	39.7	23.1	-	36.0
Urea + 12µl	46.9	38.5	-	23.5	31.0
Urea + 16µl	48.4	37.8	23.9	28.9	-
Urea + 20µl	49.6	-	24.3	-	-
Urea + 24µl	49.5	-	-	28.1	-
Urea + 28µl	49.1	-	-	-	33.4
Urea + 32µl	49.2	-	-	28.0	-
SD****	8.26	10.4	7.40	9.12	8.49

*Acid mixture was composed of an equal weight of each one of the four acids used.

**Ammonia levels are expressed as mg/100ml of incubation media.

***Acid mixtures are expressed as microliters/100ml fermentor.

****Standard deviation.

starch plus cellulose were used as sources of energy. In experiment 5 which contained the highest level of concentrate NH_3-N decreased slightly with increasing concentrations of isoacids. The levels of NH_3-N in the rumen fluid alone varied from 21 to 11 mg/100 ml from experiment 1 to experiment 5. Thus, there was some variation in the NH_3-N content of the rumen fluid from sampling to sampling. The additions of concentrates (experiments 1, 2 and 5) to the rumen fluid increased by more than 100 percent the levels of ammonia as compared to the negative control (no urea). It is obvious from the NH_3-N level of the negative controls of experiments 3 and 4 that NH_3 had been utilized during the fermentation process. In the first two experiments the amount of nitrogen equivalent from urea additions was double that in experiment 3, and 4 times that in the last two experiments (Table 1). NH_3-N was utilized by the microbes throughout the incubation process, but it is difficult to estimate the magnitude of NH_3-N used throughout the different concentrations of the isoacids in the media. The level of ammonia supplied from urea was probably in excess of that required for maximal microbial growth and, therefore, a major disappearance of NH_3-N could not be detected. Helmsley and Moir (1963) noted a slight reduction in ruminal ammonia concentration due to the addition of isoacids to a urea roughage diet *in vivo*. Using an isolated soy protein diet, ammonia levels did not appear to be affected. Umunna and associates (1975) did not show consistent differences in ammonia concentrations between

urea and urea-isoacids or casein treatments during an in vitro trial. Even though the present work differed from experiments of those workers because of many variations in the in vivo system, the similarity between results appears obvious. However, the data in Table 11 show that NH_3-N was used by the microbes.

The effects of isoacids on pH variations are reported in Table 12. The final pH for each experiment was different from the initial ph ($P<.05$). The greatest pH decline appeared in experiments 3 and 5, with a final pH of 5.2 and 5.3, respectively. This is about the pH at which microbial activity begins to cease (Brown and Tucker, 1962; Slyter et al., 1966). The higher levels of isoacids tended to decrease the initial pH measurements.

Volatile fatty acid production for the five experiments is presented in Tables 15 through 19. The statistical analysis is presented in Table 14. There were no significant differences in the concentrations of acetate, propionate or butyrate within experiments. The VFA values were slightly lower when compared to those reported by Naga and Harmeyer (1965) and Slyter et al. (1966). However, the low level of VFA in the fermentors is not necessarily an indication of low fermentation. A negative correlation coefficient was reported by El-Shazly et al. (1969) and by Valthauer et al. (1970) between DNA concentration and microbial growth. Recently Naga and Harmeyer (1975) indicated that no constant relationship exists in vitro between microbial protein

Table 12.--The effects of different concentrations of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate on rumen pH *in vitro*

	Experiment									
	1		2		3		4		5	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
No additions	6.8*	-	6.8	-	6.7	-	6.8	-	6.8	-
No urea	6.7	6.6	6.5	6.3	6.5	6.4	6.7	6.3	6.1	6.0
Urea	6.5	6.5	6.6	6.5	6.6	6.4	6.5	6.6	6.6	6.4
Urea + 2µl**	-	-	-	-	-	-	6.5	6.0	-	-
Urea + 4µl	6.0	6.0	6.2	6.0	6.3	5.6	6.3	5.9	6.3	6.0
Urea + 8µl	6.1	6.1	6.1	5.9	6.3	5.3	-	-	6.1	6.0
Urea + 12µl	6.1	6.1	6.0	5.8	-	-	6.3	5.8	6.2	5.4
Urea + 16µl	6.3	6.1	6.2	5.7	6.2	5.2	5.8	5.6	-	-
Urea + 20µl	6.2	6.0	-	-	5.9	5.5	-	-	-	-
Urea + 24µl	6.1	6.1	-	-	-	-	5.9	5.5	-	-
Urea + 28µl	6.1	6.0	-	-	-	-	-	-	6.2	5.3
Urea + 32µl	5.9	5.8	-	-	-	-	5.8	5.6	-	-
SD	.24	.24	.23	.31	.24	.54	.38	.38	.19	.42

*pH from rumen fluid at zero-time

**Acid mixtures are expressed as microliters/100ml fermentor

Table 13.--Analysis of variance for overall gas production, rumen ammonia-N and rumen pH for the *in vitro* experiment

	Degree of freedom	Mean square	Significance
Gas			
Main effects	24	67.258	.001
Treatments	9	30.290	.001**
Time	11	0.700	.999
Experiments	4	375.471	.001**
Error	395	0.992	-
Ammonia-N			
Main effects	13	423.601	.001
Treatments	9	210.347	.001**
Experiments	4	611.757	.001**
Error	20	8.619	-
Final pH			
Main effects	13	0.335	.001
Treatments	9	0.365	.001**
Experiments	4	0.313	.001**
Error	20	0.041	-
Difference between initial and final			
Main effects	13	0.173	.030
Treatments	9	0.063	.004*
Experiments	4	0.382	.999
Error	20	0.068	-

*Significant P<.05

**Significant P<.01

Table 14.--Analysis of variance for overall VFA production for the *in vitro* experiment

	Effects	Treat-ments	Experi-ments	Error
Acetate				
Degrees of freedom	21	10	4	181
Mean square	7.658	2.192	29.214	2.467
Significance	.001**	.999	.001**	-
Propionate				
Degrees of freedom	21	10	4	181
Mean square	.849	.146	3.702	.308
Significance	.001*	.999	.001**	-
Isobutyrate				
Degrees of freedom	21	10	4	181
Mean square	.748	1.024	.639	.088
Significance	.001**	.001**	.001**	-
Butyrate				
Degrees of freedom	21	10	4	181
Mean square	.317	.098	1.064	.169
Significance	.015*	.999	.001**	-
2-Methylbutyrate				
Degrees of freedom	21	10	4	181
Mean square	.186	.247	.204	.025
Significance	.001**	.001**	.001**	-
Isovalerate				
Degrees of freedom	21	10	4	181
Mean square	.134	.222	.063	.011
Significance	.001**	.001**	.001**	-
Valerate				
Degrees of freedom	21	10	4	181
Mean square	.465	.763	.375	.036
Significance	.001**	.001**	.001**	-

*P<.05

**P<.01

Table 15.--The effects of different concentrations of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate on VFA production in vitro using 10 g of concentrate as a substrate (experiment 1)

	Acetate*	Propionate	Isobutyrate	Butyrate	2-methyl butyrate	Isovalerate	Valerate	Total	C^2/C^3
No additions**	5.048	1.630	0.098	1.110	0.040	0.017	-	7.943	3.09
No urea	5.925	1.970	0.291	1.512	0.195	0.171	0.287	10.359	3.00
Urea	6.627	2.022	0.100	1.400	0.147	0.059	0.040	10.395	3.28
Urea + 2µl***	-	-	-	-	-	-	-	-	-
Urea + 4µl	8.368	2.329	0.153	1.624	0.175	0.225	0.102	13.076	3.59
Urea + 8µl	6.970	2.247	0.462	1.596	0.163	0.257	0.306	12.001	3.10
Urea + 12µl	6.348	2.073	0.580	1.488	0.236	0.290	0.470	11.485	3.06
Urea + 16µl	6.180	1.876	0.718	1.408	0.301	0.364	0.516	11.363	3.29
Urea + 20µl	6.507	2.101	0.887	1.554	0.346	0.426	0.540	12.361	3.10
Urea + 24µl	7.058	2.125	1.039	1.578	0.462	0.521	0.764	13.547	3.32
Urea + 28µl	5.970	1.839	0.956	1.478	0.484	0.479	0.785	11.991	3.25
Urea + 32µl	7.049	2.069	0.971	1.530	0.545	0.464	0.793	13.421	3.41
Standard Deviation	1.75	.52	.28	.52	.13	.10	.16		

*All values are expressed as mmole/100ml rumen fluid

**Rumen fluid only

***Acid mixtures expressed as microliters/100ml fermentor

Table 16.--The effects of different concentrations of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate on VFA production in vitro using 5 g of concentrate as a substrate (experiment2)

	Acetate*	Propionate	Isobutyrate	Butyrate	2-methyl butyrate	Isovalerate	Valerate	Total	C^2/C^3
No additions**	4.76	1.84	0.12	1.21	0.11	0.22	0.31	8.57	2.59
No urea	5.29	1.53	0.24	1.11	0.16	0.21	0.29	8.83	3.46
Urea	6.02	2.06	0.34	1.55	0.25	0.28	0.43	10.93	2.92
Urea + 4µl***	6.91	2.17	0.36	1.78	0.28	0.37	0.54	12.41	3.18
Urea + 8µl	7.38	1.87	0.67	1.56	0.41	0.42	0.69	13.00	3.95
Urea + 12µl	6.33	1.91	0.78	1.57	0.46	-	-	11.05	3.31
Urea + 16µl	7.00	1.08	0.24	1.24	0.35	0.17	0.23	10.31	6.48
Urea + 20µl	-	-	-	-	-	-	-	-	-
Urea + 24µl	-	-	-	-	-	-	-	-	-
Urea + 28µl	-	-	-	-	-	-	-	-	-
Urea + 32µl	-	-	-	-	-	-	-	-	-
SD****	1.17	.42	.14	.47	-	.06	.11	-	-

*All values are expressed as mmole/100ml rumen fluid

**Rumen fluid only

***Acid mixtures expressed as microliters/100ml fermentor

****Standard deviation

Table 17.--The effects of different concentrations of isobutyrate, isovalerate, 2-methyl butyrate and n-valerate on rumen VFA production in vitro, using corn starch and filter paper as substrates (experiment 3)

	Acetate*	Propionate	Isobutyrate	Butyrate	2-methyl butyrate	Isovalerate	Valerate	Total	C^2/C^3
No additions**	7.10	1.76	0.14	1.27	0.14	0.15	0.18	10.74	4.03
No urea	7.48	2.05	0.09	1.41	0.12	0.14	0.20	11.49	3.65
Urea	7.60	2.01	0.27	1.42	0.21	0.17	0.31	11.99	3.78
Urea + 4µl***	8.21	2.19	0.54	1.52	0.33	0.28	0.48	13.55	5.75
Urea + 8µl	7.93	2.08	0.88	1.54	0.51	0.44	0.79	14.17	3.81
Urea + 12µl	-	-	-	-	-	-	-	-	-
Urea + 16µl	7.24	2.21	1.00	1.50	0.55	0.48	0.90	13.88	3.28
Urea + 20µl	7.73	2.07	0.15	1.49	0.14	0.15	0.18	11.91	3.73
Urea + 24µl	-	-	-	-	-	-	-	-	-
Urea + 28µl	-	-	-	-	-	-	-	-	-
Urea + 32µl	-	-	-	-	-	-	-	-	-
SD****	.83	.40	.13	.18	.06	.04	.09	-	-

*All values are expressed as mmole/100ml rumen fluid

**Rumen fluid only

***Acid mixtures expressed as microliters/100ml fermentor

****Standard deviation

Table 18.--The effects of different concentrations of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate on rumen VFA production *in vitro*, using corn starch, filter paper and methionine as substrates (experiment 4)

	Acetate*	Propionate	Isobutyrate	Butyrate	2-methyl butyrate	Isovalerate	Valerate	Total	C^2/C^3
No additions**	5.89	1.87	0.11	1.15	0.07	-	-	9.09	3.15
No urea	6.06	2.05	0.11	1.24	0.08	-	-	9.54	2.96
Urea	6.16	2.21	0.17	1.29	0.11	-	-	9.94	2.79
Urea + 2µl***	5.96	1.95	0.22	1.17	0.13	0.16	0.16	9.75	3.06
Urea + 4µl	6.70	2.03	0.29	1.44	0.18	0.17	0.20	11.01	3.30
Urea + 8µl	-	-	-	-	-	-	-	-	-
Urea + 12µl	5.92	2.01	0.12	1.22	0.08	-	-	9.35	2.95
Urea + 16µl	6.16	2.04	0.11	1.23	0.08	-	-	9.62	3.02
Urea + 20µl	-	-	-	-	-	-	-	-	-
Urea + 24µl	6.57	2.15	0.18	1.12	0.14	-	-	10.16	3.06
Urea + 28µl	-	-	-	-	-	-	-	-	-
Urea + 32µl	6.14	2.05	0.16	1.28	0.11	-	-	9.74	3.00
SD****	.49	.29	.14	.15	.06	.08	.03	-	-

*All values are expressed as mmole/100ml rumen fluid

**Rumen fluid only

***Acid mixtures expressed as microliters/100ml fermentor

****Standard deviation

Table 19.--The effects of different concentrations of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate on rumen VFA production in vitro using 20 g of concentrates and methionine as substrates (experiment 5)

	Acetate*	Propi-onate	Iso-butyrate	Butyrate	2-methyl butyrate	Iso-valerate	Valerate	Total	C^2/C^3
No additions**	4.99	1.13	0.12	1.02	0.03	-	-	7.29	4.41
Nor urea	5.00	1.35	0.23	1.03	-	-	-	7.61	3.70
Urea	4.77	1.34	0.38	1.08	0.15	0.03	-	7.75	3.56
Urea + 4µl***	4.93	1.31	0.57	1.04	0.21	0.20	0.25	8.51	3.76
Urea + 8µl	8.84	1.27	0.53	1.14	0.33	0.30	0.50	12.92	6.96
Urea + 12µl	5.28	1.40	0.58	2.04	0.34	0.47	0.75	10.86	3.77
Urea + 16µl	-	-	-	-	-	-	-	-	-
Urea + 20µl	-	-	-	-	-	-	-	-	-
Urea + 24µl	-	-	-	-	-	-	-	-	-
Urea + 28µl	4.78	1.23	0.07	1.02	0.36	0.50	0.74	8.74	3.74
Urea + 32µl	-	-	-	-	-	-	-	-	-
SD****	1.2	.39	.24	.27	.14	.07	.10	-	-

*All values are expressed as mmole/100ml rumen fluid

**Rumen fluid only

***Acid mixtures expressed as microliters/100ml fermentor

****Standard deviation

FIGURE 2.--Gas production and ammonia output for experiment 1. 10 g concentrate

(O——O), gas; (●——●), NH_3.

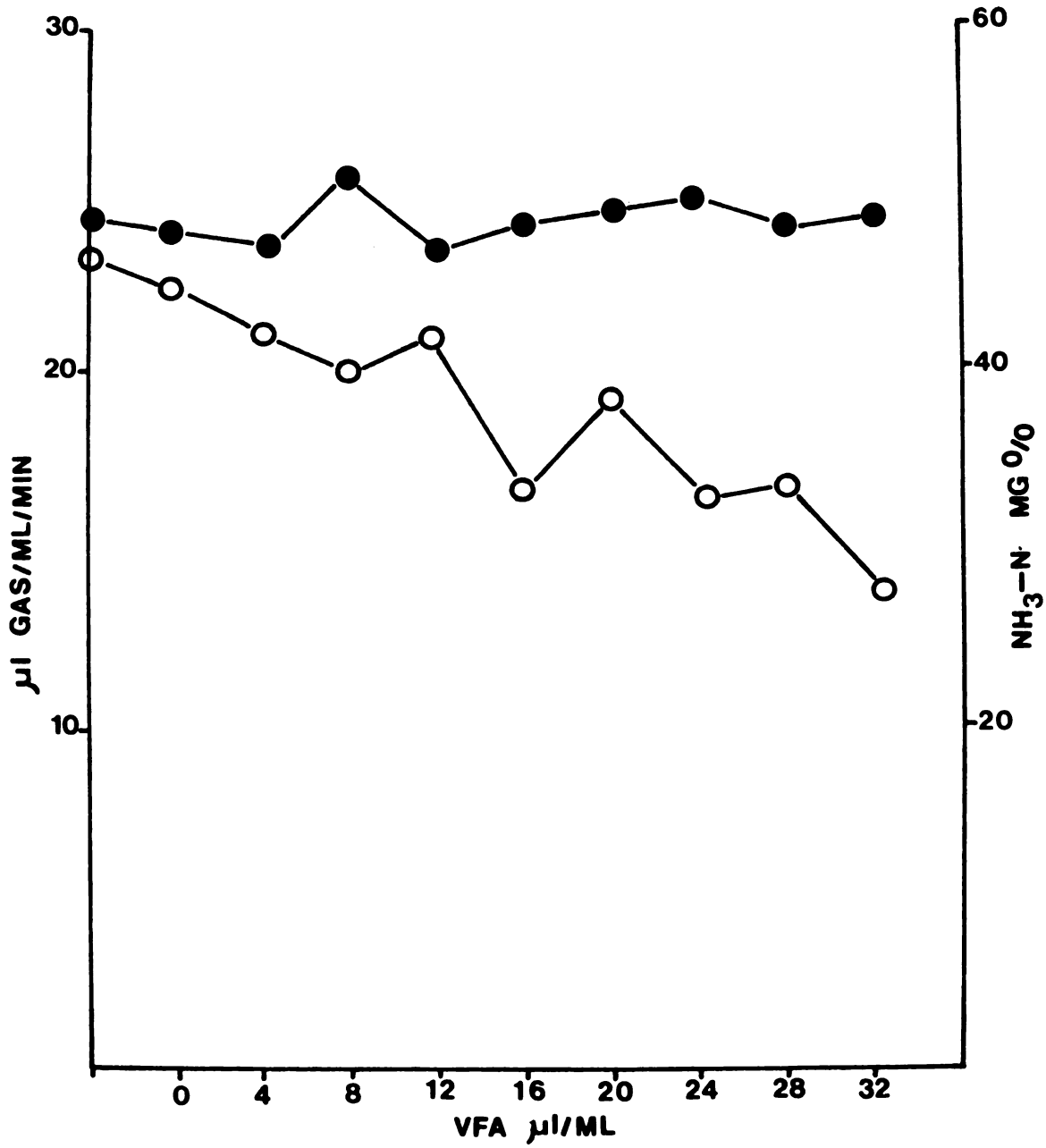

30
20
10
60
40
20
µl GAS/ML/MIN
NH3–N MG %
0
4
8
12
16
20
24
28
32
VFA µl/ML

FIGURE 3.--Gas production and ammonia output for experiment 2. 5 g concentrate

(O——O), gas; (●——●), NH_3.

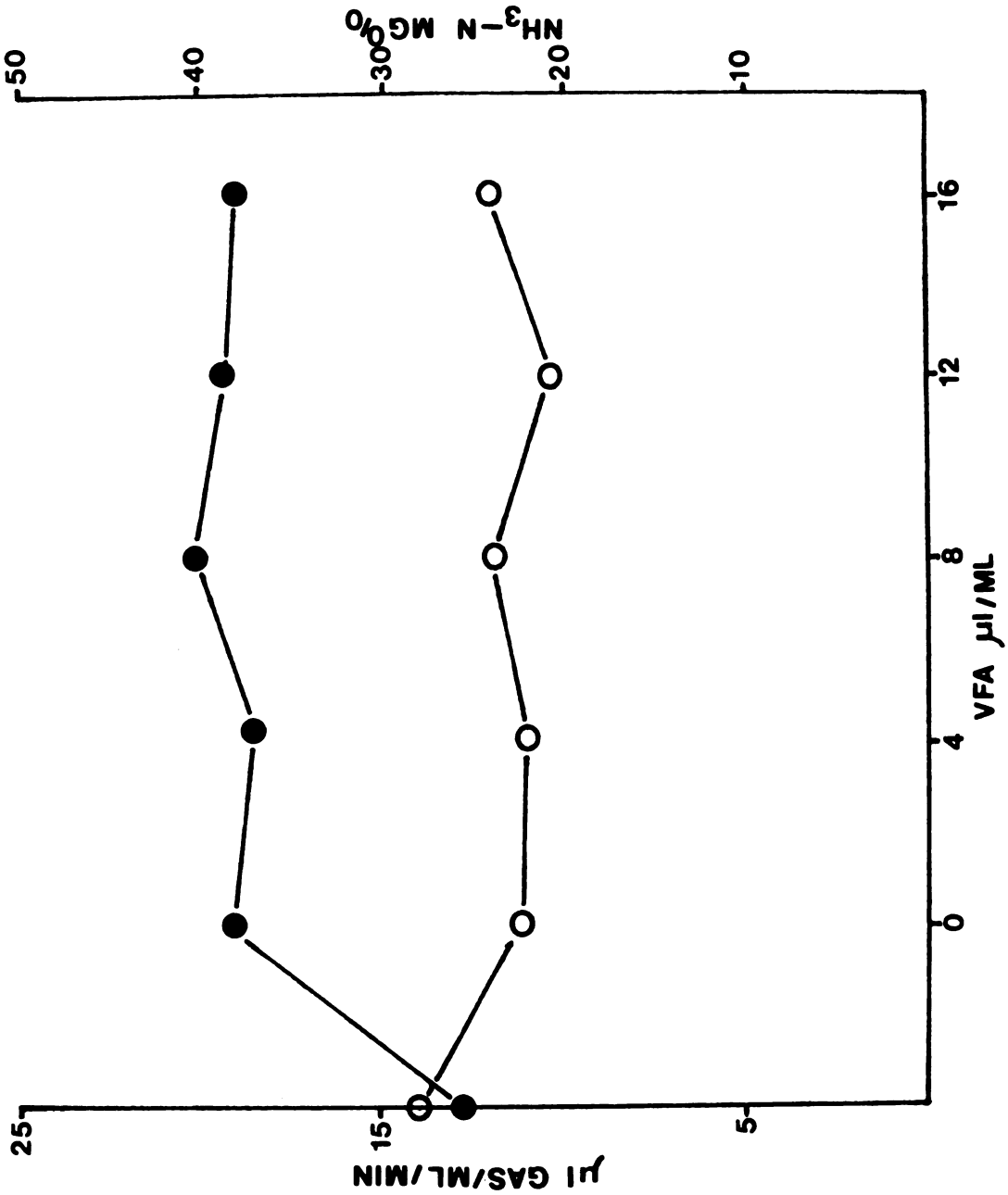

50
40
30
20
10
NH_3–N MG%
25
15
5
µl GAS/ML/MIN
0
4
8
12
16
VFA µl/ML

FIGURE 4.--Gas production and ammonia output for experiment 3. 1.5 g corn starch, 150 mg cellulose

(O——O), gas; (●——●), NH_3.

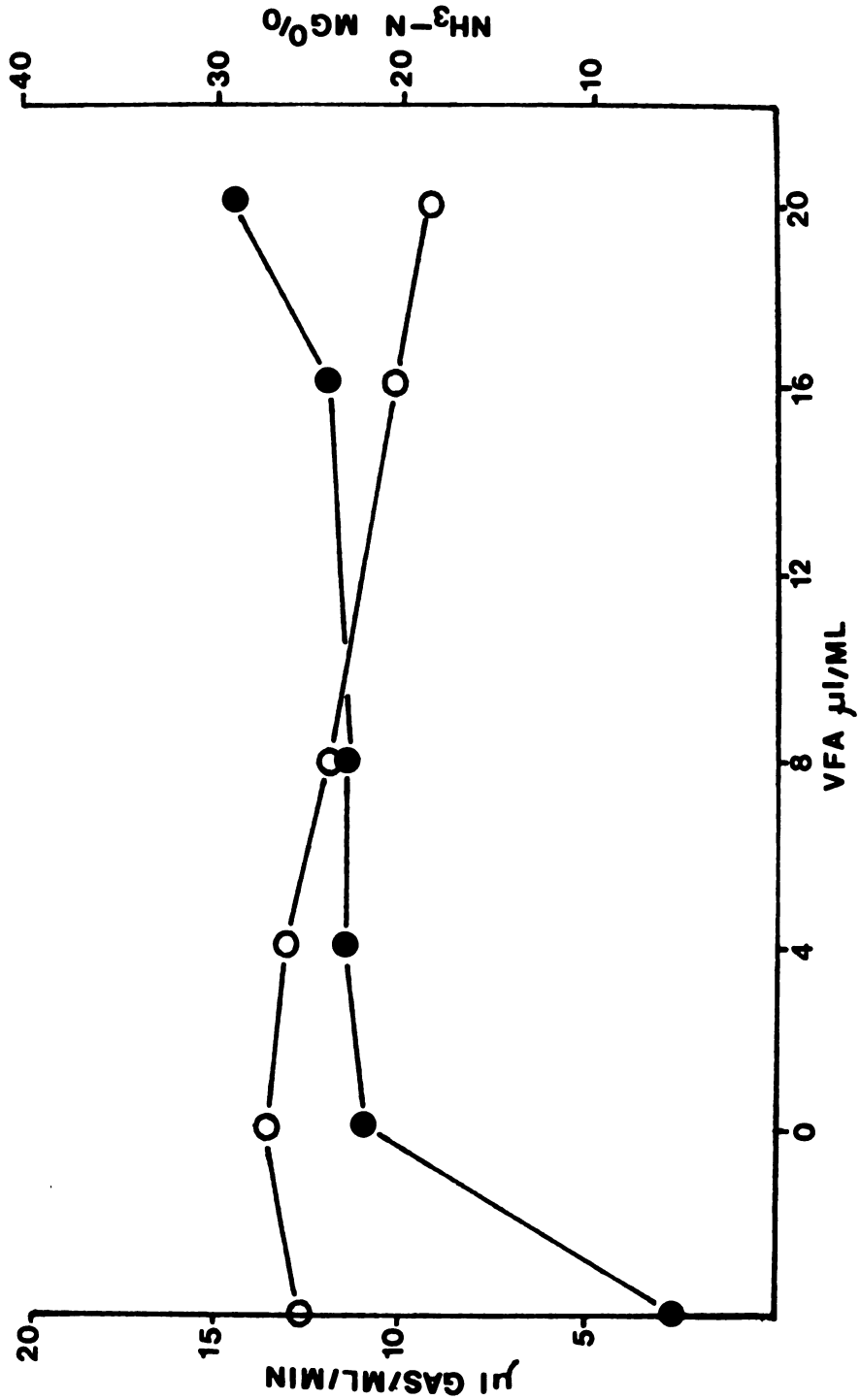

NH3–N MG%
40
30
20
10
VFA µl/ML
0
4
8
12
16
20
µl GAS/ML/MIN
20
15
10
5

FIGURE 5.--Gas production and ammonia output for experiment 4. 1 g corn starch, 1 g cellulose 1 mg methionine.

(O——O), gas; (●——●), NH_3.

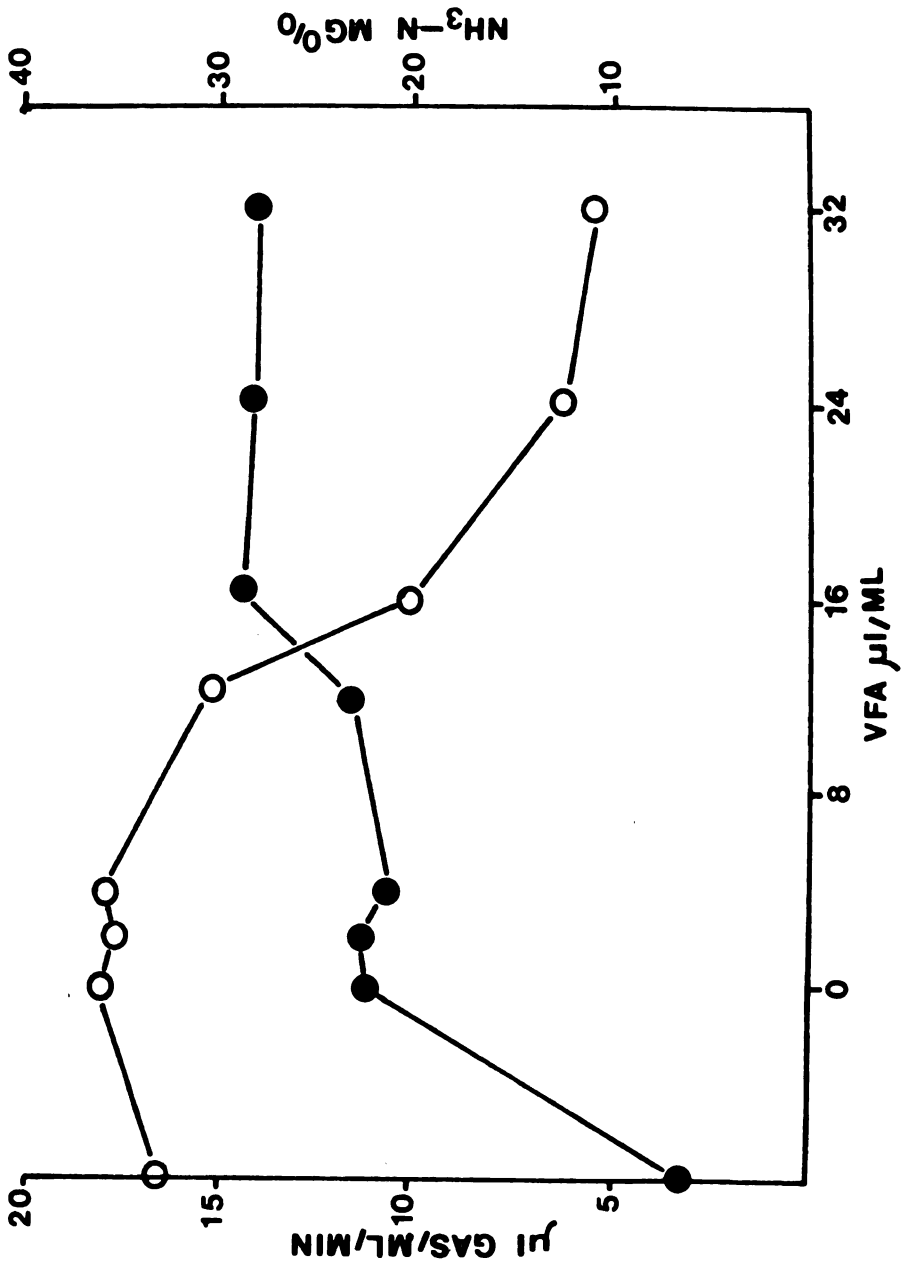
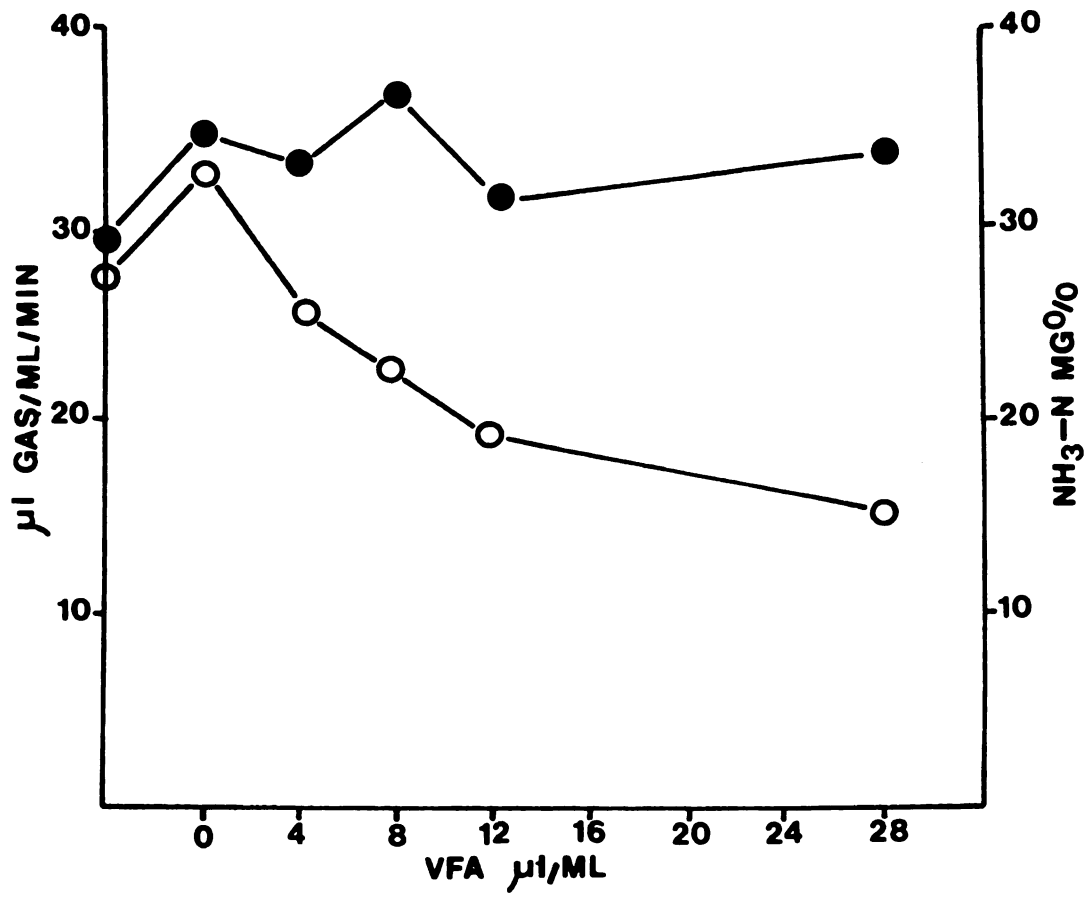

NH3–N MG%
40
30
20
10
VFA µl/ML
0
8
16
24
32
µl GAS/ML/MIN
20
15
10
5

FIGURE 6.--Gas production and ammonia output for experiment 5. 20 g concentrate 5 mg methionine (O——O), gas; (●——●), NH_3.


40
30
20
10
µl GAS/ML/MIN
40
30
20
10
NH3–N MG%
0
4
8
12
16
20
24
28
VFA µl/ML

synthesis and end product formation. These observations might explain the negative relation observed in this study between gas production and the addition of isoacids. That is, isoacids stimulate cell growth, so that more CO_2 and carbon were incorporated into cells. Since the carbon skeletons of the isoacids have been shown to stimulate cellulolytic activity of rumen microorganisms, one would have expected an increase in the rate of gas production as the concentration of the isoacids increased up to a point where maximum microbial activity would be reached. The rate of gas production would then decrease for any further addition of isoacids in the fermentors. However, the results of all five experiments showed the opposite. Gas production decreased as the concentration of the isoacids increased. The reasons for the depressing effect of the isoacids on gas production are not known. However, the general outcome of this in vitro study clearly showed that the isoacids plus urea stimulated the fermentation process. This is evidenced by the reduction of gas production, decrease of pH, reduction of ammonia level and increased molar concentration of VFA. The reduction of gas production may be a consequence of rapid microbial growth. CO_2 which is a major component of gas produced is used for the synthesis of cellular constituents. Therefore a rapid microbial growth would reduce the concentration of CO_2 in the fermentor. This is also evidenced by the reduction of ammonia nitrogen observed throughout the five experiments. Contrary to the suggestions

of some workers that isoacids may not show an effect on microbial growth when the concentration of the rumen fluid in the fermentor exceeds 20 percent, data from this study showed that even with a nearly 100 percent rumen fluid concentration in the media the isoacids stimulated fermentation. Also the utilization of the carbon skeletons from the isoacids may decrease the fermentation of natural protein resulting in less gas production.

Growth Study

This study was an evaluation of the growth rate of dairy heifers as a response to supplementing grass hay with urea plus isoacids and phenylacetate. The isoacids increased the growth rate of the younger animals but not of the older animals. There are two explanations for this. First, isoacids increase nitrogen retention in cattle. Second, younger animals are laying down more muscle than are older animals. Therefore, the nitrogen needs of younger animals are greater (Lassiter et al., 1958a). In Table 20 the first group included the five pairs of smaller animals having an average initial weight of approximately 200 kg per animal. The second group consisted of seven pairs of heifers with an average initial weight of about 225 kg. The third group included all nine pairs of animals averaging an initial weight of about 240 kg. The difference between treatment and control group was close to significant ($P<.20$) for the first two groups of heifers (200 and 225 kg). However, only

Table 20.--The effects of isobutyrate, isovalerate, 2-methylbutyrate, n-valerate and phenylacetate on growth of dairy heifers (growth study)

	Average Body Weight per Animal, kg								
	Animals per Treatment								
	5 animals			7 animals			all animals		
	Initial	Final	Gain	Initial	Final	Gain	Initial	Final	Gain
Control	204	228	.271[a]	221	249	.303[a]	241	267	.289
Treatment	207	240	.367[b]	224	258	.381[b]	241	273	.356
	Percent Increase over the Initial Weight								
Control, %		11.8			12.7			10.8	
Treatment, %		15.9			15.1			13.3	

[ab]Significant difference between control and treatment ($P<.20$), using student "t"-test comparison.

a slight difference was observed between the isoacid-treated and the urea-treated groups when the average initial weight was around 240 kg. An examination of the isoacid-treated groups shows that the younger (200 kg) animals averaged an increase of 15.9 percent over the initial weight, whereas the second group gained slightly less weight (15.1 percent). When the entire treatment group was considered (240 kg) the percentage gain was even less than that of the second group. Lassiter et al. (1958a) fed to dairy heifers corn cobs supplemented with valerate and isovalerate in a 150-day feeding trial. They observed a significant increase in growth rate by the group which was fed the acid supplement. However, the greatest effect of these acids was manifested during the first 30-day-period when the animals gained about 1.5 times the weight they gained during the remaining 120 days of the experiment.

Previous trials conducted by Beeson et al. (1964) have indicated that a combination of rumen factors including valeric and isovaleric acids improved cellulose digestion in an artificial rumen, and the growth rate of beef cattle. The present study showed that feeding a combination of isoacids and phenylacetate with urea to dairy heifers increases the growth rate. The mechanism through which the isoacid addition improves growth rate is probably by supplying their carbon skeletons to the ammonia released from urea hydrolysis.

Effects of Isoacids on Milk Production

Trial I--This feeding trial was conducted to test the effects of isoacid-urea-supplemented corn silage without any other source of energy on milk production by high-producing cows in early lactation. For this feeding trial using lactating cows the rationale was that the cows would be in negative energy balance without a concentrate supplement and any improvement caused by isoacids in extracting more energy from the roughage by the rumen microbes would be easier to detect when measuring milk production. The parameters considered in this trial were milk yields, persistency of lactation and body weight changes as summarized in Table 21. The soy protein and the isoacid treatments resulted in significantly higher ($P<.05$) milk yields as compared to the control. However, soy protein supplementation showed better performance as compared to the isoacids ($P<.05$) when milk yields are considered. The persistency with which cows maintained their milk yields was higher ($P<.05$) among cows fed soy protein supplement as compared to the control. In turn, cows fed with the isoacids maintained their milk production at a higher level than those fed with the control, but the difference between both groups was not statistically significant. Cows fed the urea treatment alone lost 486 g of body weight per day; whereas the groups on isoacids and soy protein treatments gained 94 g and 422 g weight per animal per day, respectively. The difference in

Table 21.--The effects of supplementing corn silage with soy protein, urea or urea plus isoacids and phenylacetate* on milk yields, persistency** and body weight in Holstein cows*** (trial I)

	Soy Protein	Urea	Urea + isoacids + phenylacetate
Milk yields, kg/cow/day	24.03 (6.67)^{d}	19.86 (3.45)^{e}	22.13 (2.43)^{f}
Persistency, %	93.45 (6.67)^{d}	82.31 (7.75)^{e}	86.99 (9.77)^{de}
Body weight changes**** kg/cow/day	.143 (.422)^{d}	-.486 (.845)^{e}	.094 (.372)^{d}

*Equal amounts of isobutyrate + isovalerate + 2-methylbutyrate + phenylacetate + n-valerate

**Persistency of lactation = 100 x (treatment/standardization)

***All values are means with standard deviation in parentheses

^{def}Values in same rows with different superscripts are significantly different ($P<.05$)

****Differences between control and treatments $P<.10$

body weight changes was statistically significant between the soy protein group and urea ($P<.01$), and between isoacids and urea ($P<.05$). But no significant difference was observed in body weight changes between soy protein- and isoacid-fed animals. The results of this trial show that soy protein was most effective in producing milk and maintaining milk production and body weight when corn silage was fed as the sole source of energy for high-producing cows. Data from this trial also indicated that isoacid addition to an urea-corn silage diet improved milk production and animal body weight over urea alone. This work was a preliminary feeding trial to evaluate the feeding value of isoacids as a complementary source of protein in a urea diet fed to dairy cows. The trial was designed so that the cows would be at the highest protein and energy demand. The positive response of cows fed a soy protein diet, which is a high source of natural protein, was expected. However, we are not aware of any studies concerning the use of isoacids with urea for milk production. Lassiter et al. (1958b) failed to show any difference in milk production when they supplemented corn silage and alfalfa hay with isovalerate and valerate in a complete ration for lactating cows. The results of this trial show the potential value of isoacids as a part of the supplemental crude protein for lactating cows. From literature on the nutrition of rumen bacteria, it is known that fiber-digesting bacteria require isoacids and ammonia for microbial protein synthesis. Without the carbon skeletons

from isoacids, urea or ammonia nitrogen cannot be used by rumen microbes. The major sources of carbon skeletons for biosynthesis of branched-chain amino acids (valine, leucine, isoleucine) are from degraded protein or exogenous branched-chain amino acids. With urea feeding as the sole source of supplemental nitrogen the rumen bacteria are not able to synthesize enough protein for maintainance of animal body tissues and for productive purposes. Because of lack of dietary preformed proteins, which are sources of the carbon skeletons, some exogenous branched-chain acids are needed when urea or other non-protein nitrogen sources are used. Therefore, the importance of supplying the isoacids in this particular situation of high protein demand is evidenced by the results of this trial. It is obvious from the results of this trial that the isoacids would be most effective when dietary protein is low and the protein requirement for animal performance is high.

Trial II--Since a positive response to isoacids was observed in trial I, it was decided to test the effects of addition of isoacids to a nutritionally complete ration. The ration consisted of corn silage and a supplemental concentrate. The results of this work are summarized in Table 22. Milk yields were not significantly different between animals in all four treatments. Addition to isoacids to urea improved, but not significantly, the persistency of lactation over both positive control and urea rations. Also

Table 22.--Milk yields, persistency of lactation, body weight changes, feed dry matter consumption and feed efficiency of cows fed corn silage supplemented with urea, urea plus isoacids*, soybean meal and no nitrogen supplementation (trial II)

	Negative Control	Positive Control	Urea	Urea + isoacids
Milk yields kg/cow/day	19.07 (5.54)**	21.23 (6.07)	21.27 (8.97)	21.17 (4.19)
Persistency, %	76.74 (6.35)[a]	85.71 (10.06)[b]	84.63 (4.53)[b]	87.99 (11.23)[b]
Body weight changes kg/cow/day	.626 (.41)	.670 (.43)	.626 (.40)	.786 (.55)
Silage intake DM % body weight	1.68 (.30)[a]	1.71 (.30)[a]	1.80 (.28)[a]	1.93 (.21)[bl]
Total feed intakes DM % DM body weight	3.07 (.30)	3.07 (.33)	3.26 (.45)	3.25 (.36)
Kg milk/kg DM intake	.477 (118.7)	.523 (120.1)	.523 (106.3)	.495 (63.8)

*Equal amounts of isobutyrate, isovalerate, 2-methylbutyrate and n-valerate

[ab]Values in same row with different superscripts are significantly different (P<.05) using orthogonal comparisons

[bl]Significant P<.25

**Values in parentheses are standard deviation

the isoacid treatment tended to maintain body weight at a higher level when compared to the three other groups. However, the difference was not statistically significant. Corn silage dry matter intake was slightly higher ($P<.25$) among cows fed the isoacid diet as compared to the other treatment groups. However, total feed dry matter intake per 100 kg body weight was only slightly higher for the isoacid ration over the remaining groups. The efficiency with which feed was converted to milk was essentially the same for all treatments. Therefore, the slight increase in milk yields between the negative control and the other treatment groups may not be explained on the basis of higher feed efficiency per se. In general this trial shows that the addition of isoacids to urea was most effective in maintaining body weight and the level of milk production, and in increasing corn silage dry matter intake, as compared to the other treatments. The positive effect of isoacids on increasing roughage intake is consistent with the works of Hensley and Moir (1963), and Van Gylswyk (1970). Both groups of investigators reported significant increases in voluntary hay intake when they fed isoacids with urea to sheep. The increase in hay intake was accompanied by an increase in the number of cellulolytic bacteria (Gylswyk, 1970) and an increase in the level of microbial protein (Hensley and Moir, 1963). Among several possible reasons why isoacids did not show any increase in milk production in comparison to urea alone, three explanations are notable: (1) the cows may have

been able to mobilize body tissue to meet their energy needs and the effects of the isoacids could not be detected by measuring milk production; (2) the level of protein equivalent supplied by the urea ration may have been sufficient to fulfill the protein requirements for milk production so that the addition of the isoacids could not contribute any further increment. The level of milk yields shown in Table 22 required only a ration with about 14 percent crude protein (National Research Council, 1971). This amount of protein had been reported in the current trial; and, (3) as previously suggested, isoacid addition to urea may be more effective when the level of natural protein in the diet is low and when the protein demand is high. It was reported (National Research Council, 1976) that the basal diet must be deficient in nitrogen or natural protein if supplementary urea is to be beneficial to the animal. However, even though significant differences were not uniformly observed for all parameters, the isoacid addition to urea reveals that better performance is obtained when compared even to the positive control.

Trial III--Since trial II did not result in significant differences between treatments using a randomized block design it was decided to conduct another trial using a Latin square cross-over type design which is much more sensitive than the former design (Gill, 1969). Using this design, the theoretical probability of showing a 2 kg difference in milk

production using 8 cows per treatment is about 80 percent. However it was found that when the cows were changed from one treatment to another milk production declined for about the first two weeks, recovering at the end of the fourth week, which was the duration of the treatment period. Thus, it was found that this design is not satisfactory for testing the parameters of interest. Thirty cows were used in this experiment. The milk production of 12 of them decreased more than 2 kg per day when the ration was changed. Therefore, the data from these cows were not included in the statistical analysis.

The effects of the two different mixtures of isoacids on milk yields, persistency of lactation, body weight changes, feed intake and feed efficiency are shown in Table 23. Data in this table are means of eighteen repetitions per treatment from the 18 cows which were less affected by the effect of changing diet in the cross-over experiment. Milk yields per cow per day were slightly, but not significantly, higher in both isoacid mixtures than in the control. However, fat-corrected milk (FCM) was significantly higher ($P<.05$) among cows fed the isoacid mixture 2 when compared to either the control or isoacid mixture 1, indicating that isoacid mixture 2 increased the fat content of the milk over that of the control and even over that of mixture 1. Cows fed the acid mixture 2 maintained their milk production at a higher level ($P<.10$) than those fed urea alone or the acid mixture 1. The group fed mixture 1 were slightly more persistent in

Table 23.--The effects of two different mixtures of isoacids on milk yields, persistency of lactation, body weight changes, feed dry matter intake and feed efficiency (trial III)

	Control	Acid Mixture 1*	Acid Mixture 2**
Milk yields, kg/cow/day	28.1	28.5	29.3
FCM*** kg/cow/day	25.6 (4.13)[a]	25.9 (3.24)[a]	27.1 (3.59)[b]
Persistency, %	89.3 (14.7)[c]	90.3 (9.06)[c]	94.6 (11.28)[d]
Body weight changes, kg/cow/day	- .11 (5.5)	- .27 (5.8)	- .01 (6.1)
Silage intake, kg % body weight	1.67 (.15)	1.67 (.15)	1.70 (.16)
Total feed intake, kg % body weight	3.12 (.20)	3.17 (.4)	3.18 (.22)
Kg milk/kg feed DM	1.4 (.24)	1.4 (.25)	1.5 (.4)

*Twenty-eight percent isobutyrate, plus 24 percent of isovalerate, 2-methylbutyrate and n-valerate.

[ab]Values in same row with different superscripts are significantly different ($P<.05$), using orthogonal comparisons.

[cd]Significant difference at $P<.10$

**Thirty-six percent isobutyrate, 30 percent n-valerate, and 17 percent each of isovalerate and 2-methylbutyrate.

***Fat corrected milk 4 percent at 305 days (.4 x milk + 15 x fat).

lactation than the group receiving the control ration. Cows fed isoacids 2 lost less body weight than those on acids 1 or on control. However, the difference was not statistically significant. Corn silage dry matter intake, total feed intake and feed efficiency were essentially unaffected by the acid treatments. Generally the group on isoacids 2 reveals a trend towards better performance as compared to control or isoacids 1. There is no apparent explanation for the difference between the responses resulting from isoacid mixtures 1 and 2. The difference between the composition of the two isoacids is that the molar proportions of isobutyrate and n-valerate were higher (36 and 30 percent) in mixture 2 than in mixture 1 (28 and 24 percent). This may suggest that these two acids were the most limiting. The work of Lassiter et al. (1958b) showed that cows fed with n-valerate gave better performance than when n-valerate was associated with isovalerate. However, Umunna and co-workers (1975) found much higher incorporation of isovalerate into the microbial cells than isobutyrate. In this trial II, the addition of the isoacids did not show any improvement in milk production. However, acid mixture 2 improved the persistency of lactation over the control. Lassiter's group (1958b) could not improve milk production by feeding isovalerate and n-valerate to lactating cows fed corn silage and hay. But they did not feed urea. In the current trial the isoacids probably improved utilization of nitrogen from urea. There were some similarities between these two trials.

Lassiter's trial used a Latin square cross-over design in a 28-day period. It would appear that the effect of changing diet on the appetite of the animals and the duration of the treatment period are the main explanation for results obtained in both trials.

Milk composition, plasma urea nitrogen, rumen pH, ammonia-N and VFA production are presented in Table 24. There were no significant difference among the three treatment groups as far as milk composition is concerned. However, for the isoacid groups milk protein, fat and solids nonfat were slightly higher. Rumen ammonia-N was lower in the isoacid treatments than in the control, although this difference was not significant. The same trend was observed for plasma urea-N. This suggests that there was higher utilization of ammonia by the cows on the acid treatments as compared to those on control. It is possible that carbon skeletons from isoacids have been associated with ammonia in the process of microbial synthesis. This is supported by the increase in acetate in both acid treatments over the control, indicating that fermentation is stimulated by the addition of the acids to urea. However, the addition of the isoacids did not appear to influence the molar proportions of propionate and butyrate. Isobutyrate levels increased slightly in the rumen fluid with the addition of the isoacids. But a close examination of Table 24 indicates that the isoacids were utilized since isobutyrate was the only one among the four acids added that appeared in the rumen fluid.

Table 24.--The effects of two different mixtures of isoacids on milk composition, plasma urea and rumen pH, ammonia nitrogen and VFA (trial III)

	Control	Acid Mixture 1	Acid Mixture 2
Milk total solids, %	12.14 (.80)	12.43 (.87)	12.30 (.87
Milk fat, %	3.43 (.52)	3.45 (.63)	3.55 (.58)
Milk SNF, %	8.75 (.65)	8.97 (.57)	8.75 (.73)
Milk protein, %	3.11 (.30)	3.17 (.35)	3.24 (.33)
Milk fat production g/cow/day	964.83 (173.2)[a]	976.35 (190.0)[a]	1040.15 (196.1)[b]
Milk SNF production g/cow/day	2458.75 (384.2)	2166.45 (326.9)	2563.75 (338.4)
Milk protein production g/cow/day	873.91 (114.9)	903.45 (99.9)	949.32 (117.4)
Plasma urea-N, mg/100ml	10.62 (2.64)[c]	9.68 (2.34)[d]	9.45 (2.80)[d]
Rumen pH	6.88 (.28)	6.91 (.24)	6.89 (.27)
Rumen ammonia-N, mg/100ml	11.95 (6.73)[c]	10.04 (5.46)[d]	9.27 (4.92)[d]
Rumen VFA, mmole/100ml			
Acetate	4.11 (.77)[c]	4.51 (1.16)[d]	4.63 (1.23)[d]
Propionate	1.46 (.40)	1.45 (.33)	1.47 (.33)
Butyrate	.84 (.22)	.85 (.20)	.83 (.19)
Isobutyrate	.05 (.01)	.08 (.02)	.08 (.02)
6/16/2	281	311	315

[ab]Values in same row with different superscripts are significantly different (P<.01)

[cd]Significant at P<.05

The effects of isoacids on dry matter digestibility, nitrogen digestibility, nitrogen retention and nitrogen balance are summarized in Table 25. Feed dry matter digestibility was essentially the same among the three treatment groups. However, nitrogen intake and fecal nitrogen were higher in both isoacid treatments than in the control. Nitrogen digestibility appears to be higher in the acid treatments as compared to the control. However, urinary-N was significantly higher ($P<.025$) in the control than in both acid treatments. Nitrogen retained was significantly higher ($P<.01$) for the acid treatments compared to the control. The addition of both isoacid mixtures improved ($P<.10$) the nitrogen balance of the lactating cows. The effect of isoacids on nitrogen retention is the most obvious beneficial effect showed by the use of isoacids in cattle and sheep diets (Cline et al., 1966; Lassiter et al., 1958b; Moore, 1964; Oltjen et al., 1971; Umunna et al., 1975). All previous work in which nitrogen balance studies were conducted shows that more of the available ammonia is being converted into microbial protein. In the present trial the addition of the acids reduced the urinary nitrogen loss and therefore increased the utilization of the absorbed nitrogen as was indicated by an improved nitrogen retention. Kay and Phillipson (1964) have reported an increase in nitrogen flow rate to the duodenum of sheep fed poor quality hay diets by infusing isobutyrate, 2-methylbutyrate and isovalerate; whereas similar infusion of the straight-chain acids had

Table 25.--The effects of two different mixtures of isoacids on feed dry matter (DM) digestibility, nitrogen digestibility and nitrogen retention by Holstein cows* (trial III)

	Control	Acid Mixture 1	Acid Mixture 2
DM digestibility, %	57.4	57.3	57.3
Nitrogen intake, g	338.2 (.28)	352.0 (.13)	354.2 (.09)
Fecal nitrogen, g	156.9 (6.17)	160.2 (4.8)	162.9 (5.5)
N digestibility, %	53.7 (6.28)	54.5 (4.8)	54.0 (5.5)
Urinary-N, g	96.6 (7.7)[a]	85.8 (3.7)[b]	86.4 (5.3)[b]
Urinary-N, % intake	29.5 (7.7)[a]	24.4 (3.7)[b]	24.4 (5.3)[b]
Total N excreted, g	256.5	246.0	249.3
N retained, g	81.7 (8.0)[c]	106.0 (4.4)[d]	104.9 (4.4)[d]
N retained as % of absorbed	45.1[a]	55.3[b]	54.8[b]
Milk - N, g	122.5	121.7	124.7
N balance, g	-40.7 (13.5)[c]	-15.7 (5.7)[d]	-19.8 (6.5)[d]

*All values are means per cow per day, with standard deviation in parentheses.

[ab]Values in same row with different superscripts are significantly different (P<.025), using orthogonal contrast.

[cd]Difference at P<.10

little effect on the nitrogen flow rate. This indicates an increase in microbial growth rates due to the addition of the isoacids. Although the addition of isoacids in the current trial did not improve milk yields or feed intake, the fact remains that all of these parameters showed a positive influence of these isoacids on nitrogen utilization as evidenced by the nitrogen retention. The maintenance of the level of milk production by these acids over the urea diet is additional evidence of the beneficial effect of isoacids on the nitrogen economy of ruminants fed urea. It is also possible that a greater response could have been obtained if greater quantities of the acids were fed.

Trial IV--From the results of trial III it was felt necessary to conduct another continous feeding trial using pairs of cows in a randomized block design for 90 days. Thirty animals were paired, adapted to the feeding of isoacids and high level of urea for a period of 21 days prior to the treatment period. The results of this trial are reported in Tables 26 and 27 and are the summaries of observations for 14 pairs of cows. Half of the animals were heifers in the first or second lactation. Milk yields, persistency of lactation, body weight changes and feed dry matter intake are shown in Table 26. The addition of isoacids to urea did not appear to improve significantly milk yields as compared to the control diet. However, the acid treatment showed a slight increase over the control. Fat

Table 26.--Milk yields, persistency of lactation, body weight changes, feed dry matter intake of cows fed equal amounts of isoacids*, (trial IV)

	Control	Treatment
Milk yields, kg	17.36 (3.09)	18.69 (3.42)
Fat corrected milk, kg	16.32 (3.89)	17.56 (4.35)
Persistency, %	76.87 (9.56)[a]	81.79 (10.20)[b]
Body weight changes, kg	.029 (.17)	.027 (.19)
Silage intake (DM % body weight)	2.03 (.34)	2.18 (.28)
Total feed intake (kg % body weight)	2.61 (.04)[c]	2.83 (.06)[d]

*Data are means per cow with standard deviation in parentheses.

[ab]Values in same row with different superscripts are significantly different (P<.001), using "t"-test comparisons.

[cd]Significant difference at P<.05.

corrected milk showed the same trend in favor of the acids, but without any significant difference between treatment groups. However, the persistency of lactation was significantly higher ($P<.001$) among cows fed the isoacid treatment than among those fed urea alone. This is consistently observed throughout all four trials, indicating a beneficial influence of the isoacids on urea feeding. Persistency of lactation expresses the level at which a cow maintains her production throughout the entire lactation. A cow with low persistency would indicate that the rate of milk production has been markedly reduced from one month to another, which would affect the total milk produced during the entire lactation compared with a higher persistency. Therefore information about persistency of lactation may be a more accurate parameter to estimate the effect of a diet than the actual milk production which is subjected to many variations from one animal to another. The gain in body weight was essentially the same between the two groups of animals. The isoacids increased slightly the corn silage dry matter intake over the control. But the total feed dry matter intake expressed as a percentage of body weight was significantly increased ($P<.05$) by the acid treatment over the control. This is another major benefit of the isoacid feeding as substantiated by Van Gylswyk (1970) and Hensley and Moir (1963). Although no significant difference was observed in daily milk yields, the beneficial effect of the isoacid

feeding is manifested by the significantly high persistency of lactation and the significant increase in feed dry matter consumption.

Milk composition, plasma urea nitrogen, rumen pH, rumen NH_3-N and VFA are summarized in Table 27. The addition of the isoacids to the diet did not significantly affect the milk composition, rumen pH, total rumen VFA, rumen ammonia-nitrogen or plasma urea-nitrogen. The only significant difference observed in VFA concentrations was for isobutyrate ($P<.01$), which reflects the addition of the isoacid to the ration. The molar concentrations of acetate and propionate increased slightly for the isoacid group over the control group. The level of butyrate remained essentially the same in both the control and treatment groups. It is important to note that isovalerate, n-valerate and 2-methylbutyrate were not found in detectable amounts in rumen fluid of trials III and IV. This is in contrast to the observations by Oltjen et al. (1971), Umunna et al. (1975) and Hume (1970). These workers reported significant increases in the rumen fluid of the animals to which these acids were administered. However, the significant increase in isobutyrate level observed in the current trial is in agreement with those findings. Probably the dilution factor used in our study did not permit the detection of the other acids. A close examination of the results of the present trial reveals that the addition of isoacids to urea resulted in significantly higher levels of milk production, higher feed dry matter

Table 27.--Milk composition, plasma urea nitrogen and VFA of cows fed isoacids* (trial IV)

	Control	Treatment
Milk composition total solids, %	12.18 (.46)	12.09 (.65)
Fat, %	3.60 (.36)	3.57 (.46)
Solid non fat, %	8.57 (.35)	8.52 (.42)
Protein, %	3.11 (.41)	3.11 (.38)
Fat production g/cow/day	645.25 (146.8)	650.84 (145.32)
SFN production g/cow/day	1544.77 (396.45)	1591.22 (462.54)
Protein production g/cow/day	540.01 (389.6)	581.21 (416.8)
Plasma urea-N mg/100ml	11.55 (3.47)	10.95 (3.22)
Rumen pH	7.05 (.20)	7.05 (.24)
Rumen NH_3-N mg/100ml	9.64 (5.63)	10.40 (4.28)
Rumen VFA, mmole/ 100ml		
Acetate	3.96 (.59)	4.10 (.57)
Propionate	1.06 (.17)	1.11 (.23)
Butyrate	.69 (.10)	.69 (.17)
Isobutyrate	.04 (.01)[a]	.12 (.03)[b]

*Data are means per cow with standard deviation in parentheses

[ab]Significant difference at P<.01

intake, lower plasma urea nitrogen and higher rumen acetate, propionate and isobutyrate concentrations. These data indicate a beneficial effect of isoacids on urea nitrogen utilization by dairy cows. Although statistical differences were not always observed for all parameters considered in this trial, information from persistency of lactation and feed intake are sufficient to support the evidence of beneficial effects of isoacids on the performance of the dairy animals.

SUMMARY AND CONCLUSION

One *in vitro* experiment, one growth trial and four separate milk production trials were conducted to investigate the nutritional response of dairy cattle to high-urea ration supplemented with isoacids. The results of the *in vitro* study showed that isoacids enhanced rumen microbial activity.

The growth study was conducted to investigate the effect of supplementing grass hay with isoacids and urea on growth rate of dairy heifers. This study showed that addition of isoacids to urea increased the growth rate in young animals but not in older animals.

The four milk productions were carried out to determine the performance of high-producing cows fed corn silage as the sole roughages supplemented with isoacids and urea. Those four trials showed that isoacids had a positive effect on milk production, persistency of lactation, body weight, feed intake and nitrogen balance, when added to a urea-based diet for lactating cows. It is well known that the utilization of low protein roughage by ruminants is influenced by several factors. One of these factors is unquestionally an

insufficiency of nitrogen to satisfy the growth requirements of an adequate microbial population in the rumen. The addition of urea alone to improve the nitrogen level of such dietary material has produced variable responses. This is because after the deficit in nitrogen has been removed by feeding urea, the next factor limiting the microbial growth is the lack of carbon skeletons from isoacids. One or more of these acids is required for the growth of several species of cellulose-digesting bacteria (Allison, 1965). These isoacids are normally derived from protein in the diet. The addition of the isoacids to urea in the present studies no doubt resulted in increasing the rate of proliferation of cellulose-digesting bacteria in the rumen. These studies have shown that isoacids:

a. increased rumen microbial activity in vitro
b. improved rate of growth of young animals
c. increased persistency of lactation
d. improved nitrogen retention in lactating cows

It is apparent that feed supplements must be considered not merely in terms of their gross nitrogen, mineral and energy supplements, but also in terms of their ability to satisfy the full nutrient requirements of the rumen microbiota.

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APPENDIX

Table 28.--Analysis of variance for gas production *in vitro*

	Source of variation			
	Main effects	Treatment	Time	Error
Experiment 1				
Degrees of freedom (d.f.)	20	9	11	99
Sums of square (s.s.)	222.42	207.86	14.57	20.87
Mean squares (m.s.)	11.12**	23.10**	1.32**	0.21
Experiment 2				
d.f.	16	5	11	55
s.s.	32.99	23.50	9.49	3.42
m.s.	2.06**	4.70**	0.86**	0.06
Experiment 3				
d.f.	16	5	11	55
s.s.	71.74	37.21	34.52	9.60
m.s.	4.48**	7.44*	3.14**	0.17
Experiment 4				
d.f.	16	5	11	55
s.s.	24.34	7.56	16.78	9.47
m.s.	1.52**	1.51**	1.53**	0.17
Experiment 5				
d.f.	17	6	11	66
s.s.	259.48	249.39	10.09	17.73
m.s.	15.26**	41.57**	0.91**	0.26

Overall	Experiment	Main effects	Treatment	Time	Error
d.f.	4	24	9	11	395
s.s.	1501.88	1614.19	272.61	7.70	391.76
m.s.	375.47**	67.26**	30.29**	0.70	0.99

**P<.01

Table 29.--Analysis of variance for acetate production *in vitro*

	Source of variation			
	Main effects	Treatment	Day	Error
Experiment 1				
d.f.	17	10	7	43
s.s.	63.36	34.52	27.28	131.23
m.s.	3.73	3.45	3.89	3.05
Experiment 2				
d.f.	13	7	6	19
s.s.	8.86	1.70	6.10	3.43
m.s.	0.68**	0.24	1.02**	0.18
Experiment 3				
d.f.	14	7	7	35
s.s.	8.25	1.31	7.12	5.51
m.s.	0.59**	0.18	1.02**	0.16
Experiment 4				
d.f.	12	6	6	7
s.s.	6.02	1.06	2.44	1.23
m.s.	0.50	0.18	0.41	0.25
Experiment 5				
d.f.	13	6	7	25
s.s.	10.68	0.18	9.10	3.90
m.s.	0.82**	0.03	1.30**	0.16

Overall	Experiment	Main effects	Treatment	Day	Error
d.f.	4	21	10	7	181
s.s.	116.86	160.81	21.92	27.71	446.55
m.s.	29.21**	7.66**	2.19	3.96	2.47

**P<.01

Table 30.--Analysis of variance for propionate production *in vitro*

	Source of variation			
	Main effects	Treatment	Day	Error
Experiment 1				
d.f.	17	10	7	43
s.s.	4.77	1.92	2.75	11.68
m.s.	0.28	0.19	0.39	0.27
Experiment 2				
d.f.	13	7	6	19
s.s.	8.86	1.70	6.10	3.43
m.s.	0.68	0.24	1.02	0.18
Experiment 3				
d.f.	14	7	7	35
s.s.	6.37	5.79	0.27	0.58
m.s.	0.46**	0.83**	0.40*	0.02
Experiment 4				
d.f.	12	6	6	7
s.s.	1.85	0.15	1.56	0.60
m.s.	0.15	0.03	0.26	0.09
Experiment 5				
d.f.	13	6	7	25
s.s.	10.68	0.18	9.10	3.90
m.s.	0.82	0.03	1.30	0.16

Overall	Experiment	Main effects	Treatment	Day	Error
d.f.	4	21	10	7	181
s.s.	14.81	17.83	1.46	2.48	55.83
m.s.	3.70**	0.85**	0.15	0.35	0.31

*P<.05

**P<.01

Table 31.--Analysis of variance for isobutyrate production in vitro

	Source of variation			
	Main effects	Treat-ment	Day	Error
Experiment 1				
d.f.	17	10	7	43
s.s.	9.65	8.08	0.92	3.35
m.s.	0.57**	0.81**	0.13	0.08
Experiment 2				
d.f.	13	7	6	19
s.s.	2.75	1.39	0.62	0.40
m.s.	0.21**	0.20**	0.10*	0.02
Experiment 3				
d.f.	14	7	7	35
s.s.	1.07	0.44	0.73	1.09
m.s.	0.08**	0.06*	0.10**	0.03
Experiment 4				
d.f.	12	6	6	7
s.s.	0.37	0.07	0.14	0.002
m.s.	0.03**	0.01**	0.02**	0.001
Experiment 5				
d.f.	13	6	7	25
s.s.	2.58	1.17	1.48	1.43
m.s.	0.20**	0.20**	0.21**	0.06

Overall	Experiment	Main effects	Treat-ment	Day	Error
d.f.	4	21	10	7	181
s.s.	2.55	15.71	10.24	0.70	15.90
m.s.	0.64**	0.75**	1.02**	0.10	0.09

*P<.05

**P<.01

Table 32.--Analysis of variance for butyrate production in vitro

	Source of variation			
	Main effects	Treat-ment	Day	Error
Experiment 1				
d.f.	17	10	7	43
s.s.	3.87	1.29	3.00	11.52
m.s.	0.22	0.13	0.43	0.27
Experiment 2				
d.f.	13	7	6	19
s.s.	3.59	1.36	1.75	3.44
m.s.	0.28	0.19	0.29	0.18
Experiment 3				
d.f.	14	7	7	35
s.s.	1.07	0.44	0.73	1.09
m.s.	0.08*	0.06*	0.10**	0.03
Experiment 4				
d.f.	12	6	6	7
s.s.	0.90	0.11	0.51	0.17
m.s.	0.08*	0.02	0.08*	0.02
Experiment 5				
d.f.	13	6	7	25
s.s.	5.91	2.34	4.21	1.82
m.s.	0.45**	0.39**	0.60**	0.07

Overall	Experiment	Main effects	Treat-ment	Day	Error
d.f.	4	21	10	7	181
s.s.	4.24	6.65	0.98	1.95	30.60
m.s.	1.06**	0.32*	0.10	0.28	0.17

*P<.05

**P<.01

Table 33.--Analysis of variance for 2-methylbutyrate production in vitro

	Source of variation			
	Main effects	Treatment	Day	Error
Experiment 1				
d.f.	17	10	7	43
s.s.	2.24	1.78	0.35	0.78
m.s.	0.13**	0.17**	0.05*	0.02
Experiment 2				
d.f.	13	7	6	19
s.s.	0.88	0.39	0.28	0.15
m.s.	0.07**	0.06**	0.05**	0.01
Experiment 3				
d.f.	14	7	7	35
s.s.	1.52	1.37	0.07	0.13
m.s.	0.11**	0.20**	0.01*	0.004
Experiment 4				
d.f.	12	6	6	7
s.s.	0.07	0.02	0.03	0.01
m.s.	0.01*	0.003	0.01*	0.001
Experiment 5				
d.f.	13	6	6	7
s.s.	0.95	0.42	0.57	0.47
m.s.	0.07**	0.07**	0.08**	0.02

Overall	Experiment	Main effects	Treatment	Day	Error
d.f.	4	21	10	7	181
s.s.	0.81	3.90	2.47	0.12	4.51
m.s.	0.20**	0.19**	0.25**	0.02	0.03

*P<.05

**P<.01

Table 34.--Analysis of variance for isovalerate production in vitro

	Source of variation			
	Main effects	Treat-ment	Day	Error
Experiment 1				
d.f.	16	9	7	40
s.s.	1.53	1.36	0.20	0.39
m.s.	1.00**	0.15**	0.03*	0.01
Experiment 2				
d.f.	11	5	6	11
s.s.	0.39	0.15	0.15	0.05
m.s.	0.04**	0.03*	0.03*	0.004
Experiment 3				
d.f.	13	6	7	20
s.s.	0.64	0.52	0.04	0.03
m.s.	0.05**	0.09**	0.01*	0.002
Experiment 4				
d.f.	8	1	7	1
s.s.	0.03	0.001	0.03	0.01
m.s.	0.004	0.001	0.004	0.01
Experiment 5				
d.f.	11	4	7	6
s.s.	0.45	0.25	0.14	0.03
m.s.	0.04*	0.063**	0.02	0.01

Overall	Experiment	Main effects	Treat-ment	Day	Error
d.f.	4	20	9	7	121
s.s.	0.25	2.67	2.03	0.12	1.33
m.s.	0.06**	0.13**	0.23**	0.02	0.01

*P<.05

**P<.01

Table 35.--Analysis of variance for valerate production *in vitro*

	Source of variation			
	Main effects	Treat-ment	Day	Error
Experiment 1				
d.f.	16	9	7	40
s.s.	5.09	4.67	0.53	1.05
m.s.	0.32**	0.52**	0.08*	0.03
Experiment 2				
d.f.	11	5	6	11
s.s.	1.10	0.50	0.35	0.13
m.s.	0.10**	0.10**	0.06*	0.01
Experiment 3				
d.f.	13	6	7	20
s.s.	2.28	1.99	0.06	0.18
m.s.	0.18**	0.33**	0.01	0.01
Experiment 4				
d.f.	8	1	7	1
s.s.	0.11	0.001	0.11	0.001
m.s.	0.01	0.001	0.02	0.001
Experiment 5				
d.f.	11	4	7	6
s.s.	1.70	0.86	0.99	0.06
m.s.	0.16*	0.22*	0.14*	0.01

Overall	Experiment	Main effects	Treat-ment	Day	Error
d.f.	4	20	9	7	121
s.s.	0.25	2.67	2.03	0.12	1.33
m.s.	0.06**	0.13**	0.23**	0.02	0.01

*P<.05

**P<.01

Table 36.--Analysis of variance for overall NH_3 and pH *in vitro*

	Source of variation			
	Main effects	Experiment	Treatment	Error
NH_3				
d.f.	13	4	9	20
s.s.	5506.81	2447.03	1893.13	172.38
m.s.	423.60**	611.76**	210.35**	8.62
Initial pH				
d.f.	13	4	9	9
s.s.	1.52	0.07	1.29	0.59
m.s.	0.12*	0.02	0.14**	0.03
Final ph				
d.f.	13	4	9	20
s.s.	4.36	1.25	3.28	0.83
m.s.	0.34**	0.31**	0.37**	0.04
pH difference				
d.f.	13	4	9	20
s.s.	2.25	1.53	0.56	1.37
m.s.	0.17*	0.38*	0.06	0.11

*$P < .05$

**$P < .01$

Table 37.--Analysis of variance for trial I

	Source of variation		
	d.f.	s.s.	m.s.
Milk yields			
Block	7	263.85	37.69
Treatment	2	69.48	34.74**
Error	14	30.50	2.17
Persistency			
Block	7	525.33	75.05
Treatment	2	500.44	250.22*
Error	14	874.87	62.49
Body weight change			
Block	7	2294964.	327851.96
Treatment†	2	1954393.	979696.75*
Error	14	4926948.	351924.86

*P<.05

**P<.01

†Significnace at P<.10

Table 38.--Analysis of variance for trial II

	Source of variation		
	d.f.	s.s.	m.s.
Milk yields			
Block	6	727.25	121.21**
Treatment	3	24.36	8.12
Error	18	258.64	14.37
Persistency			
Block	6	235.36	39.23
Treatment	3	501.69	167.23
Error	18	1494.60	83.03
Body weight changes			
Block	6	1.43	0.24
Treatment	3	0.12	0.04
Error	18	3.52	0.20
Silage intake			
Block	6	100999.92	16833.32
Treatment	3	55046.92	18348.97
Error	18	270718.12	15039.90
Total DM intake			
Block	6	0.27	0.05
Treatment	3	0.05	0.02
Error	18	0.39	0.02
Milk/DM			
Block	6	118047.81	19674.64*
Treatment	3	11006.52	3668.84
Error	18	145126.26	8062.57

*P<.05

**P<.01

Table 39.--Analysis of variance for milk yield, persistency of lactation and silage DM intake (trial III)

	Square	Anim/sq	Per/sq	Treatment	Error
Milk yields					
Degrees of freedom	5	12	12	2	22
Sums of squares	132684.18	248323.77	74634.74	18727.47	83317.02
Mean square	26536.84**	20693.65	6219.56	9363.73	3787.14
Persistency of lactation					
d.f.	5	12	12	2	22
s.s.	1753.56	2869.47	1190.51	280.18	1454.35
m.s.	350.71**	239.12**	99.21	140.09	66.11
Silage DM intake					
d.f.	5	12	12	2	22
s.s.	410.92	285.93	67.98	2.32	195.25
m.s.	82.18**	23.83*	5.67	1.16	8.88
Total dry matter intake					
d.f.	5	12	12	2	22
s.s.	661.96	663.67	72.79	4.56	326.64
m.s.	132.39**	55.31**	6.07	2.28	14.84

Table 39.--Continued

	Square	Anim/sq	Per/sq	Treatment	Error
Body weight					
d.f.	5	12	12	2	22
s.s.	2835.89	1350.51	19307	486.26	14962.83
m.s.	567.18	112.54	1608.97*	243.13	680.13
Feed efficiency					
d.f.	5	12	12	2	22
s.s.	0.988	0.651	0.405	0.011	0.838
m.s.	0.198*	0.054	0.034	0.006	0.038

*P< .05

**P< .01

Table 40.--Analysis of variance for nitrogen balance (trial III)

	Square	Anim/sq	Per/sq	Treatment	Error
Nitrogen intake					
d.f.	2	6	6	2	10
s.s.	0.056	0.329	0.106	0.034	0.362
m.s.	0.028	0.055	0.018	0.017	0.036
Fecal N					
d.f.	2	6	6	2	10
s.s.	146.16	86.94	343.98	10.10	155.14
m.s.	73.08*	14.49	67.33*	5.05	15.51
Urinary-N					
d.f.	2	6	6	2	10
s.s.	55.41	397.59	204.33	203.40	154.95
m.s.	27.70	66.27*	34.06	101.70*	15.50
Digestible N					
d.f.	2	6	6	2	10
s.s.	148.99	87.26	345.02	11.13	161.31
m.s.	74.49*	14.54	57.50*	5.57	16.13

Table 40.--Continued

	Square	Anim/sq	Per/sq	Treatment	Error
N retention					
d.f.	2	6	6	2	10
s.s.	117222.22	189111.11	152844.45	84155.56	191133.33
m.s.	586111.11**	31518.52	25474.08	42077.78	19113.33
N-balance					
d.f.	2	6	6	2	10
s.s.	194206.52	154199.22	149075.72	80965.63	197536.80
m.s.	97105.26	25699.87	24846.00	40482.81	19753.68

*P<.05

**P<.01

Table 41.--Analysis of variance for rumen VFA (trial III)

	Square	Anim/sq	Period/sq	Treatment	Error	Total
Acetate						
d.f.	9	20	20	2	38	89
s.s.	10.20	28.55	17.04	4.53	45.66	105.98
m.s.	1.13	1.43	0.85	2.27	1.20	
Propionate						
d.f.	9	20	20	2	38	89
s.s.	4.59	4.53	0.74	0.01	2.53	12.4
m.s.	0.51	0.23	0.04	0.05	0.07	
Butyrate						
d.f.	9	20	20	2	38	89
s.s.	0.01	0.01	0.003	0.02	0.02	0.06
m.s.	0.001	0.0005	0.0002	0.01**	0.0005	
Isobutyrate						
d.f.	9	20	20	2	38	89
s.s.	0.96	1.24	0.38	0.003	1.20	3.28
m.s.	0.11	0.06	0.02	0.001	0.03	

**P< .01

Table 42.--Analysis of variance for milk total solids, fat, protein and solids nonfat (trial III)

	Square	Anim/sq	Period/sq	Treatment	Error	Total
Total solids						
d.f.	9	20	20	2	38	86
s.s.	8.51	33.62	9.51	1.21	11.15	64.6
m.s.	0.95**	1.68**	0.48	0.61	0.29	
Fat						
d.f.	9	20	20	2	38	86
s.s.	6.47	13.30	3.88	0.25	5.73	29.63
m.s.	0.72**	0.67**	0.19	0.13	0.15	
Protein						
d.f.	9	20	20	2	38	86
s.s.	1.27	5.17	1.23	0.24	1.72	9.62
m.s.	0.14*	0.26**	0.06	0.12	0.05	
Solids nonfat						
d.f.	9	20	20	2	38	86
s.s.	3.54	13.24	10.61	1.05	9.93	38.43
m.s.	0.39	0.66**	0.53*	0.53	0.26	

*P< .05

**P <.01

Table 43.--Analysis of variance of total fat, protein and solids nonfat (trial III)

	Square	Anim/sq	Period/sq	Treatment	Error	Total
Total fat						
d.f.	9	20	20	2	38	86
s.s.	806808.14	1399123.86	292435.86	26452.91	532840.41	3057661.0
m.s.	89645.35**	69956.19**	14621.79	13226.46	14022.12	
Total protein						
d.f.	9	20	20	2	38	86
s.s.	388814.13	273701.69	136753.63	12317.65	272484.9	1084072.0
m.s.	43201.57**	13685.08*	6837.68	6158.83	7170.66	
Total SNF						
d.f.	9	20	20	2	38	86
s.s.	4349768.	3523607.	1513007.	25820.14	1313417.86	10725620.
m.s.	483307.55**	176180.35**	75650.35*	12910.07	34563.63	

*P<.05

**P<.01

Table 44.--Analysis of variance for blood urea nitrogen, rumen NH_3-N and pH (trial III)

	Square	Animal/sq	Period/sq	Treatment	Error	Total
Blood urea-N						
d.f.	9	20	20	2	38	89
s.s.	139.20	188.34	166.69	22.91	96.52	613.66
m.s.	15.24**	9.42**	8.33**	11.46*	2.46	
Rumen NH_3-N						
d.f.	9	20	20	2	38	89
s.s	894.08	815.92	511.54	113.62	659.29	2994.45
m.s.	99.34**	40.80*	25.58	56.81*	17.35	
Rumen pH						
d.f.	9	20	20	2	38	89
s.s.	2.0	1.84	1.06	0.012	1.04	5.95
m.s.	0.22	0.09	0.05	0.006	0.03	

*P<.05

**P<.01